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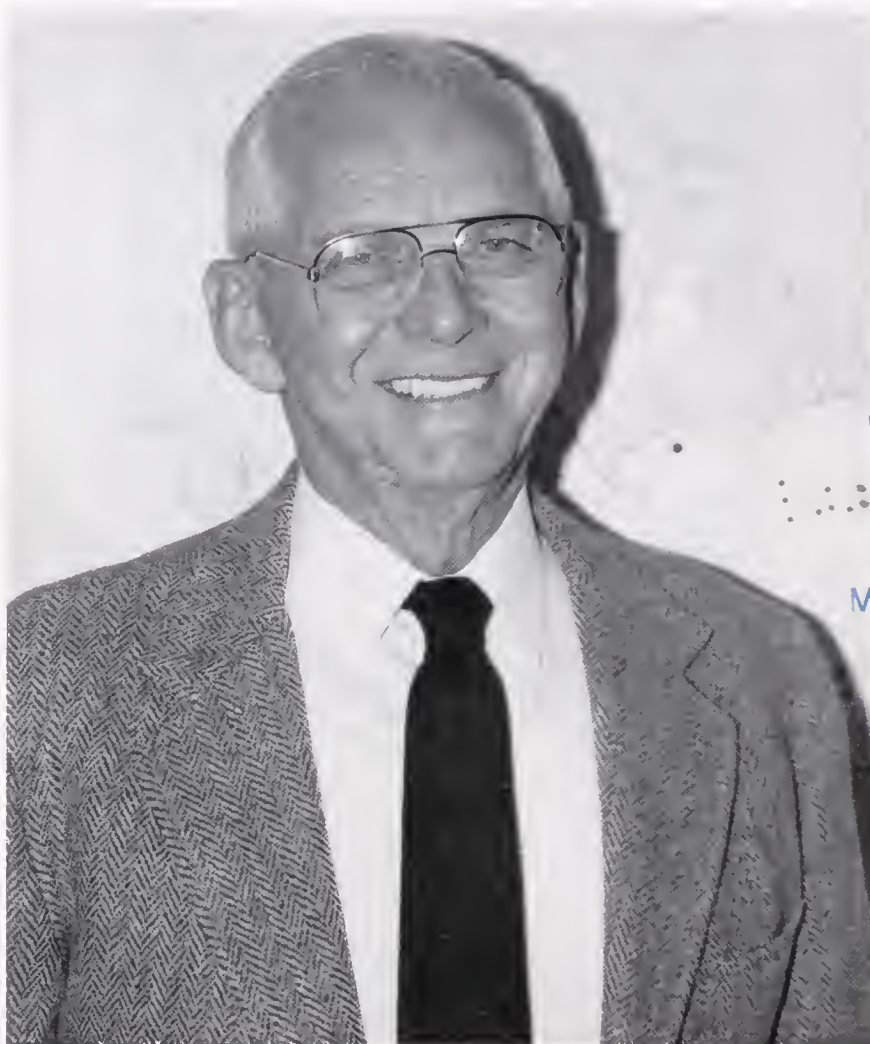
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**IN MEMORIAM  
DR. L. EUGENE CRONIN  
1917–1998**

Dr. L. Eugene Cronin, NSA member and NSA President from 1959 to 1961, died at his home in Annapolis, MD on December 18, 1998 at the age of 81. He was known nationally and internationally for his efforts on behalf of estuarine research, resource management, and habitat restoration.

A native of Aberdeen, MD, Gene Cronin received an AB in Chemistry from Western Maryland College (1938), and MS (1943) and PhD (1946) degrees in Zoology (blue crab biology) from the University of Maryland. He was a high school Biology teacher from 1938 to 1940 in Bel Air, MD. From 1943 to 1950, he was a biologist at Chesapeake Biological Laboratory (CBL) in Solomons, MD with a focus on biology and management of blue crabs, oysters, and fish. He subsequently spent five years with the University of Delaware's Department of Biological Sciences. There he established a marine laboratory in Lewes, DE, beginning in rented space in the local high school. In 1952, he and his staff of three moved into a former restaurant while making plans for a permanent research building; a converted fishing-party boat served as the University's first research vessel, *Acartia*. By the time the new research building was dedicated in 1955, Gene had laid the foundation for a program in research and education that has since grown into the University of Delaware Graduate College of Marine Studies.

Gene returned to Maryland in 1955 as Director of the Maryland Department of Research and Education, with headquarters at CBL. In 1961, the Department became the Natural Resources Institute (NRI), a 4-laboratory entity within the University of Maryland. In 1975, NRI merged with the newly-established Horn Point Environmental Laboratories (now Horn Point Laboratory) to form The University of Maryland Center for Environmental and Estuarine Studies (now The University of Maryland Center for Environmental Science). In 1977, Gene became Director of the Chesapeake Research Consortium that coordinates Chesapeake Bay-related research among the area's major research universities and institutions. In 1984, he "retired" to become a consultant, while remaining active in conservation organizations such as The Chesapeake Bay Foundation and The Alliance for the Chesapeake Bay.



During the course of his career, Gene was involved with the governance of a number of scientific societies. In addition to being President of The National Shellfisheries Association as noted above, he was the first President (1949) of the Atlantic Estuarine Research Society (AERS). His continued interactions with AERS and estuarine scientists led to his involvement in the founding of the Estuarine Research Federation (ERF), of which he was Founding President from 1971 to 1973. He was a Fellow Emeritus of the American Institute of Fishery Research Biologists. Within Maryland, he was Director of the Maryland Biology Teachers Association in his early career and a Trustee of The Natural History Society of Maryland.

Gene made numerous contributions towards understanding and conserving natural resources by serving on many State and Federal committees and commissions. He was an advisor to the Atlantic States Marine Fisheries Commission from 1955 to 1969, served on a variety of committees of the National Academy of Sciences and of the US Department of State, and sat on the Marine Board of the National Research Council. Through these activities, he played important roles in influencing fisheries policies in the Chesapeake Bay region. He had a key role in involving the US Environmental Protection Agency in a 5-year study of Chesapeake Bay and in the subsequent establishment of the Chesapeake Bay Program, a state-federal initiative.

His efforts brought him many awards. In 1994 he became the second recipient of the Mathias Medal. This biennial award "In Recognition of Scientific Excellence" is sponsored by the Sea Grant Programs of Maryland and Virginia and the Chesapeake Research Consortium to honor those who work to enhance understanding of the Chesapeake Bay ecosystem and to encourage application of this knowledge to solving environmental problems in the Bay. In 1997, ERF created an award in his name to recognize outstanding contributions to estuarine research by a young scientist, a fitting recognition of Gene's contributions to ERF. Other awards included recognition by the Oyster Institute of North America (1967), Chesapeake Bay Seafood Industries Association (1968), and the Isaac Walton League of America (1990). The Governor of Maryland appointed him as "Admiral of the Chesapeake Bay" in 1987.

Gene remained active professionally to within a few weeks of his death. He was one of seven distinguished scientists who participated in a workshop in November 1998 for university students interested in learning how research, policy, and management have been applied to Chesapeake Bay problems in the past. He was also involved in co-editing a volume on the biology of the blue crab.

Gene is survived by his wife, Alice, herself a chemist and founding member of AERS, three sons, and four grandchildren.

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**IN MEMORIAM  
TERRANCE HENRY BUTLER  
1923–1998**

Terrance (Terry) Henry Butler, a recognized world authority in the field of crustacean biology and crustacean fisheries, passed away on March 10, 1998 in Nanaimo, British Columbia, Canada. He was 74-years-old.

Terry was born in Nelson, British Columbia, where his father worked as an engineer on the Canadian Pacific ferries. He received his elementary and secondary education in the interior of British Columbia (B.C.) and, prior to World War II, entered the engineering department of Victoria College in Victoria, B.C. In 1942 he joined the Canadian army, enlisting in the engineer corps. He transferred to the Canadian Airborne Regiment, with whom he served overseas, and was stationed in the Netherlands by the end of the War. He was discharged from the army in January 1946.

Upon returning to Canada, he enrolled in the University of British Columbia where he completed a BA degree in Honors Biology in 1949. He received an MA degree from the same University in 1953; the subject of his thesis was a study of Dungeness crab biology and fisheries.

During the summer of 1948, Terry had been hired as a summer student at the Pacific Biological Station in Nanaimo, B.C., where he served as a groundfish technician conducting biological sampling of commercial groundfish catches. Thus began an association with the Biological Station that lasted for the remainder of his working career. In May 1949, he accepted a permanent position at the Pacific Biological Station as a research scientist in charge of the Crab and Shrimp Investigation. He continued in this capacity, and also served for a period as head of the Shellfish Investigation, until he retired from the Pacific Biological Station in March 1984.

During his working career, Terry undertook research studies on a wide range of subjects that included basic biology, population dynamics and stock assessment of some British Columbia crustacean species, exploratory fishing for harvestable stocks, and improvements in fishing methods. He became a recognized authority on crustacean fisheries of the West Coast of North America. As a result of his work, several crustacean fisheries were started in British Columbia. Throughout his working career, Terry maintained a close working relationship with the industry and was frequently consulted by industry for his advice.

Terry published widely in the field of crustacean biology and fisheries, having over 55 publications including his "Shrimps of the Pacific Coast of Canada," which has remained a classic reference work. He received the Queen's Silver Jubilee Medal in 1977 in recognition of his career.

Terry also had an active interest in crustacean fisheries in developing countries. From January 1957 to November 1958, he served with the Food and Agriculture Organization of the United Nations in Indonesia, assisting in the development and management of local shrimp fisheries. In 1988, he served with the Canadian Executive Services Organization to assist in the development of a southern king crab fishery in Chile.

After his retirement in 1984, Terry continued to work several days a week at the Pacific Biological Station, even when his health was failing, to continue with his studies and publications on British Columbia crustaceans. At the time of his passing, he was writing a major work, "The Crab Fisheries of British Columbia." Terry was highly respected for his research accomplishments and was an inspiration to younger scientists at the Pacific Biological Station and elsewhere. He was a kind and patient man who always had time to talk with younger staff members, to encourage them in their work, and to give them the benefit of his long years of experience.

Terry had many outside interests. He was keenly interested in plants, shrubs, and trees, and always claimed that he was a frustrated botanist. Wherever he travelled, he was very interested in the local fauna and how it compared to that in British Columbia. He had a great appreciation of classical music and he was an enthusiastic and much respected golfer and a regular member of a Saturday morning golfing group from the Pacific Biological Station.

Terry married D. Joan Abel in 1947; they celebrated their golden wedding anniversary in 1997. Together, they had six children, nine grandchildren, and a great granddaughter.

Terry was a warm-hearted, kind and generous companion. Those who knew him appreciated his understanding, his guidance and his example and these qualities served to inspire his associates and to assist them in their research and in their daily lives.

Terry will be sorely missed by his family and by all that knew him.

James Boutillier and Neil Bourne  
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## OBSERVATIONS ON THE BIOLOGY OF THE VEINED RAPA WHELK, *RAPANA VENOSA* (VALENCIENNES, 1846) IN THE CHESAPEAKE BAY

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**ABSTRACT** The recent discovery of the Veined Rapa whelk (*Rapana venosa*, Valenciennes, 1846) in the lower Chesapeake Bay provides an opportunity to observe the initial biological and ecological consequences of a novel bioinvasion. These large predatory gastropods occur in subtidal, hard bottom habitats in the lower Bay and are capable of feeding, mating, and moving while completely burrowed. Hard clams (*Mercenaria mercenaria*) are consumed preferentially in the laboratory when offered concurrently with oysters (*Crassostrea virginica*), soft clams (*Mya arenaria*), and mussels (*Mytilus edulis*). Chesapeake Bay *R. venosa* readily open and consume large hard clams (30 to 85 mm SH) leaving no visible signs of either drilling or boring behavior. Shell morphology and thickness may provide an inherent size-selective predation refuge for Rapa whelks in the Bay. These same shell characteristics may change the dynamics of shell selection by local hermit crabs, particularly the striped hermit crab, *Clibanarius vittatus*. Recent collections of striped hermit crabs from the Hampton Roads area indicate that very large striped hermit crabs are using empty *Rapana* shells as shelters.

**KEY WORDS:** *Rapana venosa*, Veined Rapa whelk, Muricidae, Thaididae, ballast water, bioinvasion, Chesapeake Bay, *Clibanarius vittatus*, *Mercenaria mercenaria*

### INTRODUCTION

The Veined Rapa whelk, *Rapana venosa*, (Valenciennes, 1846) is a large, predatory gastropod that has recently been found in the lower portion of the Chesapeake Bay. As with other representatives of the Thaididae family [Earlier classifications of the Neogastropods place *Rapana* sp. in the family Muricidae. Recent taxonomic revisions include *Rapana* in the Thaididae (R. Germon, Smithsonian Institution, Washington, D.C., pers. comm.)], this animal is a carnivore whose principal prey items include many commercially valuable bivalves. *Rapana venosa* is one of several modern *Rapana* species including *R. bezoar* and *R. rapiformis*. Although *R. thomasi* was originally described by Crosse in 1861 as a separate species (Thomas's Rapa whelk), it is currently recognized as a synonym for *R. venosa* (R. Germon, Smithsonian Institution, Washington, DC, pers. comm.).

*Rapana venosa* is native to the Sea of Japan, the Yellow Sea, the East China Sea, and the Gulf of Bohai (Tsi et al. 1983, Chung et al. 1993, Zolotarev 1996, Chung and Kim 1998). Three species of *Rapana* occur sympatrically in Chinese waters: *R. venosa*, *R. bezoar*, and *R. rapiformis* (Tsi et al. 1983). All three species are found in coastal subtidal habitats and are commercially harvested (Hwang et al. 1991, Chung et al. 1993, Morton 1994). Rapa whelks were discovered in the Black Sea in 1947 (Drapkin 1963) and have subsequently spread throughout the Black Sea and into the Sea of Azov as well as the Aegean (Koutsoubas and Voultziadou-Koukoura 1990, Zolotarev 1996) and Adriatic (Bombace et al. 1994) Seas. *R. venosa* from Korean waters described by Chung et al. (1993) ranged from 32.5 to 168.5 mm shell length (the maximum distance from the tip of the spire to the bottom of the columella, SL).

*Rapana venosa* is easily distinguished from native gastropods of the Chesapeake Bay. It has a short spired, heavy shell with a large inflated body whorl and a deep umbilicus (Fig. 1). The slightly concave columella is broad and smooth. Small, elongate teeth are present along the edge of the large, ovate aperture's outer lip. External shell ornamentation includes smooth spiral ribs that end in regular blunt knobs at both the shoulder and the periphery

of the body whorl. In addition, fine spiral ridges are crossed by low vertical riblets. Older specimens can be eroded, but the color is variable from gray to orange-brown (one specimen is atypically blonde), with darker brown dashes on the spiral ribs. The aperture and columella vary from deep orange-red to yellow or off-white. Spiral, vein-like coloration, ranging from black to dark blue, occasionally occurs internally, originating at the individual teeth at the outer lip of the aperture.

The first collection of *Rapana venosa* in the Chesapeake Bay was made in the summer of 1998 during a routine trawl collection by the Virginia Institute of Marine Science (VIMS) trawl survey in the vicinity of the Monitor-Merrimac Tunnel (Fig. 2). This specimen was positively identified as *Rapana venosa* by Drs. Jerry Harasewych (Smithsonian Institution, Washington, DC) and Yuri Kantor (Russian Academy of Science, Moscow). A subsequent sampling trip specifically for *Rapana venosa* in the same vicinity on August 24, 1998 yielded two masses of *R. venosa* egg cases (Fig. 3; a total of 50+ egg cases) but no live animals. The egg cases were returned to VIMS and maintained at ambient temperature and salinity conditions on a 14 h light:10 h dark regime. Within a week postcollection, individual egg cases began hatching with the last egg case hatching on September 21, 1998. Larvae were cultured and used in salinity tolerance experiments (Mann and Harding, in review). Given the size of the specimens collected to date from the lower Bay (68 to 165 mm SL) and the presence of viable egg cases, it seems reasonable to assume that the local Rapa whelk population is sexually mature and actively breeding.

As in the eastern Mediterranean and Black Seas (Zolotarev 1996), ballast water from commercial and/or military ship traffic is the probable source of introduction into the Chesapeake Bay. *R. venosa* larvae are planktonic for 14 to 17 days (Chung et al. 1993, Mann and Harding in review). Normal transit time to the Hampton Roads/Norfolk area from the Baltic, Black, Adriatic, or Aegean Seas is approximately 10 to 24 days (G. Ruiz, Smithsonian Environmental Research Center, pers. comm.). This time interval is well within the temporal window for survival of viable planktonic *R. venosa* larvae. At certain times during the year (e.g., May



**Figure 1.** Picture of an adult *Rapana venosa* (150 mm SL) from the Chesapeake Bay. The arrows highlight the broad columella, opercular teeth, and bright orange aperture.

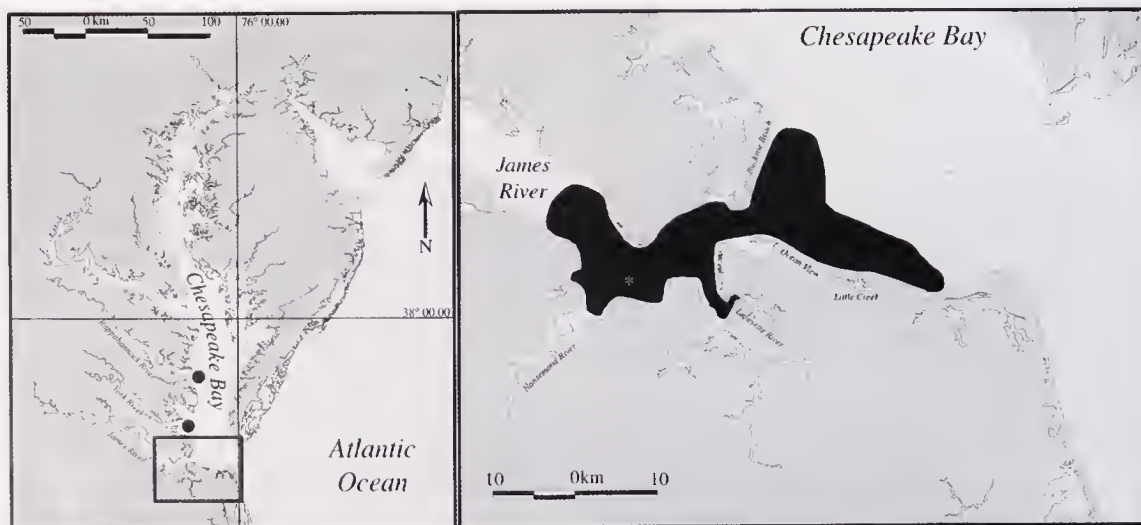
through October) temperature and salinity regimes on both ends of the trip are similar (see Mann and Harding, in review for a detailed discussion). The Hampton Roads/Norfolk area is a major foci of container, coal transport, and military ship activity. The area ranks third among U.S. ports in terms of volume of ballast discharged on an annual basis (G. Ruiz, Smithsonian Environmental Research Center, pers. comm.). Given the sheer volume of ballast water

arriving in Chesapeake Bay annually from ports with active Rapa whelk populations (15 million metric tons; G. Ruiz, Smithsonian Environmental Research Center, pers. comm.), the possibility of obtaining sufficient numbers of Rapa whelk larvae needed to eventually establish a breeding population in the Chesapeake Bay may be quite high. International traffic aside, the Hampton Roads/Norfolk area is also a major hub for coastal shipping along the eastern seaboard of North America (G. Ruiz, Smithsonian Environmental Research Center, pers. comm.). If a local population of Rapa whelks becomes established in the Bay, it is likely that the Chesapeake would eventually become a source population for other coastal ports with similar habitat conditions. This scenario places ports throughout the Middle Atlantic Bight (e.g., New York, Boston) as well as the South Atlantic Bight (e.g., Charleston) at higher risk for introduction of the species in that they would be receiving both international and local inoculations.

Since the discovery of *Rapana venosa* in the Chesapeake Bay, live Rapa whelks have been under observation in wet laboratory tanks at VIMS. To date, 412 animals have been donated to VIMS (these numbers include live animals, dead animals with shells, or shells only), mostly by commercial watermen and seafood processing companies, indicating the presence of an established population of *Rapana venosa* in the lower portion of the Chesapeake Bay. Observations to date on the basic biology and ecology of *Rapana venosa* in the Chesapeake Bay are described herein and placed in the context of potential trophic interactions of this animal in the lower Chesapeake Bay.

*Current Distribution*

The current distribution of *Rapana venosa* in the Chesapeake Bay extends from the Chesapeake Bay Bridge Tunnel northward along the western shore line in a continuous swath across Little Creek, Ocean View, Fort Monroe, and Buckroe Beach (Figs. 2 and 4). Several unconfirmed reports from the Poquoson flats area are punctuated by two confirmed discoveries of *Rapana* at Tue Marshes Light in the York River (Fig. 2). The northernmost report of a Rapa whelk in the Bay is from Butler's Hole, a small oyster rock near the mouth of the Rappahannock River; this 130 mm SL



**Figure 2.** Map showing known *Rapana venosa* distribution as of March 1999 in the Chesapeake Bay proper (A.) and the Ocean View/Hampton Roads/James River region (B.). The black circles (A.) indicate the Rappahannock and York River collection sites. The black zone (B.) shows the known distribution within the lower Bay/Hampton Roads/James River. The first collection location is indicated with an asterisk (B.).



Figure 3. *Rapana venosa* egg cases collected from Hampton Roads, VA in August 1998. The yellow egg case cluster was attached basally to a hydroid mat (A.). Note the broad phyllopodus egg case tops and egg pores shown in the top view (B.).

individual was collected by the authors during an annual oyster stock assessment dredge survey.

The majority of Rapa whelks have been collected by either commercial clambers or crab dredgers working in the lower Bay. In early September 1998, VIMS established an ongoing *Rapana* bounty system with the help of the Virginia Saltwater Commercial Fishing Development Fund and, as of January 1999, the Virginia Sea Grant program. A bounty is paid for each snail turned in to VIMS personnel, provided that collection information (i.e., location, gear, depth, and bottom type) are reported at the time of donation. The bounty program yielded an average of 8 to 10 animals per week through the end of November 1998 donated primarily by clambers working off Ocean View and Buckroe Beach

(Fig. 2). Clammers in the lower Chesapeake fish for hard clams or quahogs (*Mercenaria mercenaria*) with patent tongs. Quahogs > 50 mm shell height are abundant (approximately 1 to 11 animals m<sup>2</sup>) in portions of the lower bay (Roegner and Mann 1991), and the commercial hard clam fishery in the region is economically important, annually landing 1.1 million pounds with a dockside value of approximately \$6 million (Kirkley 1997).

The lower Bay also supports a winter crab dredge fishery targeting blue crabs (*Callinectes sapidus*) that burrow into the sand/mud bottom to overwinter. When crab dredge season opened in the lower Bay on December 1, 1998, Bay water temperatures were still 8 to 12°C. During the first 2 weeks of December 1998, over 30 Rapa whelks/day were donated to the VIMS collection by crab

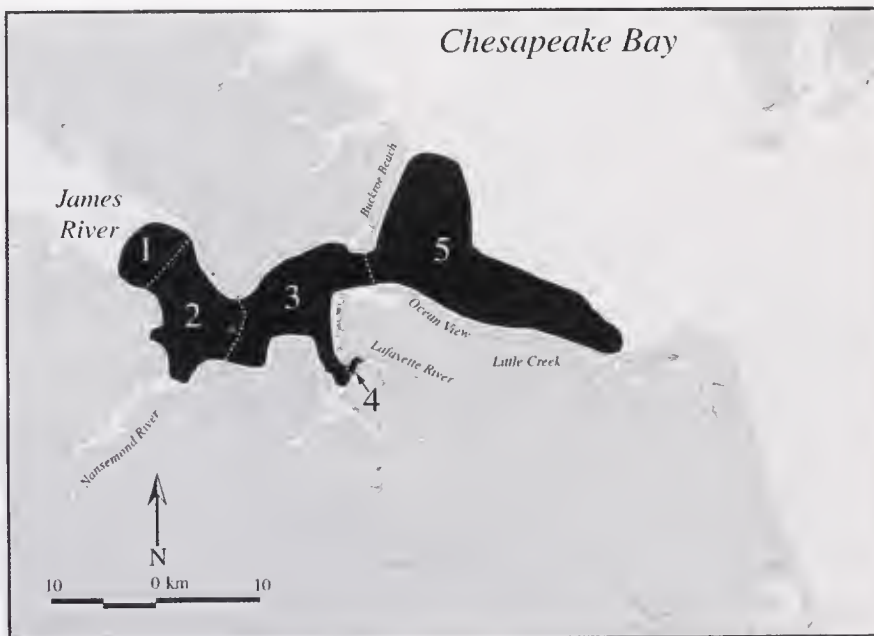


Figure 4. Distribution map of *Rapana venosa* from the lower Chesapeake Bay showing collection zones: 1.) Above the SR 258 James River Bridge, 2.) Between the James River Bridge and the Monitor-Merrimac Bridge Tunnel, 3.) Between the Monitor-Merrimac Bridge Tunnel and the Hampton Roads Bridge Tunnel, including the Hampton Bar area, 4.) the Lafayette River, and 5.) the Buckroe Beach, Fort Monroe, Ocean View, and Little Creek areas in the bay proper.

TABLE 1.

Summary of *Rapana venosa* collections through March 1, 1999 from the lower Chesapeake Bay and its tributaries.

Collection Location	n	Average Shell Length (mm)	Standard Error (SE, mm)
Lower Bay: Little Creek/Ocean View/Buckroe Beach	185	141.8	0.93
James River: Hampton Bar	7	138.8	5.23
James River: between Monitor-Merrimac Bridge Tunnel and the James River Bridge	198	132.7	0.84
James River: above the James River Bridge	11	131.3	3.63
Lafayette River	7	102.7	4.9
Nansemond River	1	135.0	—
York River: Tue Marshes Light	2	149.0	9.00
Rappahannock River: Butler's Hole	1	130.0	—

"n" refers to the number of animals collected from each location. Locations are shown in relation to the mainstem Chesapeake Bay and each other in Figures 2 and 4.

dredgers and seafood processing companies. The arrival of a cold front just before Christmas 1998 caused water temperatures to fall below 5°C and coincided with a reduction in both fishing activity and *R. venosa* donations. Throughout January and February 1999, crab dredgers working the lower Bay have reported few *R. venosa*. Presumably, the sustained colder temperatures have driven them either into deeper waters as reported in their home range (Wu 1988) or deeper into the sediment below the zone of dredging activity.

Although donations from crab dredgers in the lower Bay essentially stopped in January 1999, Rapa whelk donations from clambers working in the James River continued until the closing of the area to commercial fishing in mid-March at an average rate of 6 animals/day<sup>-1</sup>. As of this writing, there have been no *R. venosa* reported by commercial oystermen working on extant oyster beds in the James River upstream of the Route 258/17 James River bridge (Haven and Whitcomb 1983). A majority of the animals collected to date from all sources have been collected from regions with hard sand bottom in depths ranging from 10 to 60 m at salinities of 18 to 28 ppt.

The collection data from commercial sources do not lend themselves to an accurate Rapa whelk stock assessment, because it is impossible to separate the effects of fishing effort in a particular location from potential gear biases in that both crab dredges and patent tongs selectively catch larger snails (>100 mm SL) given standard ring size for both (0.06 m). However, an examination of *R. venosa* length-frequency distributions for sites in the lower Bay and Hampton Roads area yields an interesting pattern. The shell lengths (SL, mm) of animals from the five different regions with >5 confirmed Rapa whelk reports (Table 1) were compared with an ANOVA followed by Fisher's test for multiple comparisons (per Zar 1996). Data satisfied assumptions of both homogeneity of variance and normality without transformation. Animals collected from the Ocean View/Buckroe Beach/Little Creek area (Fig. 4) or from regions outside the Hampton Roads Bridge tunnel are significantly larger than animals collected from either the Lafayette River, above the James River Bridge, or between the Route 258/17 James River bridge (hereafter JRB) and the Monitor-Merrimac bridge tunnel (Figs. 4 and 5; ANOVA,  $p < .05$ ; Fisher's test,  $p < .05$ ). Animals collected from the Lafayette River are significantly smaller than Rapa whelks collected from any other site (Figs. 4 and 5; ANOVA,  $p < .05$ ; Fisher's test,  $p < .05$ ). It is interesting to note that the Little Creek/Ocean View area is immediately adjacent to

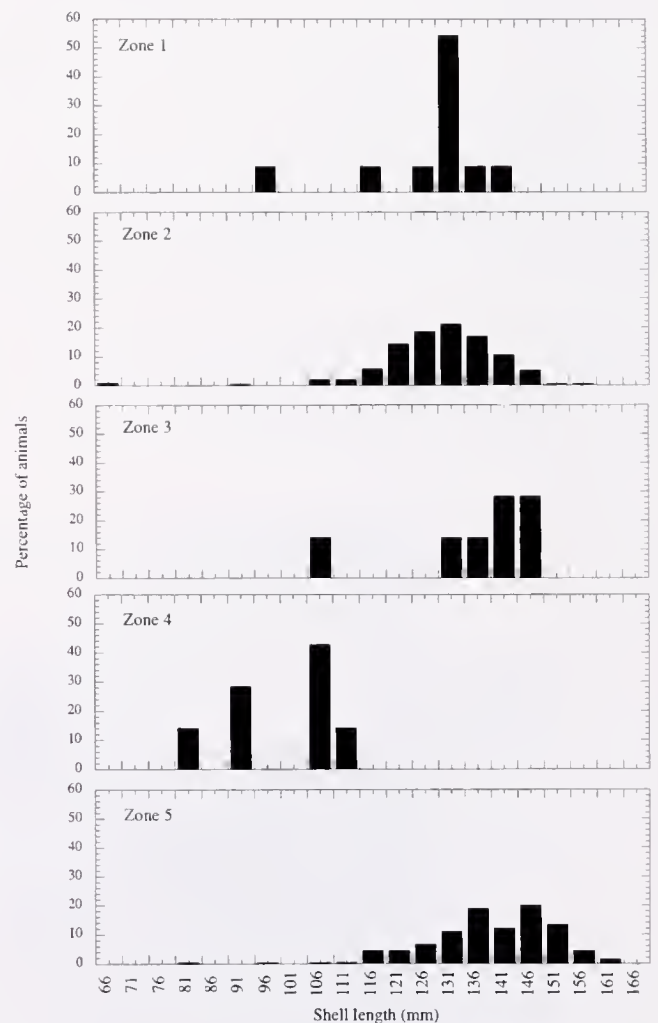


Figure 5. Length-frequency distribution of *Rapana venosa* collected from the Chesapeake Bay. Zones 1 through 5 correspond to the zones shown in Figure 4; i.e., 1.) Above the SR 258/17 James River Bridge (JRB,  $n = 11$ ), 2.) Between the JRB and the Monitor-Merrimac Bridge Tunnel ( $n = 194$ ), 3.) Between the Monitor-Merrimac Bridge Tunnel and the Hampton Roads Bridge Tunnel, including the Hampton Bar area ( $n = 7$ ), 4.) the Lafayette River ( $n = 7$ ), and 5.) the Buckroe Beach, Fort Monroe, Ocean View, and Little Creek areas in the Bay proper ( $n = 181$ ).

both the anchorage for commercial and military ships awaiting pilots and clearance to enter the port and the Thimble Shoals shipping channel (Figs. 2 and 4). The area between the JRB and the Monitor-Merrimac bridge tunnel includes the Newport News coal container terminal, a major site of deballasting for international ships awaiting coal.

#### Age Estimates

In the absence of age and growth estimates for Chesapeake Bay *Rapana venosa*, age and growth estimates for a Knobbed whelk (*Busycon carica*) population from Virginia's Eastern Shore may offer a conservative estimate of potential Rapa whelk growth rates. Kraeuter et al. (1989) and Castagna and Kraeuter (1994) provide growth and length-at-age estimates for *B. carica* from both laboratory and field studies extending over a 14-year period. Growth rates for *B. carica* were greatest during the first year (approximately 32 mm/y<sup>-1</sup>) and then subsequently decreased to 14.4 mm/y<sup>-1</sup> for the first 10 years followed by growth rates of 6.5 to 9.5 mm/y<sup>-1</sup> for animals older than 10 y and/or greater than 160 mm SL (Fig. 6; Kraeuter et al. 1989, Castagna and Kraeuter 1994). In the absence of any data on *Rapana* growth rates in the Chesapeake Bay, it is reasonable to consider growth rates of such sympatric species as *B. carica* for initial estimates of Rapa whelk age. Ninety-five percent of all *R. venosa* collected thus far in the Chesapeake Bay are between 110 and 160 mm SL. If this range of *Rapana* shell lengths is overlaid onto the *B. carica* growth curve presented by Kraeuter et al. (1989) and Castagna and Kraeuter (1994), the resulting age distribution extends from approximately 7.5 to 13 years (Fig. 6). These are conservative growth estimates when considered in relation to the growth rates for Black Sea *Rapana* reported by Chukhchin (1984).

Rapa whelk length-at-age relationships have been described by

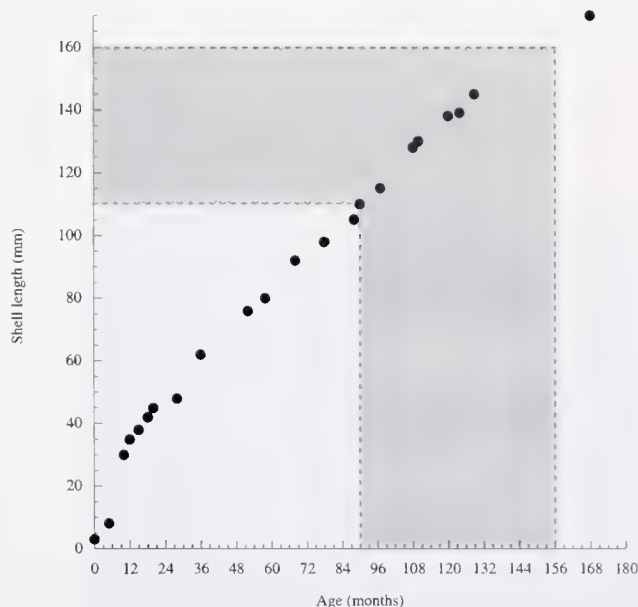


Figure 6. Plot of *Busycon carica* length-at-age relationship from laboratory and field observations of an Eastern Shore, VA *B. carica* population after Kraeuter et al. (1989) and Castagna and Kraeuter (1994). The shaded zone indicates the size range (SL, mm) and corresponding age estimate for 95% of the *Rapana venosa* collected in the Chesapeake Bay thus far.

Chukhchin (1984) for animals from the Black Sea. Chukhchin (1984) estimates reports growth rates for individuals in Sevastopol Bay of 20 to 40 mm during year 1, with mean shell length (SL) values of 64.6 mm, 79.4 mm, 87.5 mm, and 92.1 mm in years 2 through 6, respectively. This terminal size is smaller than the maximum SL of 120.1 mm reported by Smagowicz (1989) for a specimen in a collection from Bulgaria and Georgia, whose exact collection location was not reported. Chukhchin (1984) correlates shell thickening with spawning events and notes that the first spawning occurs in the second year at sizes ranging from 35 to 78 mm SL with a mean value of 58 mm SL.

#### Habitat Preferences

Both field collections and laboratory observations confirm that *Rapana venosa* prefers hard sand bottom habitats. These animals are avid burrowers and remain completely burrowed for more than 95% of the time in the laboratory. A 150 mm SL *R. venosa* can burrow into a sand bottom so that its shell is completely covered in less than 1 h. The only visible sign of a burrowed Rapa whelk is the maroon U-shaped siphon that is usually extended 1 to 3 cm above the surface of the sand. Rapa whelk siphons are sensitive to both light and motion and are retracted immediately at the slightest disturbance. Siphonal sensitivity combined with the animal's burrowing speed and low visibility conditions in the Bay may make conventional benthic survey methods that rely on direct observation of the animal (diver transects, video surveys) difficult as non-invasive stock assessment techniques. Bombace et al. (1994) observed an apparent increase in *R. venosa* biomass after artificial reef deployment in the Adriatic Sea. It is possible that there were burrowed *Rapana* at the sites at the time of reef deployment and that increases in *Rapana* sightings after reef construction are attributable to the emergence of local snails to feed on the reefs not the arrival of snails from other areas.

Laboratory observations indicate that Rapa whelks are capable of both feeding and mating while burrowed. They move reasonably quickly while burrowed (approximately 1 body length per minute). Hard sand bottom habitat is relatively common in the lower Chesapeake Bay (Fig. 7) and is not likely to be a limiting factor for potential range expansion of the animal in the bay.

#### Prey preferences

*Rapana bezoar* was described by Morton (1994) as "a generalist predator of subtidal molluscs." This description is certainly apt for *R. venosa* in the Chesapeake Bay. In laboratory feeding studies, Chesapeake Bay *R. venosa* prefer hard clams to oysters (*Crassostrea virginica*), soft clams (*Mya arenaria*), or local mussels (*Mytilus edulis*), although they will eat these other bivalves when hard clams are rare or unavailable (Fig. 8). A 140 mm SL Rapa whelk is capable of consuming a 75 to 80 mm hard clam in less than 1 h.

Previous reports on the feeding behavior of *R. thomasi* (now recognized as *R. venosa*) from the Black Sea place *R. venosa* among the gastropods that drill their prey (Gomoiu 1972, Carrier 1981) or use paralytic toxins during feeding (Chukhchin 1984). Morton (1994) describes feeding behavior of *R. bezoar* in terms of boring or crude rasping usually on the posteroventral shell margin. Similar rasping behavior has been observed for Chesapeake Bay *Rapana venosa* feeding on small hard clams [ $<30$  mm shell height, SH (distance from hinge to the opposite shell margin)]. Small chips or rasp marks are visible on the posteroventral shell margin

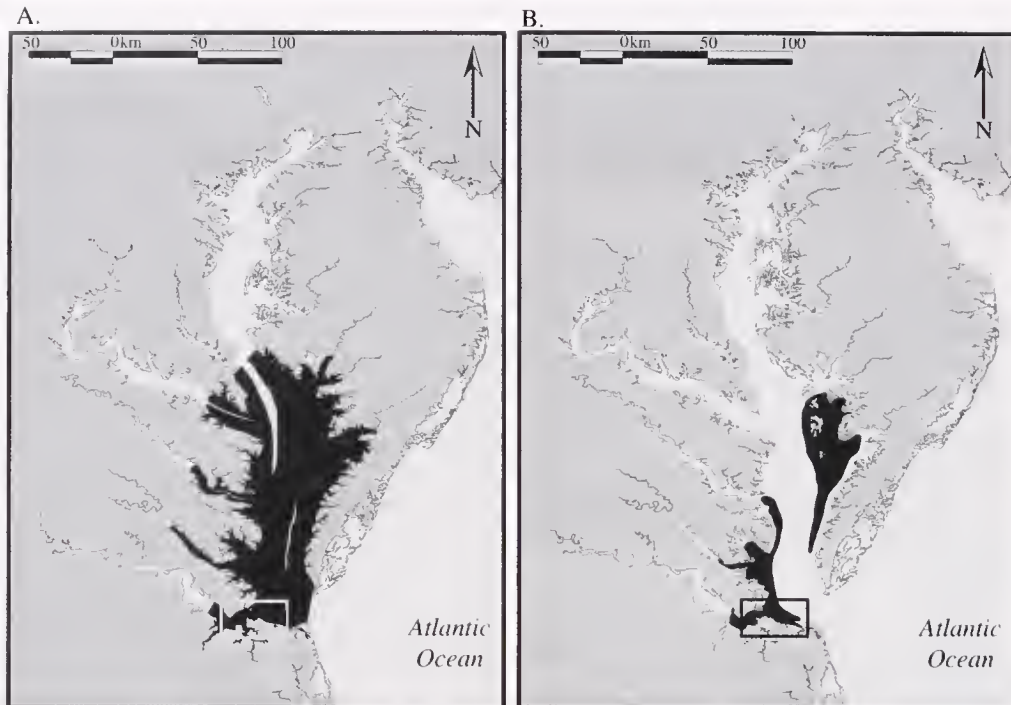
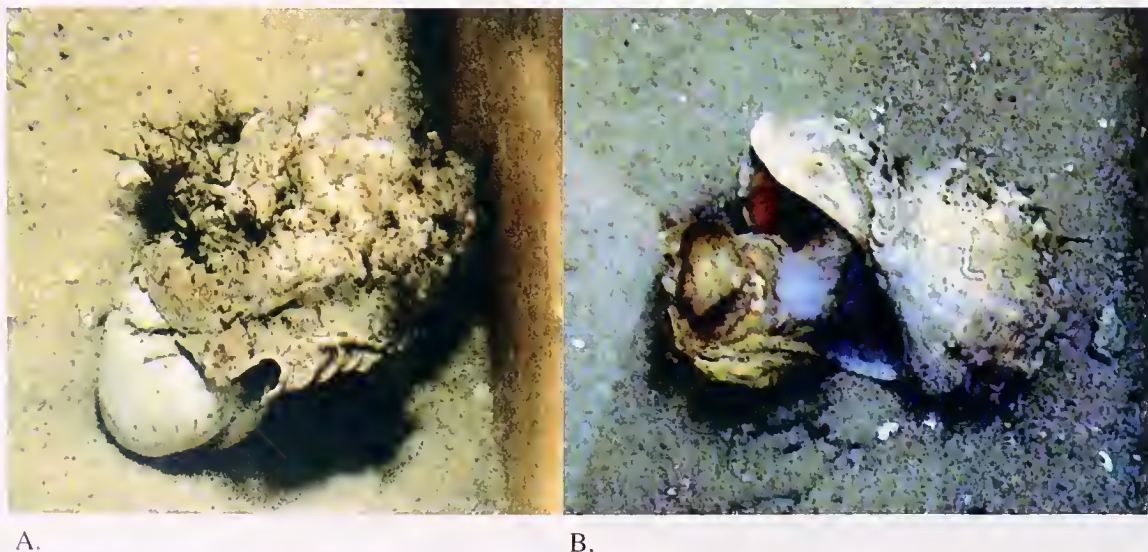


Figure 7. Maps of the lower Bay showing sand bottom habitat (A.) and hard clam populations (B.) in black per Roegner and Mann (1991). The Ocean View/Hampton Roads/James River region is indicated by a square in both maps.

of some small clams attacked and eaten by large *R. venosa*. However, Chesapeake Bay *R. venosa* readily open and consume large hard clams (30 to 85 mm SH) leaving no visible signs of either drilling or boring behavior. *R. venosa* grasps its prey along the shell margin and covers the clam with its foot until the clam gapes slightly (Fig. 8). When the clam gapes, the *Rapana* inserts its proboscis between the clam valves and begins feeding. The entire clam is consumed leaving clean, empty, articulated valves with no visible predation signature as the end product. Food is not likely to be a limiting factor for *Rapana venosa* in the Chesapeake Bay. Rapa whelks seem to share habitat preferences with their favored food item; the preferred habitat for both hard clams and Rapa

whelks is sand bottom. The known *Rapana venosa* distribution overlaps regions of moderate to high hard clam densities in the lower bay (Fig. 7).

The absence of a predation signature on large hard clams consumed by Rapa whelks is troubling in light of recent conversations with commercial clambers working in the Ocean View and Hampton Roads area (Fig. 4). The clambers report an increase in the number of empty shell valves caught within the last 1 to 2 years and attribute the increase in empty valves to a corresponding increase in natural clam mortality. Given the number and size of *Rapana venosa* reported from these same areas during 1998 and the absence of a predation signature on large hard clams consumed



A.

B.

Figure 8. Adult *Rapana venosa* consuming a hard clam (A.) and an oyster (B.).



in the laboratory, it is possible that the recent increase in empty, articulated shell valves observed by local watermen is attributable to Rapa whelk predation and not natural mortality.

Rapa whelks have also been described as scavengers consuming carrion (Chukhchin 1984, Morton 1994). Laboratory observations indicate that Rapa whelks prefer to capture and kill their own food; they will not feed on carrion in the presence of live prey. However, Chesapeake Bay *Rapana* have been caught incidentally by recreational fishermen that were using fresh squid as bait.

#### Potential Predators: Rapa Whelks

*Rapana venosa* are prey for native octopods in their native waters. Few of the habitats that *Rapana* have invaded include resident octopods as upper-level predators enabling *Rapana* populations to grow quickly and inflict considerable damage on local shellfish resources; for example, the decimation of the Black Sea oyster population as described by Chukhchin (1984). Within the Chesapeake Bay, the only upper-level or apex predators that might be capable of using Rapa whelks as a food resource are those that currently eat the local whelk species; that is, Channeled whelks (*Busycotypus canaliculatus*) and Knobbed whelks (*Busycon carica*). Crabs and other gastropod species are potential predators for very small *Rapana*. Sea turtles may be capable of eating Rapa whelks <100 mm SL.

Benthic communities in the lower Chesapeake Bay include several crustacean species that may be capable of crushing very small (<30 to 40 mm) Rapa whelks. Blue crabs, mud crabs (e.g., *Eurypanopeus depressus*), and two species of hermit crabs [flat clawed (*Pagurus pollicaris*) and striped (*Clibanarius vittatus*)] are common in lower Bay intertidal and subtidal habitats. Drapkin (1963) suggests that hermit crabs may be able to kill or remove very small Rapa whelks [Although Drapkin (1963) describes habits of *R. bezoar* from the Black Sea, it is now recognized that *R. venosa* (also previously described as *R. thomasiana* and *R. thomasiana thomasiana*) is the only *Rapana* species that has ever been introduced into the Black Sea.] from their shells. Similarly, Magalhaes (1948) describes stone crabs (*Menippe mercenaria*), blue crabs, and hermit crabs as potential predators on *Busycon* sp. from Beaufort, NC. Oyster drills (*Urosalpinx cinerea*) and moon snails (*Neverita duplicata*) may be able to catch and drill small Rapa

whelks in areas where they co-occur. However, neither crushing nor drilling are realistic threats once a Rapa whelk exceeds a certain size, given the thickness and strength of a *Rapana* shell. Magalhaes (1948) reports old growth shell thicknesses for 160 mm SL specimens of *Busycotypus canaliculatus* and *Busycon carica* as 1.6 mm and 4.5 mm, respectively. Preliminary observations on shell thicknesses for Rapa whelks 145 to 155 mm SL indicate that Rapa whelk shells are twice as thick as Knobbed whelk shells and up to six times thicker than Channeled whelk shells.

Small to medium Rapa whelks (40 to 100 mm SL) may be vulnerable to predation by sea turtles (e.g., loggerhead sea turtle, *Caretta caretta*). Sea turtles in the lower Bay consume local whelk species as indicated by the presence of Knobbed and Channeled whelk opercular plates in sea turtle gut contents. Similar crushing of a Rapa whelk shell would be possible, provided that turtle gape width was sufficient to reach around the shell. Given that *Rapana* have thicker shells (see above), are morphologically more compact or "boxy" than either Knobbed or Channeled whelks (Fig. 9), *Rapana* are probably vulnerable to predation by sea turtles for a shorter temporal window than local whelks. The maximum necessary "bite" or gape shell dimension on a Channeled (150 mm SL) or Knobbed whelk (165 mm SL) is less than the same dimension on a 140 mm SL *Rapana* (Fig. 9). Both local whelk shells have numerous locations that are vulnerable to turtle predation, because the bite or gape width is smaller than the maximum dimension; whereas the *Rapana* profile is essentially square, with all dimensions greater than the maximum bite dimension of a local whelk (Fig. 9). Chesapeake Bay *Rapana venosa* probably reach a size-selective predation refuge from all potential predators at a reasonably small size (e.g., 100 mm) because of their shell morphology and thickness.

#### Potential Predators: Egg Masses

Female *Rapana venosa* seasonally produce mats or masses of individual egg cases that are 30 to 40 mm tall and basally attached to firm substrate (Fig. 3). The egg cases are <3 mm in diameter at the dorsal or basal end and taper to a wide, phyllopodus-looking top or ventral surface with an anterior opening or pore through which the veliger larvae emerge (Chung et al. 1993, Mann and Harding, in review; Fig. 3). Immediately after deposition, indi-

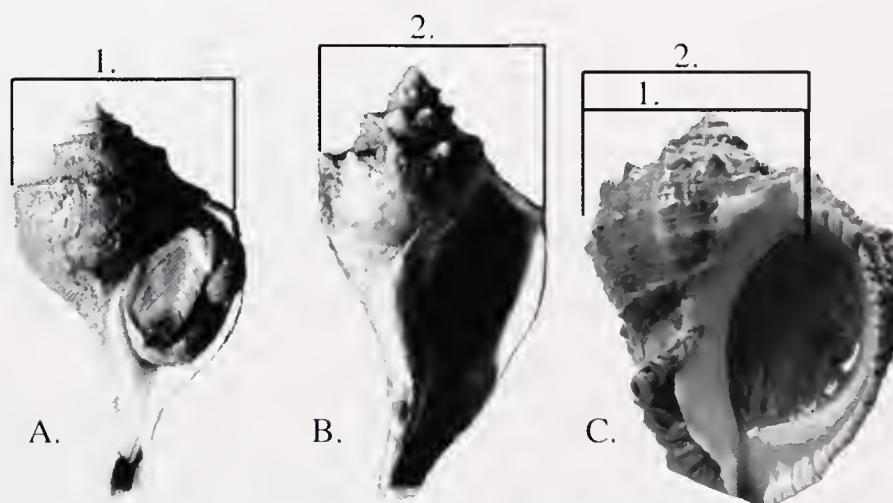


Figure 9. Profiles of Channeled (A.), Knobbed (B.) and Rapa (C.) whelks showing the maximum horizontal or "bite" dimension for a 150 mm SL Channeled (1.) and a 165 mm SL Knobbed (2.) whelk in relation to the same dimension of a 150 mm SL Rapa whelk.

vidual egg cases are lemon yellow. During development (12 to 17 day per Chung et al. 1993), egg cases change from lemon yellow to pale gray, then black, and finally deep purple when the egg case is dying. The gray-black color shift occurs as the shells of the individual veligers within develop before hatching (Mann and Harding in review). Hatching usually occurs during the black color phase but may occur successfully up until the completion of the black-to-purple color transition (Mann and Harding in review).

The egg cases that were collected from Hampton Roads, Virginia in August 1998 (see above) were attached to a hydroid mat. Commercial watermen and divers have observed egg cases attached to bridge pilings and commercial crab pots deployed in the Hampton Roads/Ocean View area (Fig. 4). Freshly laid egg cases, with their lemon yellow color and three-dimensional extension above the substrate, are unlike any native egg cases or organisms. The combination of egg case morphology and coloration may attract benthic feeding fishes that are seasonally abundant in the lower Chesapeake Bay including Atlantic croaker (*Micropogonias undulatus*), spot (*Leiostomus xanthurus*), white perch (*Bairdiella chrysoura*), and yearling striped bass (*Morone saxatilis*). These fishes are probably capable of consuming whole egg cases or at least dislodging them from the mat and damaging them. Local crab species (blue crabs, mud crabs, hermit crabs) and cownose rays (*Rhinoptera bonasus*) may also disturb or damage egg cases while feeding, accidentally or otherwise. Damage to an egg case may be as lethal to the enclosed veliger larvae as complete consumption, because successful veliger release depends upon a functional egg pore (Fig. 3). If the egg pore is damaged or blocked, the larvae have no other exit route and will suffocate as the egg case dies.

#### *Effects of Rapana venosa shells in the Chesapeake Bay*

The presence of a novel large gastropod in the lower Chesapeake Bay provides a new supply of shells for species of local hermit crabs; for example, flat-clawed (*Pagurus pollicaris*) and striped (*Clibanarius vittatus*) hermit crabs. Striped hermit crabs from the Lafayette River have been collected in Rapa whelk shells ranging from 80 to 110 mm SL. These striped hermit crabs are large and will readily consume oyster spat, mussels, mud crabs, and blue crabs (<50 mm carapace width) in laboratory experiments. [We have collected four live striped hermit crabs living in *Rapana* shells. One crab died postcollection and has been measured; it has a shield length of 13.7 mm. The other three are still alive and removing them from their shells to measure shield lengths is impossible without killing the animals. Chelae measurements (from the tip of the chela to the joint) on all four crabs are in the range of 12 to 13 mm.]

The particular morphology of Rapa whelk shells may make them more attractive to striped hermit crabs than to flat clawed hermit crabs. Of the 71 hermit crabs collected thus far from the lower Chesapeake Bay, 93% have been flat-clawed. Flat-clawed hermit crabs have been collected in Channeled whelk (13%), Knobbed whelk (61%), and moon snail shells (26%) ranging from

50 to 190 mm SL (whelks) or 30 to 50 mm shell diameter (maximum dimension across the shell; moon snails). All of the striped hermit crabs observed thus far have been collected in Rapa whelk shells. Previous studies have shown that large shells may be a limiting resource for hermit crabs (Bach 1976, Lively 1989, Lancaster 1990, Borwn 1992). Rapa whelk shells are thicker, have taller spires and are less elongate than local whelk or moon snail shells (see above). The favorable characteristics of larger shells in relation to hermit crab occupancy that were summarized by Brown (1992); that is, larger shells are less vulnerable to predators, increase female clutch sizes, and allow further growth may enhance the success of local striped hermit crabs. Similar increases in hermit crab size subsequent to the introduction of *Rapana* have been reported from the Black Sea by Drapkin (1963).

#### SUMMARY

Although current distribution estimates of *Rapana venosa* in the Chesapeake Bay are biased by both fishing effort and gear, it is clear that a substantial population is present in the lower Bay. The post-World War II invasions of the Black, Adriatic, and Aegean Seas as well as the Sea of Azov by this animal indicate that it is capable of significantly affecting local shellfish and benthic communities in relatively short periods of time (e.g., Drapkin 1963, Bombace et al. 1994, Zolotarev 1996). Given that the lower Chesapeake Bay supports both hard clam and oyster fisheries, the presence of an animal credited with the "destruction of the Gudauta oyster bank" per Drapkin (1963) has significant economic and ecological ramifications. Continuing research on the basic biology of this animal in the Chesapeake Bay in combination with a fishery-independent stock assessment program for the Chesapeake Bay *Rapana* (both in progress at VIMS) will provide necessary information for successful management and control strategies.

#### ACKNOWLEDGMENTS

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## MOLLUSCAN AQUACULTURE IN CHINA

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**ABSTRACT** Molluscan aquaculture in China has been growing rapidly in the past decade. China produced 6.4 million metric tons (MMT) of mollusks from aquaculture in 1996, an eightfold increase over that of 1986. At least 32 species of marine mollusks are cultured commercially in China. The 1996 production included 2.3 MMT of oysters, 1.6 MMT of clams (mostly *Ruditapes*, *Meretrix*, razor clams, and blood cockles), 1 MMT of scallops, 0.4 MMT of mussels, 700 tons of abalone, and 20 tons of marine pearls. Shandong province is the largest producer of cultured mollusks, followed by Guangdong, Fujian, Liaoning, Guangxi, and Zhejiang provinces (ranked 2–6, respectively). As a generalized pattern, molluscan aquaculture in China is characterized by scallops, abalone, and Manila clams in the northern provinces (Shandong and Liaoning), oysters and pearl oysters in the south (Fujian, Guangdong, and Guangxi), and various clam species in the middle (Zhejiang and Jiangsu). The production technology ranges from simple gathering and stocking of wild seeds for several clam species to sophisticated hatchery and growout operations for abalone and pearl oysters. The rapid development of intensive mariculture during the past decade may have exceeded the carrying capacity of some areas and contributed to deterioration of the culture environment. Abalone and scallop cultures in the north have been seriously affected by diseases and mortalities in recent years.

**KEY WORDS:** China, aquaculture, mariculture, molluscan aquaculture, hatchery, oyster, scallop, mussel, clam, razor clam, blood cockle, pearl oyster, abalone, disease, environment

### INTRODUCTION

China has a long history of aquaculture, and its aquaculture industry is the world's largest, accounting for 63% of the world total in 1995 (FAO 1997). Thus, aquaculture is an important industry for China. Because of its large population and limited farmland, China has been resolutely promoting the utilization of aquatic resources. Several traditional capture fisheries declined because of overfishing during the 1970s and 1980s, which led to the recognition of aquaculture as the point of growth in aquatic food production. Chinese aquaculture production surpassed wild fisheries in 1988 (Fig. 1A). In 1996, aquaculture production reached 18.6 million tons, which is 31% higher than production from wild fisheries (MAC 1997).

Freshwater finfish culture, which dates back thousands of years, has been the traditional form of aquaculture, and it remains the most important form of aquaculture in China today. China also has a long history of mariculture, particularly of mollusks. For example, historical records show that oysters were cultured in China over 2,000 years ago. Blood cockles were cultured in the Zhejiang area over 1,700 years ago. Razor clams and *Ruditapes* clams are the other two major mollusks that have been traditionally farmed in China.

Modern mariculture in China started in the 1950s. The first major development was seaweed culture during 1950s, prompted by breakthroughs in breeding technology. By the end of the 1970s, annual seaweed production reached 250,000 metric tons in dry weight (approximately 1.5 million tons of fresh seaweed). Shrimp culture developed during the 1980s because of advances in hatch-

ery technology and economic reform policies. Annual shrimp production reached 210,000 tons in 1992. Disease outbreaks since 1993, however, have reduced shrimp production by about two-thirds. Mariculture production increased steadily between 1954 and 1985, but has been exponential since 1986, mostly driven by molluscan culture (Fig. 1B).

Molluscan culture in China began to expand beyond the four traditional species (oyster, cockle, razor clam, and *Ruditapes* clam) in the 1970s. Mussel culture was the first new industry to emerge, followed by scallop aquaculture in the 1980s. Abalone culture has become a major industry in recent years. Traditional oyster and clam cultures have also advanced and expanded during the last decade. The destruction of shrimp culture by diseases in recent years has led to intensified efforts in molluscan culture. Because of the rapid development in recent years, molluscan culture has become the largest sector of the Chinese mariculture industry, accounting for 84% of total production in weight. The rapid development of the Chinese molluscan aquaculture industry has not been well documented, and misinformation is common in the literature outside China. This paper presents a current overview of molluscan aquaculture in China, which should be useful to the aquaculture community. This review is primarily based on findings during two month-long visits, literature reviews, and personal knowledge of the authors, and is limited to distribution and general culture practices for major mollusks.

### DATA COLLECTION

In September 1996, the first two authors were invited by the Chinese State Bureau of Foreign Experts to advise on molluscan aquaculture, diseases, and broodstock management. The visit

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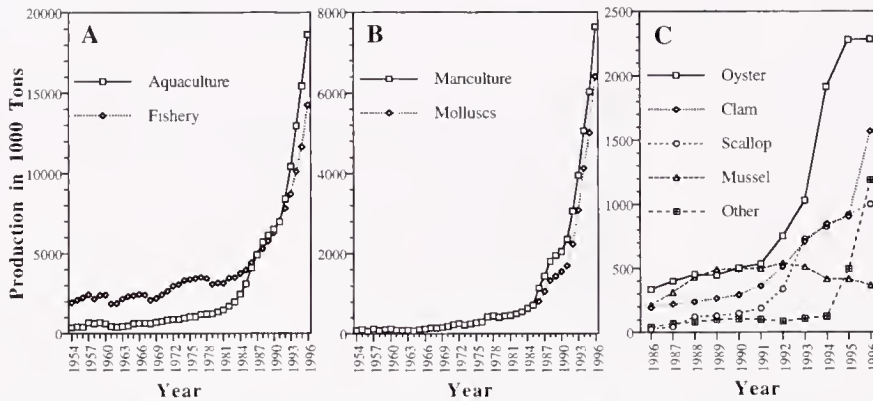


Figure 1. Production trends in China (MAC 1985–97). A, aquaculture versus capture fishery; B, total mariculture production versus molluscan aquaculture; and C, aquaculture production of major molluscan groups from 1986 to 1996. Productions are in wet, whole body weight.

lasted for 4 weeks and covered much of the central coast of China, from Qingdao in Shandong province to Wenzhou in Zhejiang province (Fig. 2). It consisted of on-site visits to aquaculture facilities and discussions with local scientists, managers, and farmers. Major aquaculture sites we visited included those at and around Qingdao, Rizhao, Ganyu (Jiangsu), Lianyungang, Dafeng (Jiangsu), Qidong (Jiangsu), Wenzhou, and Yueqingjiang. In October 1997, the first author visited the north and south coasts of China, which were not covered by our 1996 visit. The 1997 visit was part of a study on the Chinese molluscan aquaculture industry

conducted by the first and third authors under sponsorship of the US–China Living Marine Resources Exchange Program. The 1997 visit covered major aquaculture facilities and research institutions in Liaoning and Shandong provinces in the north, and Fujian, Guangdong and Guangxi provinces in the south. Cities and areas covered by the 1997 visit included Dalian, Yantai, Rongcheng, Qingdao, Wenzhou, Xiamen, Guangzhou, Shenzhen (bordering Hong Kong), and Beihai.

Production figures cited in this paper are mostly from official statistics published by the Ministry of Agriculture of China (MAC 1986–1997). Collecting accurate production data for molluscan aquaculture is always challenging, which is particularly true in China for several reasons. First, production of most molluscan species in China is estimated by multiplying the total culture area by an average yield per unit area. This method alone contributes a large degree of uncertainty. Second, the culture of a large number of species in various forms and across culturally diverse regions is a source of confusion and error. For example, the word “clam” (Ge in Chinese) may include different species in different areas. Oyster production from south China is traditionally reported in meat weights and that from the north in whole weight (with shells). Certain types of culture practices may be considered as aquaculture in one area, but not in another. Finally, production may be over- or underestimated by local officials for management and/or political reasons. Despite all these factors, the official figures are still the best or only available statistics. Some Chinese scientists and managers believe that the official statistics may overestimate the overall production by 20 to 30%. For some species, such as blood cockles and razor clams, the official statistics are very close to estimates from local scientists. The decline of the shrimp aquaculture industry because of diseases is reflected in the official statistics, which corresponds well to expert estimates. (We should see a decline in scallop production, because of mortalities, in 1997 to 1998 if the official statistics are accurate.) Starting in 1996, a new reporting system, or standard, was implemented, which corrected some problems. For example, oyster and some clam production before 1996 were reported as meat weight, and the new statistics converted all molluscan production to whole body weights. For this report, oyster production data prior to 1996 were converted to whole body weight using a factor of 6.11, as recommended by the Ministry of Agriculture of China (MAC 1997).

Scientific names are presented for all species discussed in this paper. The English common names, if available and generally accepted, are also used. For species with no common English



Figure 2. A map of China's coastal provinces showing major cities and areas visited during this study.

names or conflicting ones, the Chinese common name is given in standard pinyin to avoid confusion with different translations.

### OVERVIEW OF PRODUCTION

Mollusks are cultured all along China's 18,000-km coastline. The scope of molluscan aquaculture is reflected by the large number of species cultured. A quick survey indicates that at least 32 species of marine mollusks are cultured commercially in China (Table 1), and the list is probably incomplete and growing. The list contains five gastropods and 27 bivalves. The bivalve species include three oysters, four scallops, five mussels, one pearl oyster, and 14 clams. The list in Table 1 is arbitrarily divided into "major" and "minor" species. Most of the 14 major species are well-known mollusks that support large aquaculture industries in China. The minor species are also cultured at commercial scales, but with less significant production.

In 1996, China produced 6.4 million metric tons of mollusks (whole body wet weight) from aquaculture, which is about eight times that of 1986 (Table 2). Oysters topped the species list, with production of 2.3 million tons, or more than one-third of the total. Clam production ranked second, with 1.6 million tons. The actual clam figure may be much higher, because some clam species from certain areas are reported under "others mollusks." Most of the 1.2 million tons of "other" mollusks listed in Table 2 are probably

TABLE 2.  
Aquaculture production (whole weight in metric tons) of mollusks in China: 1986 vs. 1996.<sup>a</sup>

Species Group	1986	1996
Oyster	336,013	2,284,663
Clam	191,951	1,568,325
Scallop	23,686	999,573
Mussel	210,657	366,251
Abalone	—	700
Pearl Oyster	—	10,000 <sup>b</sup>
Others	41,592	1,187,083
Total	803,899	6,416,595

<sup>a</sup> From MAC (1987, 1997) Oyster production in 1986 was converted to whole weight using a factor of 6.11. Clam statistics include razor clam and cockle species.

<sup>b</sup> Estimated from the production of 20 tons of pearls.

miscellaneous clams, and the total clam production from aquaculture could be anywhere between 1.6 to 2.5 million tons. Scallops ranked third, with a production of 1 million tons. Abalone production was negligible in weight but significant in value. An estimated 20 tons of marine pearls was produced, corresponding to about 200 million pearl oysters or 10,000 tons in whole weight.

TABLE 1.  
A list of marine mollusks cultured in China.

English Name	Scientific Name	Major Culture Areas, Notes
<b>Major species</b>		
Zhe oyster	<i>Crassostrea plicatula</i> Gmelin	Fujian, most important oyster, wild seeds
Suminoe oyster	<i>Crassostrea rivularis</i> Gould	Guangdong, Fujian, low salinity, wild seeds
Pacific oyster	<i>Crassostrea gigas</i> Thunberg	Liaoning, Shandong, hatchery seeds, longlines
Manila clam	<i>Ruditapes philippinarum</i> Adams and Reeve	All coast, most common clam, mostly wild seeds
Colorful clam	<i>Ruditapes variegata</i> Sowerby	Fujian, Guangxi, some hatchery seeds
Meretrix clam	<i>Meretrix meretrix</i> Linnaeus	Jiangsu, Shandong, extensive culture
Mud cockle	<i>Tegillarca granosa</i> Linnaeus	Zhejiang to Guangdong, high-value, some hatchery
Razor clam	<i>Sinonovacula constricta</i> Lamarck	Zhejiang, Fujian, wild seeds, intertidal flats
Zhikong scallop	<i>Chlamys farreri</i> Jones and Preston	Shandong, Liaoning, wild seeds, cage culture
Bay scallop	<i>Argopecten irradians</i> Lamarck	Shandong, Liaoning, Fujian, hatchery seeds, cage
Wrinkled abalone	<i>Haliotis discus hannai</i> Ino	Liaoning, Shandong, hatchery seeds, cage, raceway
Colorful abalone	<i>Haliotis diversicolor</i> Reeve	Guangdong, Fujian, hatchery seeds, cage culture
Pearl oyster	<i>Pinctada martensii</i> Dunker	Guangdong, Guangxi, hatchery seeds, cage culture
Blue mussel	<i>Mytilus edulis</i> Linnaeus	Shandong, Liaoning, wild seeds, longline culture
<b>Minor species</b>		
Huagui scallop	<i>Chlamys nobilis</i> Reeve	Guangdong, Fujian
Japanese scallop	<i>Patinopecten yessoensis</i> Jay	Liaoning
Thick-shell mussel	<i>Mytilus coruscus</i> Gold	Zhejiang, Fujian
Green-jade mussel	<i>Perna viridis</i> Linnaeus	Fujian, Guangdong
Senhouse mussel	<i>Musculus senhousei</i> Benson	Fujian, Guangdong
Penshell	<i>Pinna pectinata</i> Linnaeus	Zhejiang, Fujian
Xishishe surfclam	<i>Macra antiquata</i> Spengler	Shandong, Jiangsu, Fujian
Square surfclam	<i>Macra veneriformis</i> Reeve	Jiangsu, Liaoning
Chinese surfclam	<i>Macra chinensis</i> Philippi	Jiangsu
Cyclina clam	<i>Cyclina sinensis</i> Gmelin	Jiangsu, salt
Chinese glaucomya	<i>Glaucomya chinensis</i> Gray	Guangdong, Fujian
River corbula	<i>Potamocorbula rubromuscula</i> Zhuang and Cai	Jiangsu
Morella clam	<i>Moerella iridescens</i> Benson	Zhejiang
Hairy cockle	<i>Scapharca subcrenata</i> Lischke	Hebei, Liaoning, Shandong
Giant cockle	<i>Scapharca broughtonii</i> Schrenck	Hebei, Liaoning, Shandong
Mud snail	<i>Bullacta exarata</i> Philippi	Zhejiang
Red conch	<i>Rapana venosa</i> Valenciennes	Liaoning, Shandong
Sea hare	<i>Notarchus leachii cirrosus</i> Stimpson	Fujian

With the exception of mussels, production in all species groups increased several fold over the 10-year period between 1986 and 1996 (Fig. 1C). Production statistics before 1986 did not separate species groups, so it is impossible to make comparisons with earlier years. Economic reform and government promotion are probably the dominant factors behind the rapid growth. Mussel production has been declining since 1992 probably because of competition from high-value species such as scallops, oysters, and abalone.

Molluscan aquaculture is widely distributed among the Chinese coastal provinces (Table 3). Shandong province in the north, with a production of 2.1 million tons, or one-third of the national total, is the largest producer of cultured mollusks and leads other provinces in scallop, clam, and mussel culture. Yantai, Rongcheng, and Rizhao are the major mariculture areas in Shandong. Liaoning is the leader in abalone aquaculture and also contributes significantly to scallop and mussel production. Jiangsu province cultures a variety of clam species and little else. Zhejiang province leads the nation in cockle and razor clam culture. Fujian, Guangdong, and Guangxi are major oyster culture provinces. Fujian is also a major producer of clams, especially razor clams. Guangdong and Guangxi are the dominant producers of pearl oysters. Hebei and Hainan are relatively poor in coastal resources and contribute little to molluscan production. As a generalized pattern, the Chinese molluscan aquaculture can be characterized, with some exceptions, by the scallop, Manila clam, and abalone culture in the north, oyster and pearl oyster culture in the south, and a variety of clams in the middle.

We could not find any statistics on the trading of cultured mollusks. Most of the abalone facilities we visited ship a large portion of their product to markets in Hong Kong and Japan. A significant portion of cultured scallops is also exported. Some Meretrix and Manila clams are exported to Japan, and some oysters are sold to Hong Kong. Most of the cultured mussel, razor clams, Ruditapes clams and blood cockles, oysters, and scallops are consumed in China. China also imports some mollusks, such as geoduck and squid from North America, green mussel from New Zealand, and blood cockle from Korea. A hotel in Wenzhou, where we stayed in 1996, was selling geoducks from Canada at US\$33/kg. We were told by sources that China has become a net seafood importer in recent years, which may be partly because of the large quantities of fish and fishmeal imported for processing.

## OYSTERS

China produced over 2.3 million tons of cultured oysters in 1996. By weight, oysters are the largest molluscan group cultured in China, and most of the output comes from three species. The most important species is the zhe oyster, *Crassostrea plicatula* Gmelin. Official statistics do not distinguish individual oyster species, but experts estimate that the zhe oyster accounts for 50–60% of the total oyster production. The second largest production comes from the Suminoe oyster, *C. rivularis* Gould (or *C. ariakensis* Fujita), which accounts for 20–30% of the total. The other major species is the Pacific oyster, *Crassostrea gigas* Thunberg, which may account for 10–20% of the national production.

China has about 20 recorded species of oysters occurring along its coast, and the classification is sometimes problematic. There are three points of uncertainty concerning aquaculture species. First, oyster farmers in southern China recognize two forms of oysters traditionally regarded as *C. rivularis*. One is called the "white" oyster, and the other is called the "red" oyster (referring to the meat color). Some experts now believe that the "white" oyster is actually true *C. rivularis*, and the "red" oyster may be *C. iredalei* (Li et al. 1988). The two oysters are usually present in the same area at variable proportions. Another uncertainty is about the species status of the Dalianwan oyster, *C. talienwhanensis* Crosse, which is cultured in the Dalian area. Some people believe that the Dalianwan oyster is actually a variety of *C. gigas*. Also, people disagree on whether the monk-hat oyster, *C. cucullata*, is synonymous with the zhe oyster (*C. plicatula* Gmelin), and both names are used in the literature.

### Zhe Oyster

The zhe oyster is found along the entire coast of China. It is small as compared with the Suminoe and Pacific oysters, and thin-shelled. The left shell is deeply cupped, similar to that of Kumamoto oyster (*C. sikamea*), but even more pronounced (Fig. 3A). The right shell is flat and covered with radial plates. The zhe oyster grows rapidly during the first year, after which shell growth usually stops.

Zhe oysters are cultured primarily in Fujian province and other parts of the southern coast. In Fujian alone, 23,000 hectares are used for oyster culture, 80% of which are for zhe oysters. Traditionally, the zhe oyster is cultured on stone pilings, vertical stone

TABLE 3.

Aquaculture production of major molluscan groups (whole weight in metric tons) from nine coastal provinces of China.<sup>a</sup>

Province	Total	Oyster	Clam <sup>b</sup>	Scallop	Mussel	Razor	Cockle	Other
Liaoning	871,657	95,969	224,787	223,286	104,888	7,012	1,119	214,596
Hebei	51,568	—	31,591	8,882	1,900	—	9,195	8,493
Shandong	2,144,166	338,209	481,974	753,902	140,983	39,997	31,356	357,745
Jiangsu	122,012	—	105,820	—	—	3,338	4,361	8,493
Zhejiang	357,861	62,459	19,121	877	11,826	189,521	56,288	17,769
Fujian	1,138,226	804,845	106,605	12,325	64,281	102,651	3,363	44,156
Guangdong	1,204,212	558,950	39,534	130	40,352	—	20,222	545,024
Guangxi	511,885	422,555	82,765	15	2,021	—	4,529	—
Hainan	5,008	1,676	1,751	156	—	—	1,425	—
Total	6,406,595	2,284,663	1,093,948	999,573	366,251	342,519	131,858	1,187,783

<sup>a</sup> From MAC (1997).

<sup>b</sup> Clam here refers to Ruditapes clams for some provinces and may include meretrix and other clams (excluding razor clams and blood cockles) for other provinces.



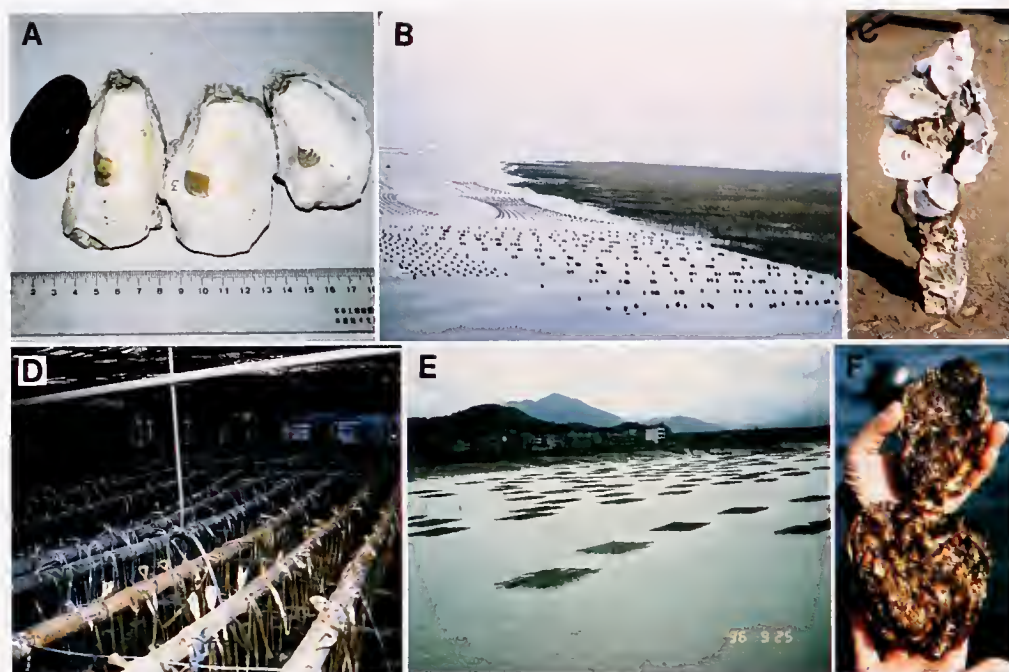


Figure 3. Oyster culture. A, The deeply cupped left shells of the zhe oyster cultured in Fujian and Guangdong. B, intertidal racks and stakes and suspended longlines used for the culture of zhe oyster in Xiamen. C, Suminoe oysters cultured on cement stakes in Beihai area. D, a hatchery in Wenzhou area where Pacific oyster spat are being set on shell-strings. The large concrete tanks (20–50 m<sup>3</sup>) are typical for all hatcheries and are used for larval culture of a variety of species. E, bamboo rafts used to culture Pacific oysters on shell-strings in Yoeqingjiang, Wenzhou. F, Pacific oysters cultured in Dalian reach 8–10 cm in 6 months.

strips (over 1-m tall), and bamboo or wooden stakes. These materials are arranged in a variety of configurations and formations in intertidal areas (Cai and Li 1990), and oyster-growing areas filled with these structures cover miles of coastline (Fig. 3B). Culture of zhe oysters depends entirely on natural seeds. Large stone strips are permanent structures used for both seed collection and grow-out. Often, seeds are collected on stones, shells, bamboo, and cement blocks in one area and moved to another area for culture. Recently, suspended longlines (Fig. 3B) and raft culture of shell strings has gained popularity with farmers, because it results in better growth and allows for utilization of open-water areas. Stone and bamboo racks in traditional oyster fields have been abandoned in some areas because of their lower productivity. Seeds are usually collected in May and September. Seeds collected in May are usually harvested starting in December. Production peaks in February, around the Chinese New Year, and ends in March. The total culture time is under 1 year. For the fall seeds, 14 to 17 months are needed to reach a market size of 6–7 cm.

#### Suminoe Oyster

The Suminoe oyster occurs naturally along most of the Chinese coast, where it is called the Jinjiang (“close-to-river”) oyster in Chinese. It tolerates a wide range of salinity, but prefers low salinity estuaries and riverbeds, especially for settlement. Local oyster farmers recognize two forms of the oyster: the white and red oysters. The former is preferred for its flavor and valued more than the red oyster.

The Suminoe oyster is cultured primarily in two southern provinces, Guangdong and Guangxi. Culture methods are similar to those used for the zhe oyster. At the Guangxi site we visited,

concrete stakes, 50-cm long and 6 × 6 cm at the cross section, are used for both spat collection and growout (Fig. 3C). The stakes are transported to upper river sites for spat collection in the spring. After the spat set, the stakes are planted along the lower river beds for growout. A dozen oysters per stake are considered optimal. About 30,000 stakes are planted per hectare, which produce about 12,000 pounds of oyster meat at harvest. At low tide, the river beds are covered with a forest of oyster-carrying stakes. Suminoe oysters are also cultured on shell strings hanging on rafts and longlines. Unlike the zhe oyster, which usually stops shell growth after the first year, the Suminoe oyster maintains rapid growth throughout the first 3 years. Oysters are usually harvested in 2 to 3 years at a size of 10–15 cm. In some areas, oysters are moved to productive areas for fattening before being harvested.

#### Pacific Oyster

Pacific oysters occur naturally along the Chinese coast. However, most of the Pacific oysters being cultured in China were originally introduced from Japan. This species is cultured in all parts of the coast, but the major producers are Liaoning and Shandong provinces in the north, and Guangdong in the south.

Pacific oyster culture depends exclusively on hatchery-produced seeds (Fig. 3D). As with most other bivalves, larvae are cultured in large concrete tanks (10–100 m<sup>3</sup>). Vitamin supplements and antibiotics are often used during larvae culture to maximize yields. Spat are set on strings of scallop or oyster shells. The recommended density is about 20 to 30 spat per shell, but, in practice, the density is often two to three times that. Farmers may break the shell in half for growout if the density is high. Spatted shells sell for about US\$0.01–0.02 per piece, depending upon sea-

son. They are inserted into nylon ropes and cultured on suspended longlines (Fig. 3B) or rafts (Fig. 3E). Bottom culture is also practiced in certain areas.

Pacific oysters grow rapidly. Oysters at most sites we visited, including Dalian in the north, reach 8 to 10 cm after the first growing season (Fig. 3F). One factor is that the seeds are produced early in the spring so that they have a full season to grow. Another factor may be the high productivity of the culture sites. Depending upon demand, oysters may be harvested within the first year. Oysters cultured in the intertidal areas may need 2 to 2.5 years to reach market size. Triploid Pacific oysters, which undergo little or no gametogenesis, are used for production in Shandong and Liaoning provinces because of their superior growth and improved survival against "summer mortality," a syndrome linked to reproduction (Perdue et al. 1981).

Some oysters are sold live on local markets or to restaurants. Vendors in the market would shuck the oysters if requested. Oysters are cooked in a variety of recipes, but rarely consumed raw in China. In southern China, most oysters are cooked and dried for storage, and the broth from processing is condensed into oyster sauce, a popular seasoning in Chinese cooking.

#### CLAMS

At least 14 species of clams are cultured in China (Table 1). The major species include the Manila clam (*Ruditapes philippinarum* Adams and Reeve), the colorful clam (*R. variegata* Sowerby), the Meretrix clam (*Meretrix meretrix* Linnaeus), the mud cockle (*Tegillarca granosa* Linnaeus), and the constricted razor clam (*Sinonovacula constricta* Lamarck). In addition to the five major species, at least nine others are commercially cultured in

China (see Table 1). The official figure for clam production in 1996 is 1.6 million tons, but the actual figure may be higher, because some clam species may not be listed as clams in official figures. On the other hand, clam production listed as from aquaculture may not be cultured in a strict sense. Culture of most clams, such as razor clams, cockles, and most of the *Ruditapes* clams, involves seed collection or hatchery production, nursery, and planting, and can be clearly classified as aquaculture. In some areas, however, culture of *Meretrix* and some other clams may simply mean protective enhancement of natural resources. The culture fields are protected from predators and poachers, but no seed collection and planting are involved. In some cases, a mixture of culture and wild harvest is practiced. Seeds of one species are planted, but multiple species are harvested from the same planting ground. All considered, China's clam production from aquaculture may be anywhere between 1.6 and 2.5 million tons.

#### *Ruditapes* Clams

*Ruditapes* clams are the most widely cultured clams in China and account for probably 60–70% of the total clam production. Two species predominate: the Manila clam, *R. philippinarum* Adams and Reeve and the colorful clam *R. variegata* Sowerby (Fig. 4A). The two are often not distinguished in China, and both are referred to as "Gezi" in Chinese. The Manila clam is cultured along most of the coast and accounts for most of the production. The colorful clam is cultured in the southern provinces.

Most seeds are collected from the wild, with Shandong and Fujian provinces being the major producers. Seed collection involves selection and building of seed-collection beds, eradication of predators, and routine maintenance. In southern China, clam

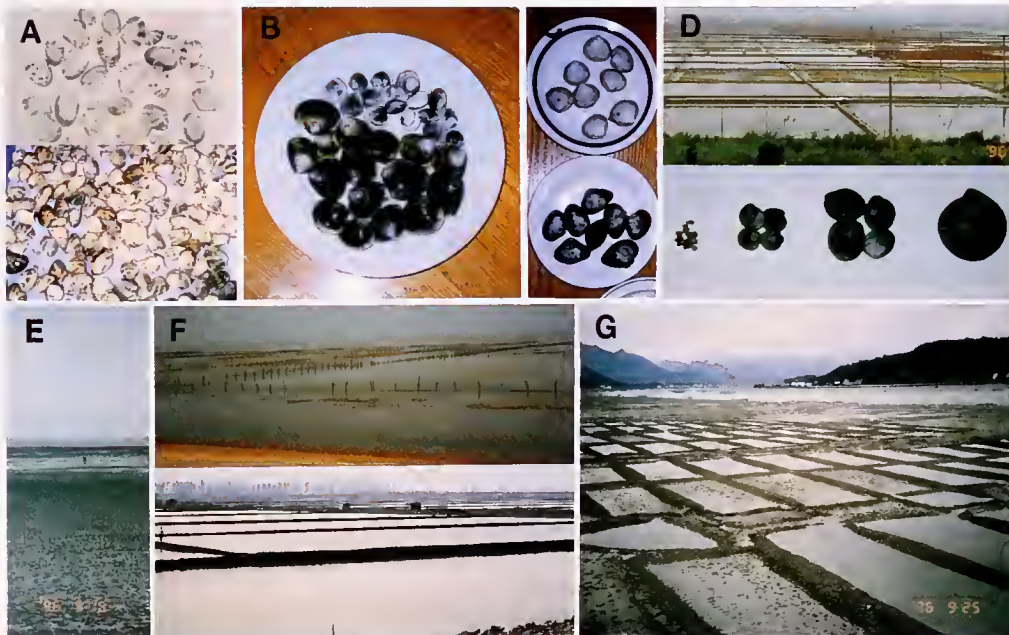


Figure 4. Clam culture. A, Manila (upper) cultured in Jiangsu and the colorful *Ruditapes* clam (lower) cultured in Fujian. B, the cyclina (upper) and meretrix (lower) clams cultured in Daxing, Jiangsu; the dark coloration is characteristic of clams cultured in shrimp ponds. C, the square surfclam (upper) and hairy cockle (lower) cultured in Ganyu, Jiangsu. D, salt ponds (upper) in Qidong used for the production of cyclina clam seeds (lower), which usually reach a commercial size of 1 cm in a year. E, unprotected clam beds in Qidong used to culture meretrix, cyclina clams, square, and xishishe surfclams. F, net-fenced clam plots (upper) in Ganyu, Jiangsu, used to culture various clam species; large shrimp ponds (lower) in Wenzhou area, used for polyculture of shrimp, mud cockle and other clams. G, heds for the collection of the constricted razor clam seeds in Wenzhou area; the heds should be completely drainable during collecting season.

seeds are also produced from hatchery production. Clam seeds, 5 to 10-mm in size, are planted at a density of about 35 million per hectare, but density may vary, depending upon the size of seeds and sediment type. In most cases, clam beds are not protected with nets. Clams are harvested at a size of 30 mm or larger, which is usually reached in 10 to 16 months.

Manila clams are one of the most common seafoods in coastal regions of China. Most clams are sold live in local markets, for about US\$0.5/kg. They are either stir-fried or used in soup with shells on. Ruditapes clams are usually not depurated before reaching the local market. "De-sanding" by placing clams in saltwater for an hour or two is usually the first step in the kitchen. Now there is a frozen product that uses depurated clams, vacuum-packed and frozen in a microwave oven-ready plastic bag. Some frozen clams and clam meat are sold to Japan.

#### Meretrix and Other Clams in Jiangsu

The meretrix clam (Fig. 4B) is found in most parts of China's coast. It resembles the hard clam (*Mercenaria mercenaria*) in biology. It prefers sandy substrates and Jiangsu province, which has a long sandy coast, is the major producer of meretrix clams. Several other minor clam species are also cultured in Jiangsu using the same culture system as for meretrix clams, including the cyclina clam (*Cyclina sinensis* Gmelin) (Fig. 4B), the square surfclam (*Macra veneriformis* Reeve) (Fig. 4C), and the xishishe surfclam (*M. antiquata* Spengler).

Seeds of meretrix and most other clams are collected from the wild. Most cyclina clam seeds are artificially produced in earth ponds (Fig. 4D). Earth pond seed production is a low-tech and low-cost substitute for hatcheries and is effective for certain mollusks, including the Ruditapes, the cyclina clam, razor clam, and oysters. Large earth ponds, usually 1–3 hectares in area and 1-m deep, are dried and treated with bleach or herbal poisons before filling with filtered seawater (to 0.1 mm). Brood stocks are induced to spawn either in the pond or inside the hatchery. A density of 1–4 D-stage larvae/mL is desired. Ponds are fertilized to boost algae growth. No water is discharged before larvae are completely settled. Seeds that settle in the ponds may be thinned if the density is too high. About 3,000 seed clams/m<sup>2</sup>, at a commercial size of 10 mm, can be expected from earth pond production by the end of the first year.

The seed clams are mostly planted in unprotected intertidal flats (Fig. 4E), although planting areas are usually treated with herbal pesticides to remove predator species before planting. After the seed stage is passed, predation is not a major problem. Meretrix and surfclams live close to the surface and are capable of moving and relocating, particularly during storms; therefore, some clam beds are protected with net fences to prevent escape (Fig. 4F). In Jiangsu, the same bed may be used for meretrix and other clams. The clam beds are continuously harvested by manually picking out clams that have reached market size. Another major form of culture for meretrix and other clams is in shrimp ponds (Fig. 4F). Because of the shrimp disease problem, most shrimp ponds are now used for other species or polyculture with other species, especially clams. In the polyculture ponds, shrimp are stocked at a low density and harvested at a small size before mortality starts. One or two clam species are selected and planted in the shrimp ponds. One Jiangsu farm we visited grows both meretrix and cyclina clams in their shrimp–clam polyculture system. Each shrimp pond is about 3.3 hectares and produces about 10 tons each of

meretrix clams and shrimp. Because the harvested shrimp are small, clams constitute a significant proportion of the farm's income. Abandoned salt ponds are also used for clam culture. Mortality has been reported for meretrix clams cultured in ponds, which coincides with spawning of the clams in August when the water temperature reaches 30–31°C. The mortality is usually 30–40%, but may reach 80–90%. Growers believe the problem is related to high temperature and poor water quality (e.g., low oxygen and, possibly, high bacterial loads) adding to the "stress" of spawning. Mortality in other clam species is rare.

#### Blood Cockles

Three species of blood cockles are cultured in China: the mud cockle (*Tegillarca granosa* Linnaeus), the hairy cockle (*Scapharca subcrenata* Lischke), and the giant cockle (*Scapharca broughtonii* Schrenck). The mud cockle is the most desired and widely cultured of the three. Total cockle production from aquaculture was about 131,858 tons in 1996, mostly from the mud cockle. The mud cockle is found in muddy sand beaches of the Shandong peninsula and south. Larval settlement is found to be best on substrates that are more on the sandy side, but adult mud cockles usually grow faster on muddy sediments. The mud cockle is a small and slow growing species, usually taking 2 to 3 years to reach a market size of 2.5 cm.

Both wild and hatchery-produced seeds are used in cockle culture. Considerable experience in seed collection and nursery rearing has been accumulated throughout the history of cockle culture (1,700+ years). Cockle seeds, called "cockle-sand" (about 0.5 mm), are usually collected in September and October, using cloth bags or nytex screens. Cockle sand sells for about US\$1,200/kg, but prices vary greatly from year to year. Seeds of the cockle-sand size are usually cultured in nursery beds for a year before reaching the next stage—"cockle-beans" (about 2,000 cockles/kg). Nursery beds are elevated so water can drain off completely at low tide. Predator species are eradicated before planting by applying herbal poisons before spat fall. A layer of fine sand is added to overly muddy substrates. Growout planting density varies greatly among different areas and depending upon seed size, ranges from 150 to 1,500 seeds/m<sup>2</sup> (Wang et al. 1993). Hatchery techniques have been developed to meet the increasing demand for cockle seeds. The Zhejiang Mariculture Institute in Wenzhou we visited is a leader in cockle hatchery technology. Larval culture is the same as for other bivalves, but a unique practice in cockle hatcheries is that mud is added as a substrate to induce settlement. Mud cockle is cultured in intertidal plots and ponds. The yield from cockle ponds is about 7 to 10 tons/hectare.

The mud cockle is a delicacy in the Shanghai and Zhejiang areas. It is sold for about US\$10/kg in Zhejiang area, but imports from South Korea drove the price down by about 30% in 1997. Cockles are prepared by briefly dipping them in boiling water, after which they are consumed semiraw. Because of incomplete cooking and poor sanitation in some areas, cockle consumption, particularly with hairy cockle, has occasionally been associated with Hepatitis infection.

#### Constricted Razor Clam in Zhejiang and Fujian

The constricted razor clam (*Simonovacula constricta* Lamarck) is found in most parts of the Chinese coast. It tolerates wide temperature and salinity ranges and prefers substrates with a muddy top layer and fine sand bottom. This species is primarily

cultured in Zhejiang and Fujian provinces and is probably the most important mollusk for Zhejiang province, where its production is greater than all other mollusks combined. Zhejiang produced 189,521 tons of razor clams in 1996, which represents 55% of the national total. Zhejiang and Fujian together account for 85% of all razor clams produced in China.

Most razor clam seeds are collected from the wild. Hatchery and earth pond production are used when wild seeds are insufficient. Wild seed collection has a long history and well-established protocols. Collection beds are elevated plots with drainage channels so that the beds are completely exposed at low tide (Fig. 4G). Newly settled razor clam spat would move away from areas that are submerged at low tide. Larval settlement occurs in the fall, and seeds (1–2 cm) are harvested 3 month later and planted at a density of 1,000–1,500 kg/hectare for growout. The razor clams typically grow to about 5 cm in 5–8 months after planting (1 year of age). Large clams are harvested at 1 year of age, and small ones are cultured for a second year. Razor clams have several predators, including moon snails, crustaceans, and several fish, especially eels. Herbal pesticides are used to control predators. The razor clam is the first intermediate host of a parasitic worm (*Vesisocellium solenophagum* Tong) and suffers frequent mortalities as a result (DFC 1979; Wang et al. 1993). Several finfish species are the final host of this parasite.

### SCALLOPS

China produced 1 million metric tons of scallops from mariculture in 1996. Most of the production was from two major species: the native zhikong scallop (*Chlamys farreri* Jones and Preston) and the introduced bay scallop (*Argopecten irradians* Lamarck) (Fig. 5A). The native zhikong scallop accounted for about

three-quarters to four-fifths of the total, and the bay scallop accounted for one-fifth of the total or about 200,000 tons annually. There are two other species, *Patinopecten yessoensis* Jay (Fig. 5B) and *Chlamys nobilis* Reeve, being cultured along the coast with little output. *P. yessoensis* was introduced from Japan. Its life history resembles that of the sea scallop, *Placopecten magellanicus*, of the North Atlantic Ocean. It is a low-temperature species and is cultured only in the northern provinces, Liaoning and Shandong. It is larger in size and commands a higher market price than the zhikong and bay scallops, although its yield is low. *C. nobilis* occurs naturally in the South China Sea and southern Japan. It is cultured in southern China on a limited scale.

### Zhikong Scallop

The zhikong scallop is naturally found in north China, Korea, and Japan. It can survive at  $-1.5^{\circ}\text{C}$  but does poorly when the temperature exceeds  $25^{\circ}\text{C}$  (DFC 1979; Wang et al. 1993). Most of the zhikong scallop culture is in Shandong and Liaoning provinces. Rizhao, in southern Shandong, probably represents the southernmost extent of its range. Shandong province is the leading producer, accounting for more than 80% of the national total.

Zhikong scallop culture was first developed, between 1973 and 1983, using hatchery seed. Large-scale culture has led to the establishment of breeding populations in many areas of Shandong. Now zhikong scallop culture uses almost entirely natural seed. Breeding grounds on Shandong peninsula currently provide sufficient seed for the scallop culture industry. One of the most productive bays in Shandong produced about 130 billion scallop seeds in 1996. The zhikong scallop has two natural spawning seasons in most areas, one in the early summer and one in the fall. Protocols for seed collection have been well established through years of

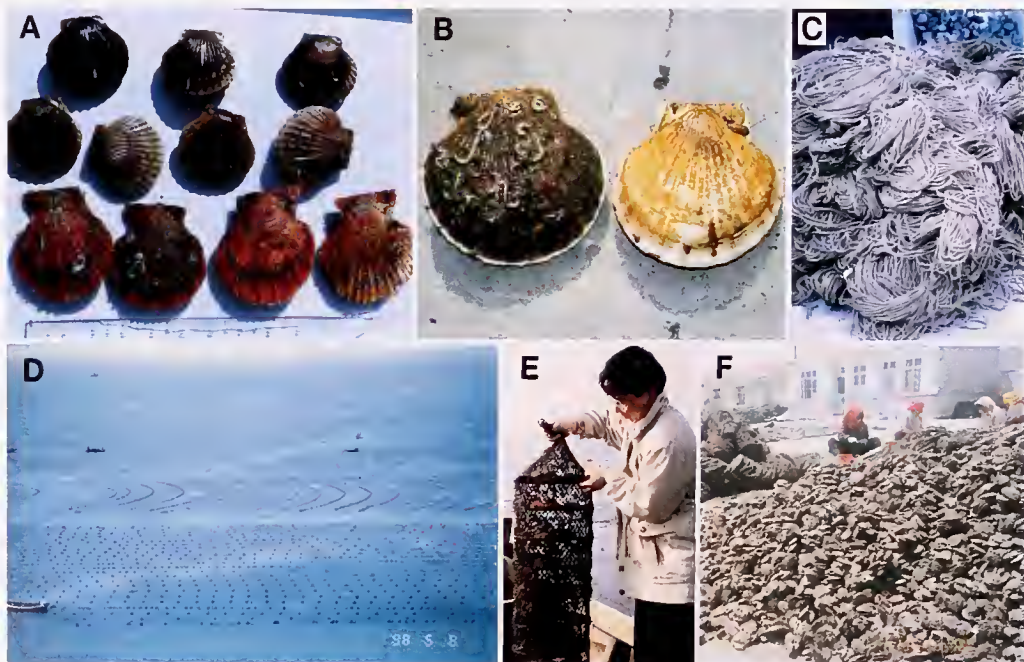


Figure 5. Scallop culture. A, the introduced bay scallop (upper) and the native zhikong scallop (lower) cultured in Shandong (photo by H. Yang). B, the Japanese scallop cultured in Dalian. C, ropes made from palm tree fibers used for the collection of scallop spat and seaweed seedlings in hatcheries. D, suspended longlines used for the culture of scallops, abalone, Pacific oyster, and seaweeds in Rongcheng, covering most of the bay. E, lantern nets used for scallop culture. F, zhikong scallops are harvested at 1.5 years of age (photo by H. Yang).

research and experience (DFC 1979, Wang et al. 1993). Successful collection involves site selection, preparation of collection material, and forecasting collection dates by obtaining data on gonadal development, larval stages, and density at the collection site. Seeds are usually collected using bags (30 × 40 cm) stuffed with nylon screens. Each bag may collect 100 to 1,000 spat, depending upon location, season, and year.

Scallop culture primarily uses summer seeds, which set between late May and early July. Commercial seeds, about 1-cm in size, are harvested and sold in October. They are then put in lantern nets and reared in a nursery area until the following March, when they reach about 3 cm and are ready for growout. Lantern nets on suspended longlines are used for scallop culture (Fig. 5D). The lantern nets are about 35-cm in diameter with 8–10 layers (Fig. 5E). In the nursery, about 200–300 seed scallops are stocked in one layer, or 2,000–3,000 per cage. The growout density is usually 30 to 50 scallops per layer, although higher densities are often used by farmers. One of the scallop farms we visited in Rizhao produces about 2,500 tons of whole zhikong scallops per year. Total annual production in the Rizhao area is about 80,000 tons. Typically, 1-cm scallop seeds are bought from northern Shandong in October. The seeds are maintained in a nursery area at five times growout density until the following March, when the scallops have reached 2.5–3.0 cm. Young scallops are thinned to 50–80 per layer or 400–500 per cage for growout. They usually reach market size, 6–7 cm, by December (Fig. 5F). The lantern nets are hung on longlines, which are usually 80 to 100-m long and supported by rubber floats. The water depth at this site is about 20 m. At the time of our visit (September), the lantern nets were heavily fouled by a variety of organisms.

Scallop culture in Shandong is experiencing a mortality problem. Mortality generally begins in early August as the water temperature reaches and exceeds 28°C. It lasts for about 20 days and ends when the temperature begins to decrease. Mortalities at the Rizhao site are in the 20 to 30% range each year, but they may reach 80% farther north, in sites nearer to where the seeds originate. To the north, the timing of the deaths is similar to that in Rizhao, and the temperature is about the same or perhaps slightly (~1°C) warmer. The mortalities were first observed in 1994 at the Rizhao site and have continued since then. Both 1994 and 1995 were warmer (by 2 and 1°C, respectively) than normal. Temperatures in 1996 were more typical, and the death rate lessened. Mortality worsened in 1997 to 1998, reaching 80% or more in many areas throughout Shandong.

There are several suspected causes for the scallop mortality, although they have not been studied extensively. Most scientists in China believe that the mortalities are caused by a combination of overcrowding, high summer temperature, and deteriorating water quality. Scallop farmers often culture scallop at 2 to 3 times the density (30 to 50/layer) recommended by local scientists. The number of longlines and culture plots (not just for scallop culture) has been increasing rapidly in recent years and may have exceeded the carrying capacity of many coastal areas. Overcrowding at both cage and bay level may have added considerable stress to the culture environment. A haplosporidan parasite of the type responsible for extensive mortalities in oysters in the United States was identified in bay scallops in China, but there was no evidence that it was causing mortalities in that species or that it had been transferred to zhikong scallops (Chu et al. 1996). Finally, there is also suspicion that the scallop stock is deteriorating. Although all seeds are collected from the wild, they are collected from a restricted

area where the wild population is believed to have originated from hatchery production during the late 1970s and early 1980s.

#### Bay Scallop

The bay scallop is not native to China. It was introduced from the United States in 1982 by the third author and colleagues. Of the original shipment, a total of 26 bay scallops survived and were spawned in January 1983, producing the first generation of bay scallops in China (Zhang et al. 1986). The juveniles reached an average 6.9 mm in May and were transferred to culture sites in Shandong and Fujian provinces. The scallops grew to 50 mm by September and 59 mm by December 1983. Market size, 50 to 60 mm, can therefore be reached within a year. This is a major advantage over the zhikong scallops, which usually take 1.5 to 2.0 years to reach market size. The shorter turn-around time of bay scallops is partly attributable to its faster growth, and partly to the fact that they are spawned in the early spring (or late winter) so that they catch a full growing season. Because zhikong scallop seeds are collected in the fall, they miss most of the first growing season. Because of the short growout time, bay scallops quickly gained acceptance by scallop farmers, and aquaculture expanded rapidly after 1984. By early 1990, the annual production of bay scallops had reached about 200,000 tons. Bay scallop culture production has declined somewhat in the past few years. Because of the recent summer mortality problem in zhikong scallops, bay scallop culture may increase again.

Bay scallop seeds are produced exclusively in hatcheries, where thousands of mature adults, which are hermaphroditic, are placed in lantern nets and induced to spawn in large concrete tanks, ranging from 10 to 100 cubic meters. When the desired egg density is reached (about 50/mL), the adults are moved to the next tank to continue spawning. The first water change is made as soon as the larvae reach D-stage, which is usually 24 h after spawning. Larvae reach the eyed stage in 10 to 14 days at a size of 170–190 µm, at which time spat collectors are placed in the tanks. Two common types of spat collectors are used. One is a rope curtain made from natural palm tree fiber (Fig. 5C). The other is polyethylene or nylon nets/screens. Spat, which attach to the collectors by byssal threads, are cultured in the hatchery until they reach 500–600 µm, after which they are transferred to shrimp ponds or nursery areas. Commercial seeds are sold at a size of 0.5 to 1.0 cm. Lantern nets on suspended longlines is the predominant form of culture for all scallops. The culture density of bay scallops is about the same as the zhikong scallops, 30 to 50 scallops per layer, 250 to 400 per net. Hatchery production of larvae usually occurs between March and May, and scallops are harvested between November and December.

Most of the bay scallop production continues to be from the 26 scallops first introduced in 1982, and there have been signs of inbreeding, such as larval and juvenile mortality. Several new broodstock introductions have been made to expand the gene pool, but offspring of the recently introduced scallops have not yet entered the mainstream production.

Most of the scallops are processed upon harvest. Adductor muscles are either individually frozen, or cooked and then dried. A small fraction of scallops is sold live to local restaurants.

#### MUSSELS

Mussel culture is relatively new in China. It started with blue mussel in the 1950s as a byproduct of seaweed (*Laminaria*

*japonica*) culture. Five species of mussel are cultured commercially. The most widely cultured is the blue mussel, *Mytilus edulis* Linnaeus (Fig. 6A), which is produced chiefly in Shandong and Liaoning provinces. Four other species, the thick-shell mussel (*Mytilus coruscus* Gould), the Senhouse mussel (*Musculus senhousi* Benson), the green-jade mussel (*Perna vividis* Linnaeus) (Fig. 6B), and the penshell (*Pinna pectinata* Linnaeus) (Fig. 6C), are also cultured in Guangdong and other southern provinces on limited scales. The Senhouse mussel is primarily used for shrimp, fish, and chicken feed. All five species are native to China.

For blue mussel culture, hatchery-produced seeds were used to supplement wild set during the 1970s. Now wild mussel seeds are abundant, and hatcheries are used for other molluscan species. Seeds are usually collected in May and June, occasionally also in the fall. When the juveniles reach about 10 mm, they are thinned and reattached to ropes for growout. Culture on suspended longlines is the predominant form of growout for the blue mussel. Bottom culture is also used for the green and thick-shell mussels in the south.

A portion of the juveniles that set in May and June are harvested between October and December, at a size of 60 to 70 mm. Smaller mussels are returned and cultured for longer periods, but most mussels are harvested within a year. Mussels with full gonads are considered most desirable on the market. Some mussels are sold fresh on local markets, but most are steamed and dried into a traditional product called "Dan-cai." Mussel meat that is dried without cooking is called "butterfly meat." Some mussels are cooked to produce "oyster sauce." Mussels are also used as feed for cultured shrimp and *Rapana* snails. Mussel culture has been in decline in recent years probably because of competition from other high-value species, such as scallops, oysters, and abalone.

#### ABALONE AND OTHER GASTROPODS

Abalone culture is new in China. Large-scale culture started in the late 1980s, and it has been developing rapidly in the past decade. Now it is one of the largest components of the molluscan culture industry by value. It is probably also the most sophisticated industry in terms of production technology. Abalone is not individually listed in official statistics, and production estimates vary greatly. The annual production is probably between 500 to 900 tons.

Two species of abalone are cultured: the wrinkled abalone, *Haliotis discus hannai* Ito (Fig. 7E) and the colorful abalone, *Haliotis diversicolor* Reeve. The colorful abalone is a southern

species and is cultured mainly in Guangdong and Fujian. The wrinkled abalone is the major species and accounts for about three-quarters of the total production. It is a northern species and is cultured mainly in Shandong and Liaoning provinces. Liaoning's Dalian area is the leader in abalone aquaculture, and much of the technology has been developed there. There are three major abalone culture companies in Dalian: the Bilong Seafood Co., Pacific Seafood Co., and Xinda Products Co. We visited all three facilities. The Bilong Seafood Co. has been the leader in abalone production for some time. The Pacific Seafood Co., which was established in 1993 with an investment of US\$8.5 million, is poised to become the largest abalone culture company in China. It is designed to produce 600 tons of abalone per year, although it had not produced the first crop when we visited in 1997. Xinda has successfully used hybrid abalone to combat disease problems. Abalone culture in Shandong province is catching up fast. Over 40 large abalone facilities with investments of more than US\$1 million each, have been built in Shandong's Rongcheng area. Most of the new facilities have not reached their full production capacity, and many are running at a loss because of disease problems. On the other hand, one facility in Rizhao that we visited has recovered all its investment in 3 years. The Rizhao facility is primarily designed for hatchery production of seed and has little growout production.

All abalone seeds are hatchery produced with well-developed technology. Production starts in early spring with broodstock conditioning at elevated temperatures. After about 1,000 degree-days of conditioning (at about 18–20°C), abalone are ready to spawn. To induce spawning, they are left without water for 1 hour and then exposed to UV-treated seawater ( $600 \mu\text{W}/\text{h}/\text{m}^3$ ). Males usually begin to spawn 1 hour after being in UV-treated water, and females within 30 to 40 min after the males. Fertilized eggs are incubated at density of 15–20 mL. Eyed larvae are set on corrugated plastic plates, which are precoated with a layer of diatoms to induce settlement (Fig. 7A). Abalone spat remain on the settlement plates until they reach 3 mm. After that, they are separated from the settlement plates and transferred to large punctured plastic plates for nursery culture (Fig. 7B). The holes allow for better water circulation and for the young abalone to move from side to side. The plates are supported in net-pans and placed in raceways, typically 0.5-m deep, 1 to 2-m wide, and 10 to 20-m long (Fig. 7C). Commercial diets (formulated) are used during the nursery phase. Juvenile abalones are cultured to a size of 1 to 2 cm in the nursery before growout. Abalone seeds sell for about \$0.25 each.

Three major forms of growout are used in abalone culture. The



Figure 6. Mussel culture. A, the blue mussel harvested as a byproduct from seaweed and oyster longlines in Dalian. B, green-jade mussel cultured in Guangdong and Fujian. C, the penshell cultured in Zhejiang.

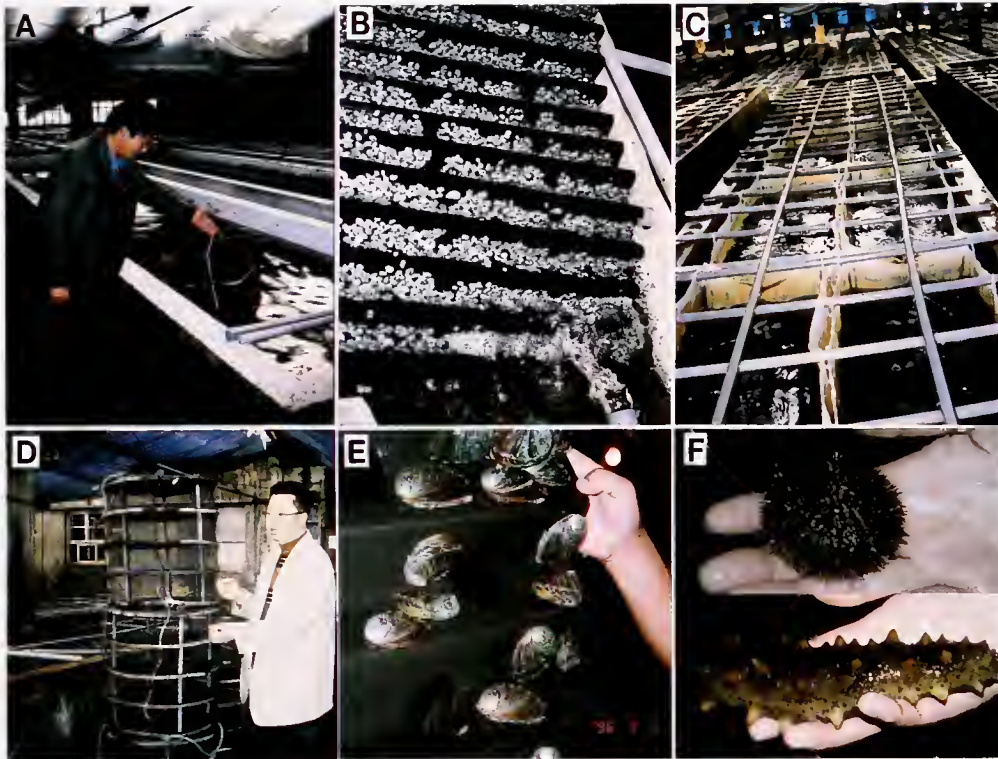


Figure 7. Abalone culture. A, settlement plates are coated with diatoms before use in a Dalian hatchery. B, juvenile abalones are cultured on plates in indoor raceways in a Dalian hatchery. C, typical indoor raceways used for abalone larval culture and nursery. D, large and specially designed cages for abalone culture on suspended longlines, Dalian. E, close to market size abalone cultured in raceways inside an abandoned air-raided bunker in Lianyungang. F, sea urchin and sea cucumber are alternative species cultured in abalone hatcheries.

first, and most widely used, is culture in cages on suspended longlines. Most abalone cages are much larger and more sophisticated than the lantern nets used for scallops (Fig. 7D). They are about 70-cm in diameter and 1-m tall with 4–5 layers. One type of cage is made from large plastic tubes (35-cm in diameter and 60-cm long), with screens on each end and 1-cm holes on the side. Some farmers also use scallop lantern nets for abalone culture. The second form of culture is on plastic plates placed in indoor concrete raceways (Fig. 7E). Abandoned air-raided bunkers are now popular places for indoor abalone culture. The third form, which is not widely used, is in intertidal ponds. Intertidal net fences are also used in some areas for abalone culture. Fresh kelp, mostly *Laminaria japonica*, is the primary food during growout, and artificial diets are used when algae are not available.

Abalone is considered to be one of the best and most valuable seafoods in Chinese culture and other parts of Asia. Commercial size abalone (7 to 9 cm) is priced at \$3 to \$4 per animal, and a large proportion of the abalone produced in China is sold live to markets in Hong Kong and Japan. Some abalone are sold to local restaurants. Abalone is also valuable as an ingredient in Chinese medicine. Abalone culture was highly profitable in the late 1980s and early 1990s, but recent disease problems have been plaguing abalone culture in the north. Many abalone facilities in Shandong and Liaoning have reduced or stopped abalone culture and started growing sea urchins and sea cucumbers (Fig. 7F), which have similar culture requirements.

One of the diseases is known as the pustule disease and is caused by *Vibria fluvialis*-11 (Li et al. 1998). Another major dis-

ease affecting the wrinkled abalone has a distinct syndrome. It includes a long incubation period and slow disease progression and is characterized by a shrinking of the meat within the shell. The condition is reminiscent of "Withering Syndrome", which affects wild black abalone (*Haliotis cracherodii* Leach) along the California coast. This disease is transmissible and is strongly associated with a rickettsial infection (Gardner et al. 1995, Friedman et al. 1997). A similar syndrome of *Nordotis discus discus* in Japan is associated with a virus (Otsu and Sasaki 1997). Whether these syndromes are related is presently unknown.

Several other gastropods are also cultured in China, including the red conch (*Rapana venosa* Valenciennes), the mud snail (*Bullacta exarata* Philippi) and the sea hare (*Notarchus leachii cirrosus* Stimpson). For the red conch, juveniles are collected from the wild and cultured in cages or lantern nets. They are fed with blue mussels. The mud snail is a small gastropod, belonging to Order Cephalaspidea (bubble shells), with an adult size of 2 to 3 cm. It has a large foot that covers much of its thin shell. The culture of mud snails involves the selection of muddy flats that have heavy larval settlement. The flat is treated with pesticides to remove predators and fertilized to stimulate diatom growth just before settlement occurs. No other management is required. Production at harvest usually ranges between 35–75 metric tons per hectare. The mud snail, pickled in liquor, is a delicacy in the Zhejiang and Shanghai areas and is priced at about \$1/lb. Mud snail culture is a significant industry in Zhejiang and is highly profitable. The sea hare has been cultured for hundreds of years in southern China, where juveniles are collected from the wild and cultured in ponds.

Sea hares are not cultured for their meat, but for their egg cases, which when processed, constitute a valuable remedy in Chinese medicine, referred to as “Sea Powder.”

### PEARL OYSTER

Pearls are produced from both freshwater and marine bivalves. China has a long history of culturing freshwater pearls and currently produces about 800 tons annually, mostly from the freshwater mussel *Hyriopsis cumingii* Lea. Freshwater pearls are not only marketed as jewelry, but also used as ingredients in Chinese medicine and cosmetic creams. Marine pearls have a higher market value than freshwater pearls. Wild marine pearls have been harvested for several thousand years in China, but artificial culture is less than 50 years old. China now produces about 20 tons of marine pearls annually, second to Japan’s production of about 40 tons.

Marine pearls are produced by species of Pteriidae. In China, almost all cultured marine pearls are from the Martensii pearl oyster, *Pinctada martensii* Dunker (Fig. 8A). This species is also the major species cultured in Japan and accounts for over 95% of worldwide marine pearl production by weight. Other species such as *Pinctada maxima* Jameson and *Pinctada margaritifera* Linnaeus are also cultured experimentally in China. Pearls from the latter two species are more valuable because of their larger size, unique coloration, or both.

Marine pearls are primarily cultured in provinces on the South China Sea. Guangdong and Guangxi provinces produce over 90% of China’s total. We visited Beihai, the pearl city of China, where the famous Hepu pearls are produced. This area has about 360 pearl oyster hatcheries and 2,000 farms, and produces about 40–50% of the national total. All seeds are hatchery produced using

methods similar to those used for bay scallops. As in other molluscan hatcheries, simple technologies are often practiced. One example is the culture of age in plastic bags (Fig. 8B). Hatchery production usually starts in April. When spat reach 1 mm, they are put into fine-mesh bags and moved to nursery areas in the sea. Seeds are transferred to growout cages at a size of 5–8 mm and a density of about 5,000 per cage. Unlike the multiple-layer lantern nets used for scallops, cages used for pearl oyster culture are small (25 × 25 × 10 cm), single compartment units (Fig. 8C). They consist of metal frames with nylon net sides. Some cages are made with a metal ring, about 30-cm in diameter (Fig. 8D). These cages are hung on suspended rafts, longlines, or intertidal longlines that are supported by wood stakes about 60-cm tall. Juveniles are thinned five to seven times during a 2 to 3-year period to a final density of 30–50 adults per cage.

Pearl oysters are cultured for 2 to 3 years to a size of 50–70 mm before being used for pearl production. Marine pearls are produced by inserting a nucleus (5–8 mm) attached with a small piece of mantle (2–3 mm) from a donor oyster into the mantle of the recipient pearl oyster. The attached mantle tissue will grow, encapsulate the nucleus, and produce nacre. Freshwater pearls are usually produced by inserting a piece of mantle only. Nucleus pearls have recently been introduced to freshwater pearl production and have become strong competitors with marine pearls. Nuclei are usually made from molluscan shells or synthetic materials. The number of nuclei inserted per oyster depends upon the size of the nucleus and the oyster, but on average, about two nuclei per oyster are inserted. Nucleus insertion is performed in spring or fall. Summer is avoided because of the additional stresses of high temperature and reproduction. When nuclei are inserted between February and April, pearls are harvested in November and December of the



Figure 8. Pearl oyster culture. A, the outside (upper) and inside (lower) appearance of the Martensii pearl oyster used for the production of marine pearls in southern China. B, plastic bags are used for algae culture (second stage) in many molluscan hatchery; the last stage culture is usually done in shallow concrete tanks. C and D, single compartment cages used to culture pearl oysters on intertidal longlines in Beihai. E, pearls are harvested by sacrificing oysters; one pearl per oyster is expected, on average. F, pearl necklaces on display at markets in Beihai, the pearl city of China.



same year. The recovery rate is about 50% or one pearl per oyster. It takes about 10 million pearl oysters at harvest to produce one ton of pearls.

At harvest, pearl oysters are sacrificed to collect the pearls (Fig. 8E). The meat is used for chicken and duck feed. Raw pearls come in various shapes and colors. They are sorted and processed in a series of chemical treatments before reaching the jewelry market (Fig. 8F). They are classified into four size categories: large (>8 mm), medium (6–8 mm), small (5–6 mm), and fine (<5 mm). Large pearls are worth considerably more than smaller ones. Nucleus-free pearls are produced by inserting only donor tissue and are intended for use in Chinese medicine. Pearl powder from pearls and shells is used in toothpaste and facial creams.

One problem in pearl production is sexual maturation. When pearl oysters are full of gametes, nucleus insertion is difficult, and the survival and pearl recovery rates are low. Some farmers inhibit maturation in the fall by placing oysters at high density and in deep water. Mature oysters are sometimes induced to spawn beforehand to improve their condition for nucleus insertion. Sterile triploids are being tested for pearl production at the South China Sea Institute of Oceanology in Guangzhou and Guangxi Institute of Oceanology in Beihai, and preliminary results are encouraging.

#### PERSPECTIVES

Molluscan aquaculture in China is impressive in both scope and magnitude. It is practiced in almost all inhabited parts of the coast and covers all major molluscan species. A wide range of production technology is used, ranging from sophisticated intensive culture of abalone, scallop, and pearl oysters, to primitive extensive culture of certain clams and snails. Some practices are distinctive. One example is the "semiartificial" collection of clam seed, which involves site selection, bed construction, substrate modification, larval forecast, and predator control. Another example is seed production from earth ponds, which seems to be an effective approach under low-tech conditions. Polyculture of mollusks, shrimp, and/or fish in ponds is widely practiced.

The past decade represents the fastest growing period for molluscan aquaculture in China. This period coincides with rapid growth in the overall Chinese economy and is primarily influenced

by China's economic reforms. The replacement of central planning with a market economy is probably the leading force responsible for the rapid growth. Marine mollusks are among the best-loved seafood in China. As the Chinese economy grows and people's income rises, demand for mollusks will continue to increase and prompt further growth of the aquaculture industry.

The rapid development of molluscan aquaculture has brought with it some problems, the most pressing of which is the deterioration of the culture environment. The expansion of mariculture during the past decade has put considerable stress on the marine environment, and the carrying capacity of the coastal water may be exceeded in many areas. Longlines and rafts often cover much of a bay (Fig. 3B, E; Fig. 5D). Large shrimp ponds are densely situated (Fig. 4F). Scallops are often cultured at excessive densities at both cage and baywide levels. Excessive feeding from shrimp and abalone culture leads to accumulations of tremendous waste. Red tides have become more frequent along China's coast. Overcrowding and poor water quality, coupled with high water temperature, are believed to be the leading causes for the massive scallop mortalities in 1997 to 1998. Abalone culture has been seriously affected by diseases. There is a great need for the development of new management strategies to maintain yields while minimizing conditions that degrade the environment, cause disease, or both. Future growth of the molluscan aquaculture industry may largely depend upon technological advances that make molluscan aquaculture more efficient and environmentally friendly, instead of crude expansion in scale.

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## SETTLEMENT OF THE BLUE MUSSEL *MYTILUS GALLOPROVINCIALIS* LAMARCK ON ARTIFICIAL SUBSTRATES IN BAHÍA DE TODOS SANTOS B.C., MÉXICO

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**ABSTRACT** The culture of the blue mussel *Mytilus galloprovincialis* in Bahía de Todos Santos is a growing economic activity. This culture depends upon mussel seed collection from artificial collectors; however, there are no studies on time and duration of the mussel settlement season, collector material, and settlement pattern. Between December 1994 and November 1995, pieces of about 163 cm<sup>2</sup> of nylon ropes, polypropylene ropes, ropes made with polypropylene and cotton, pads of synthetic fibrous material, and dried *Luffa*, sp. were used as collectors and were deployed at 2- and 5-m depths. Mussel settlement occurred during all the period of study, and its fluctuation was similar for all collectors tested. Major settlement occurred in December and January for all collectors and depths studied. The observed settlement pattern indicates that direct settlement of competent pediveligers from the plankton onto the substrates is the main source of recruitment in the area (92%). There was a trend of greater settlement on pads of synthetic material than on the other collectors. This material seems to be appropriate for scientific studies; whereas, for commercial activity, any filamentous rope collectors are recommended.

**KEY WORDS:** settlement, *Mytilus galloprovincialis*, mussel seed collectors, dispersion and culture

### INTRODUCTION

The culture of the blue mussel *Mytilus galloprovincialis* using submerged longlines in Bahía de Todos Santos, Baja California (Fig. 1) started in 1991 (Cáceres-Martínez 1997). At present, the annual production is around 150 metric tons and it is marketed in México and in the United States. Submerged longlines, 200-m long, are suspended from 200-L plastic floating barrels and are anchored with 0.8 or 1.2 ton concrete anchors. The main line is placed at a 5-m depth, from which culture ropes, 7-m long, are suspended. Mussel seed is obtained from nature on artificial collectors placed from late November to December. The collectors consist of a polypropylene rope placed inside a thin plastic net and suspended from surface longlines (Cáceres-Martínez 1997). This practice and type of collectors were established on the basis of the experience of mussel growers. On the other hand, it is known that colonization on natural and artificial substrates by

mussels, *Mytilus* sp. may occur by settlement of competent pediveliger larvae and/or by settlement of drifting postlarvae (Davies 1974, King et al. 1989, Cáceres-Martínez et al. 1993, Cáceres-Martínez et al. 1994). This settlement pattern could have practical importance for the mussel grower, because its knowledge in a given area may improve the chance of collecting mussel seed from nature (Cáceres-Martínez and Figueras 1998). However, there are no scientific studies in the area to corroborate or improve mussel seed collection practices.

The aim of the present study is to determine the time and duration of the mussel settlement season and the relative percentage of competent pediveligers to postlarvae during settlement on different artificial collectors deployed at two depths in Bahía de Todos Santos.

### MATERIALS AND METHODS

The study was performed in the mussel culture area of Bahía de Todos Santos, which is approximately 18-km long and 14-km wide and has a surface area of 252 km<sup>2</sup>; it has a sandy bottom, and it is partially separated from the ocean by the two small islands, Islas de Todos Santos. The study was carried out from December 1994 to November 1995. Pieces of 25-cm long and 2-cm diameter (163 cm<sup>2</sup>) of nylon ropes, material similar to that used by mussel growers (FN); polypropylene ropes (FP); ropes made with polypropylene and cotton (FPC); pads of 25-cm long and 6.5-cm wide (163 cm<sup>2</sup>) of synthetic fibrous material (Commercial Scotch Brite™) (SF) and similar pieces of dried fibrous *Luffa* sp. (Cucurbitacea) (L) were used as collectors. Ropes were unraveled by passing them through a grinding machine to increase their filamentous nature. A comparison among surface area of the substrates was relative because of the difficulty for determining their exact surface area.

The five collectors were attached to a PVC tube and hung at 2- and at 5-m depths, covering part of the depth at which mussel growers place their collecting ropes (1 to 7 m-depth). Collectors

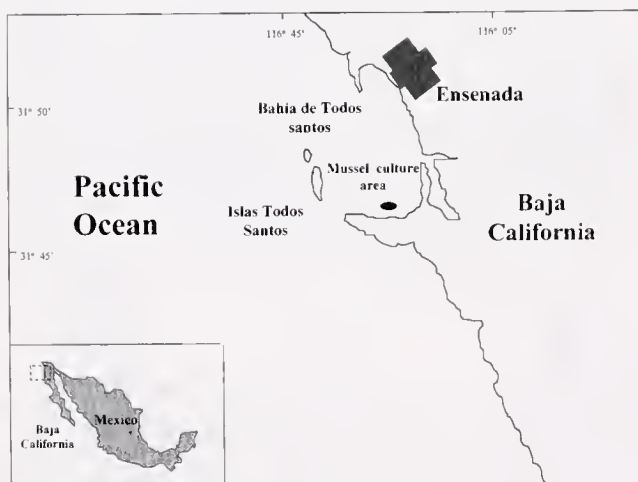


Figure 1. Map showing the study area, filled oval indicates the culture area where collectors were deployed.

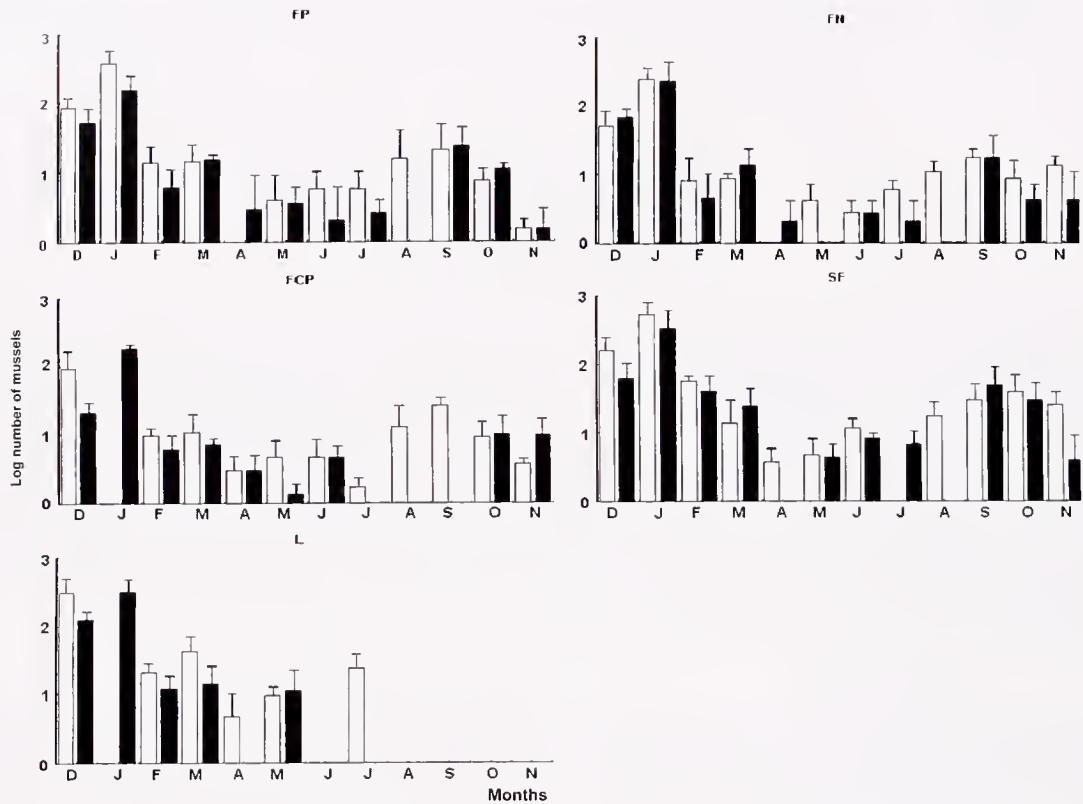


Figure 2. Logarithm of the mean number of mussels settled (+ standard error) on different collectors placed at 2- (open bars) and 5-m (filled bars) depth, during a study period in Bahía de Todos Santos, México. This graphic representation allows better visualization of results during low settlement periods.

were replaced after periods of  $30 \pm 5$  days. Each collector was taken to the laboratory in one plastic bag, then the collectors were immersed individually in a 10% solution of commercial sodium hypochlorite (NaClO) for 5 min. (to dissolve organic material and to facilitate detachment of mussels) and were rinsed with running water directly onto a 0.09-mm sieve. After that, the seed was dried in an oven at 70°C for 24 h and passed through a series of sieves

of 0.09 to 0.7-mm mesh to facilitate mussel separation by size. Three subsamples of mussels were obtained from each sieve and counted. For graphic representation, data for Fig. 2 were log transformed. Thirty individuals per fraction were measured with an ocular micrometer under a stereoscopic microscope or with an electronic caliper if the mussel size was  $>5$  mm to determine size distribution.

Competent pediveliger larvae were separated considering mussels with shell lengths from 0.250 to 0.470 mm (mean 0.360 mm) according to the minimum and maximum size values recorded for this stage of *Mytilus* sp. (Rees 1954, Bayne 1965, Widdows 1991). Moreover, the growth rate of recently settled *Mytilus galloprovincialis* [ca.  $25 \mu\text{m d}^{-1}$  at 15 to 17°C (Aguirre 1979)] was considered to estimate the probable increase in shell length of mussels during the sampling period, mussels greater than expected will confirm the attachment on collectors of drifting postlarvae. Water temperature was recorded during samplings. The number of spat from different collectors and depths studied were compared using an analysis of variance (ANOVA).

## RESULTS

Settlement fluctuations throughout the study period on different collectors are shown in Figure 2. In general, in all the collectors, a similar fluctuation was detected at the two depths studied: the settlement began to rise in December, with a peak in January (in these months, the 78% of the total spat obtained during all the

TABLE 1.

Expected size of mussels during time intervals studied.

Date of Collector Replacement	Temperature (°C)	Time for Colonizing (days)	Theoretical Expected Size (mm) 2-m depth	Theoretical Expected Size (mm) 5-m depth
15.12.94		35	1.212	1.210
08.02.95		54	1.687	1.685
27.02.95		19	0.812	0.810
29.03.95	17	30	1.087	1.085
25.04.95	18	27	1.012	1.010
18.05.95	17.6	23	0.912	0.910
16.06.95	20	29	1.062	1.060
13.07.95	20.7	27	1.012	1.010
24.08.95	21.4	42	1.387	1.385
28.09.95	18.6	35	1.212	1.210
30.10.95	18.6	32	1.137	1.135
22.11.95	17	23	0.912	0.910

study was recorded), and it remained low throughout the other months, except September, when a light increase was detected. This general fluctuation was best recorded on collector SF where the greater quantity of spat was detected. Particular differences are observed among the obtained spat on the studied collectors and depths; settlement seems to be more abundant at 2- rather than 5-m depth. This occurs during December and January in FP, January in FN, December in FPC, December and January in SF, and December in L. The loss of FPC and L in January prevent a comparison in these collectors. During the months of low settlement, this difference is not clear. The weakness of L favored their frequent loss during the study period. Statistical comparison of the spat among collectors showed that differences were neither significant ( $F = 2.18$ ,  $p = .7$ ) during the study period, nor between depths ( $F = 0.92$ ,  $p = .34$ ).

Expected sizes of spat during permanence time on collectors and the temperatures recorded are shown in Table 1. Size distributions of the spat in different collectors are shown in Figures 3 to 7. In general, a similar distribution was recorded in all collectors and depths. The maximum size recorded in December was 1.59 mm in all the collectors and at both depths; whereas, in January, it was 5.99 mm in FP at 2-m depth, 3.74 and 3.70 in FN and L, respectively, at 2-m depth, and <3.59 in the other collectors

and depths. During December and January, about 7% of mussels in all the collectors and depths reached a size greater than expected, lately this percentage accounts for 1%. During the low settlement period, the maximum sizes recorded were 12.49 mm during April in SF at 2-m depth, 10.32 mm during February in FP at 5-m depth, and 5.33 during August in FPC at 2-m depth. In the other collectors and depths, the maximum sizes were <3.59 mm. Sizes of <0.470 mm were recorded during all the year and in all collectors.

DISCUSSION

The presence of mussels  $\leq 0.470$  mm throughout the year and their abundance in December and January reflects the presence of spawning mussels throughout the year and the occurrence of a major spawning period during late autumn. The reproductive cycle of *Mytilus galloprovincialis* in Bahía de Todos Santos has not been studied; however, in the west coast of North America, the major spawning season of *M. edulis* (after the works of Harger (1972) in Santa Barbara, California, the *Mytilus edulis*-like in southern California was identified as *M. galloprovincialis* (see Mc Donald and Koehn 1988, Koehn 1991)) takes place in winter (see Suchanek 1981). It is known that in populations of *M. edulis* and *M. gallo-*

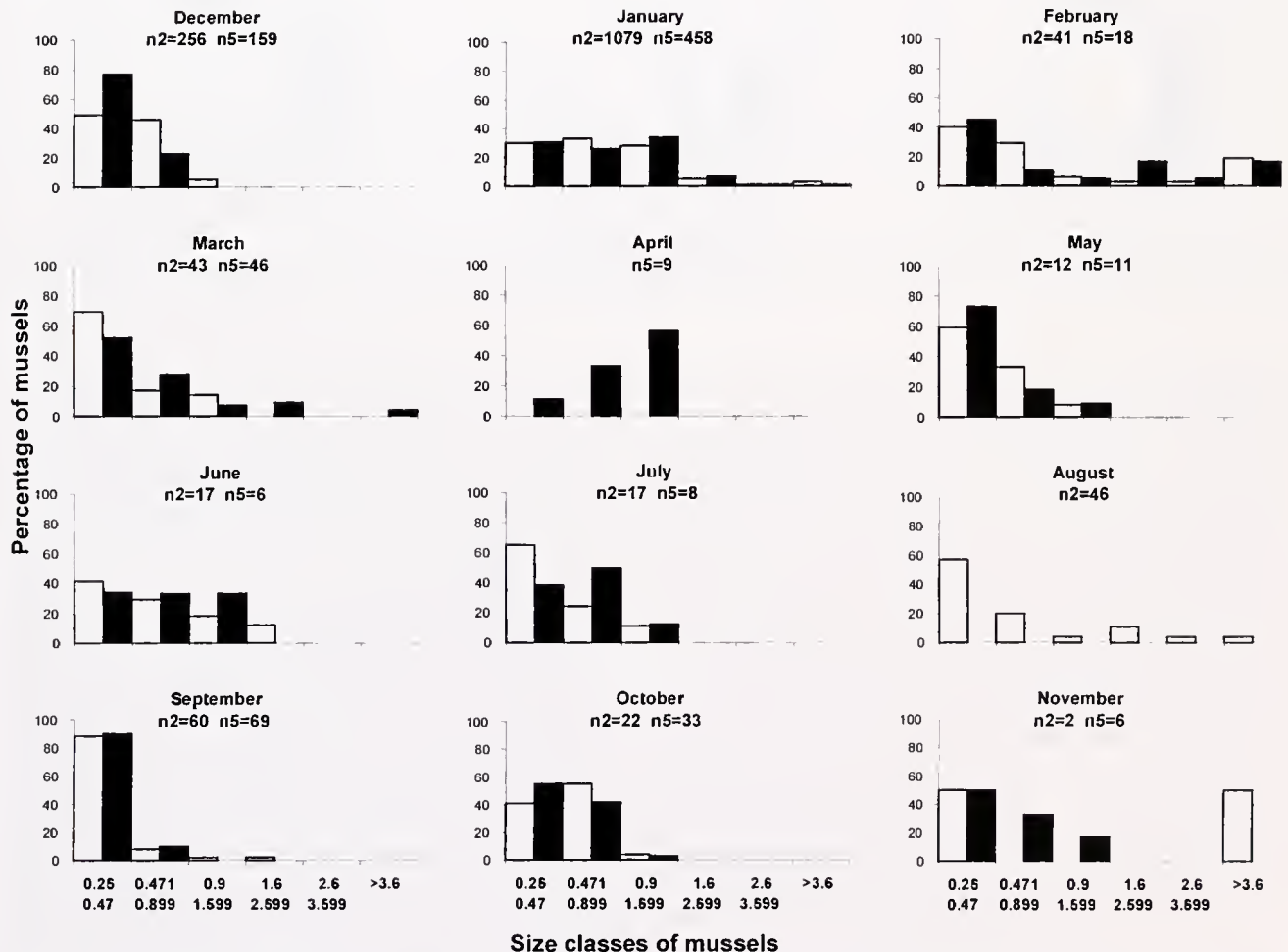


Figure 3. Size distribution in percentage of mussels settled on collectors FP placed at 2- (open bars) and 5-m (filled bars) depth, during a study period in Bahía de Todos Santos, México.

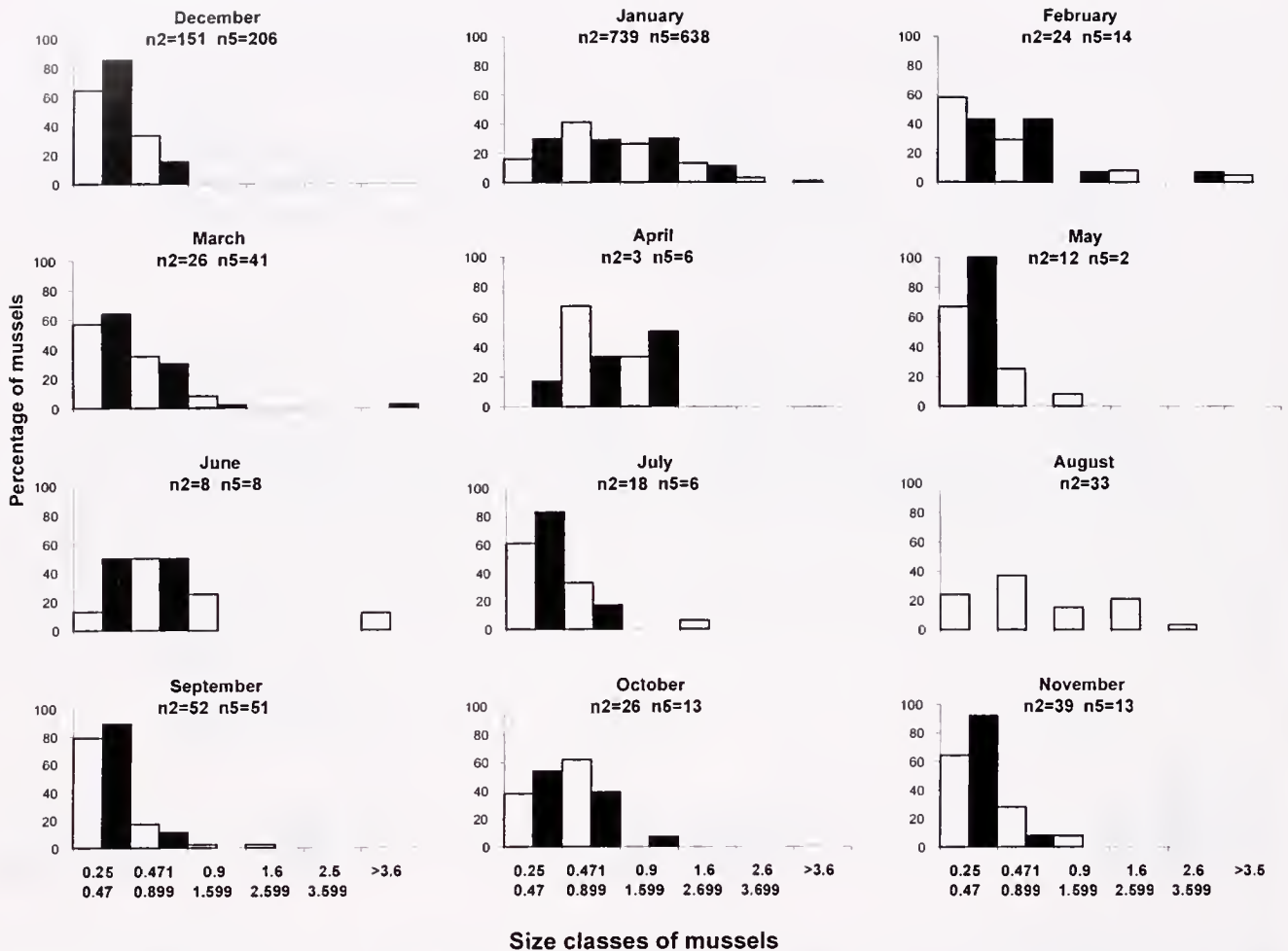


Figure 4. Size distribution in percentage of mussels settled on collectors FN placed at 2- (open bars) and 5-m (filled bars) depth, during a study period in Bahía de Todos Santos, México.

*provincialis* from different areas of the world, after a main spawning season, minor spawning during the year may occur (Seed 1976, Ferrán 1991, Villalba 1995, Cáceres-Martínez and Figueras 1998). Differences between reproductive cycles from mussels located in different environmental conditions could be explained by temperature, salinity, photoperiod, food, nutrient reserves, hormonal cycle, and genotype differences (Seed 1976, Devauchelle and Mingant 1991, Robinson 1992, Seed and Suchanek 1992, Couturier 1994).

It is important to note that *M. californianus* is present in the exposed rocky shores of the ocean side of the Bahía de Todos Santos, where *M. galloprovincialis* is found only rarely. To the contrary, *M. galloprovincialis* is clearly dominant in the protected areas of the bay and obviously in culture area, where *M. californianus* is rarely seen. However, the occasional presence of *M. californianus* in the culture area, suggests that some larvae of this species may reach and survive culturing conditions, then it is reasonable to assume that some settled larvae may belong to this species. However, morphological identification of mussels <3 mm is practically impossible (Rees 1950, Loosanof et al. 1966, Hines 1979, personal observation). To resolve this point, it is necessary to do detailed studies on identification throughout the molecular genetics of mussel larvae and postlarvae. In this study, we assume

that the major recorded spat corresponds to *M. galloprovincialis*, taking into account the prevalence of *M. galloprovincialis* and that its reproductive cycle presents one major spawning season in the west coast of North America with respect to the reproductive cycle of *M. californianus* without a major spawning season (Young 1946, Suchanek 1981, Hines 1979, Curiel-Ramirez and Cáceres-Martínez unpublished data).

The similarities found in the settlement of mussels in different collectors and depths suggest a relatively uniform presence of competent pediveligers and postlarvae in the study area. A trend similar to a major settlement in surface collectors was found by Fuentes and Molares (1994) and Molares and Fuentes (1995) in Ria de Arosa, Spain, but a contrary tendency was found by Cáceres-Martínez and Figueras (1998) in Ria de Vigo, Spain. These results have been explained by the presence of the thermocline and by the behavior of larvae and postlarvae settlement (Fuentes and Molares 1994, Cáceres-Martínez and Figueras 1998). The preference of mussels to settle in substrate has been widely studied (de Blok and Geelen 1958, Bohle 1971, Davies 1974, Dare et al. 1983, Eyster and Pechenik 1987, King et al. 1990, Cáceres-Martínez et al. 1994), and it has been found that rugose and filamentous substrates are the best for settlement, because they are related to the

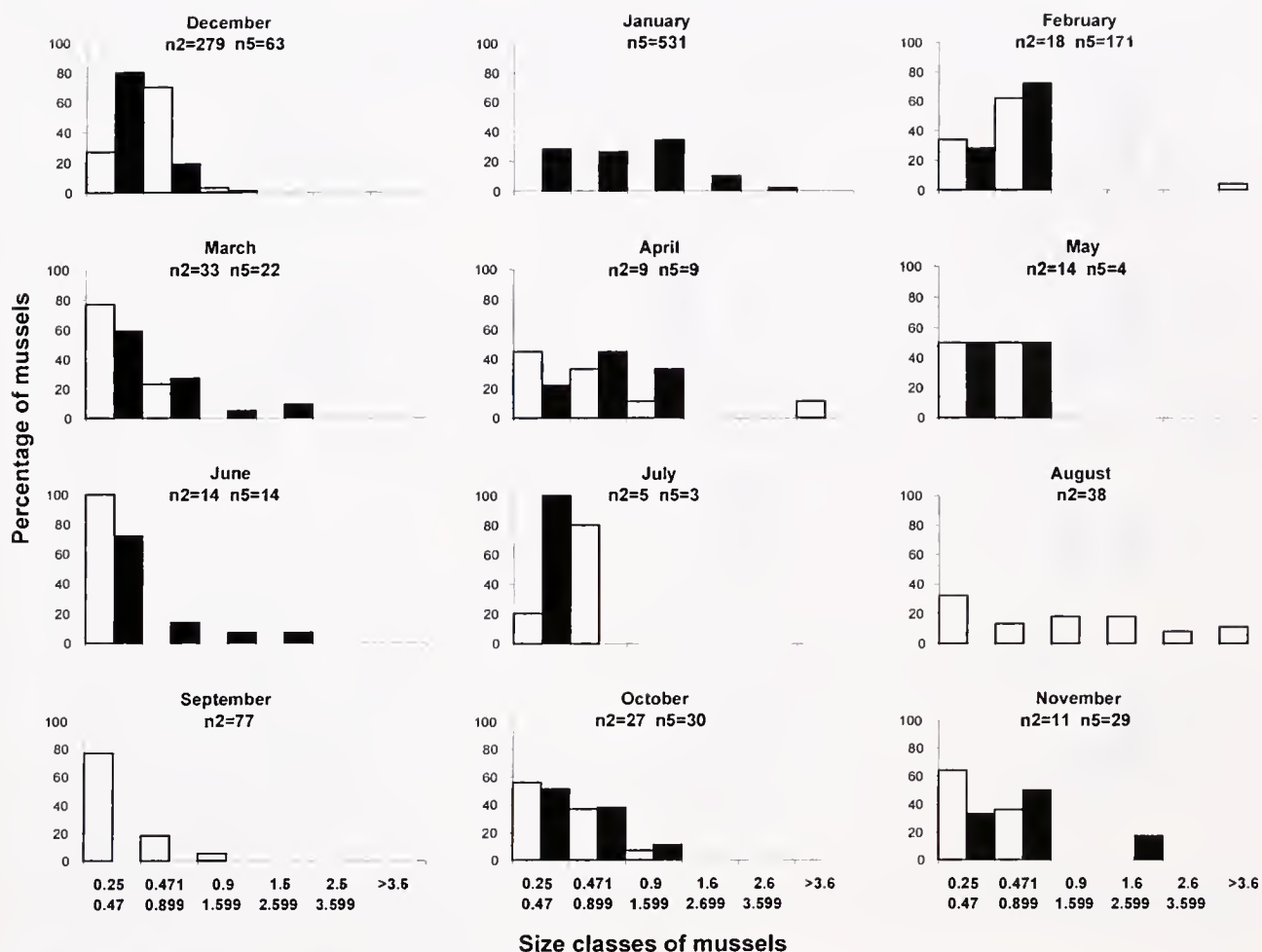


Figure 5. Size distribution in percentage of mussels settled on collectors FPC placed at 2- (open bars) and 5-m (filled bars) depth, during a study period in Bahía de Todos Santos, México.

use of long contact mucous threads of competent pediveliger and postlarval stages that are easily jammed between filaments and rugosities (de Blok and Tan Mass 1977, Cáceres-Martínez et al. 1994). However, comparison among efficiencies of filamentous substrates is difficult to do, not only because of the enormous surface variability of filamentous artificial substrates, but also because these substrates are colonized immediately in the water by filamentous algae, hydroids, and debris that modify their surface. In laboratory studies where small pieces of substrates are used and environmental conditions are under control, image analysis has proved to be a useful tool to calculate the substrates surface area (Cáceres-Martínez et al. 1994). However, this is hardly applicable to large collectors used in field studies. In these circumstances, a useful comparison among filamentous substrates is related to durability of collectors, handling, cost, nature of the study, and commercial use. Despite the fact that statistical analyses indicate that there were no differences among the spat recorded in the collectors studied, there was a clear trend of major settlement on commercial pads (SF). The observed tendency allows us to suggest the use of SF for scientific studies in the field or laboratory; however, their handling for commercial purposes is limited because of the difficulty of extracting the seed, in comparison with the use of fila-

mentous ropes. Therefore, FP, FN, and FPC could be useful for commercial purposes. The use of L is limited because of its weakness for handling and its low durability in the sea.

Although 92% of total spat was considered as competent pediveliger that arrived and grew on the collectors during the permanence of the substrates underwater between the sample collection, the presence of mussels larger than expected confirm the recruitment of at least a small proportion of postlarvae by dispersion. However, it is important to mention that temperatures over 17°C and other environmental conditions may have a direct effect on mussel growth rates. Offshore scarcity of drifting mussels agrees with the fact that extension of postlarvae dispersion is more or less limited to the vicinity of mussel beds and high current areas (Newell et al. 1991, Cáceres-Martínez et al. 1994, Cáceres-Martínez and Figueras 1997, Cáceres-Martínez and Figueras 1998). The presence of very large postlarvae (12.49 mm) supports the notion that the higher limit in the size of drifting postlarvae is around 10 mm (Beukema and Vlas 1989, Cáceres-Martínez and Figueras 1997).

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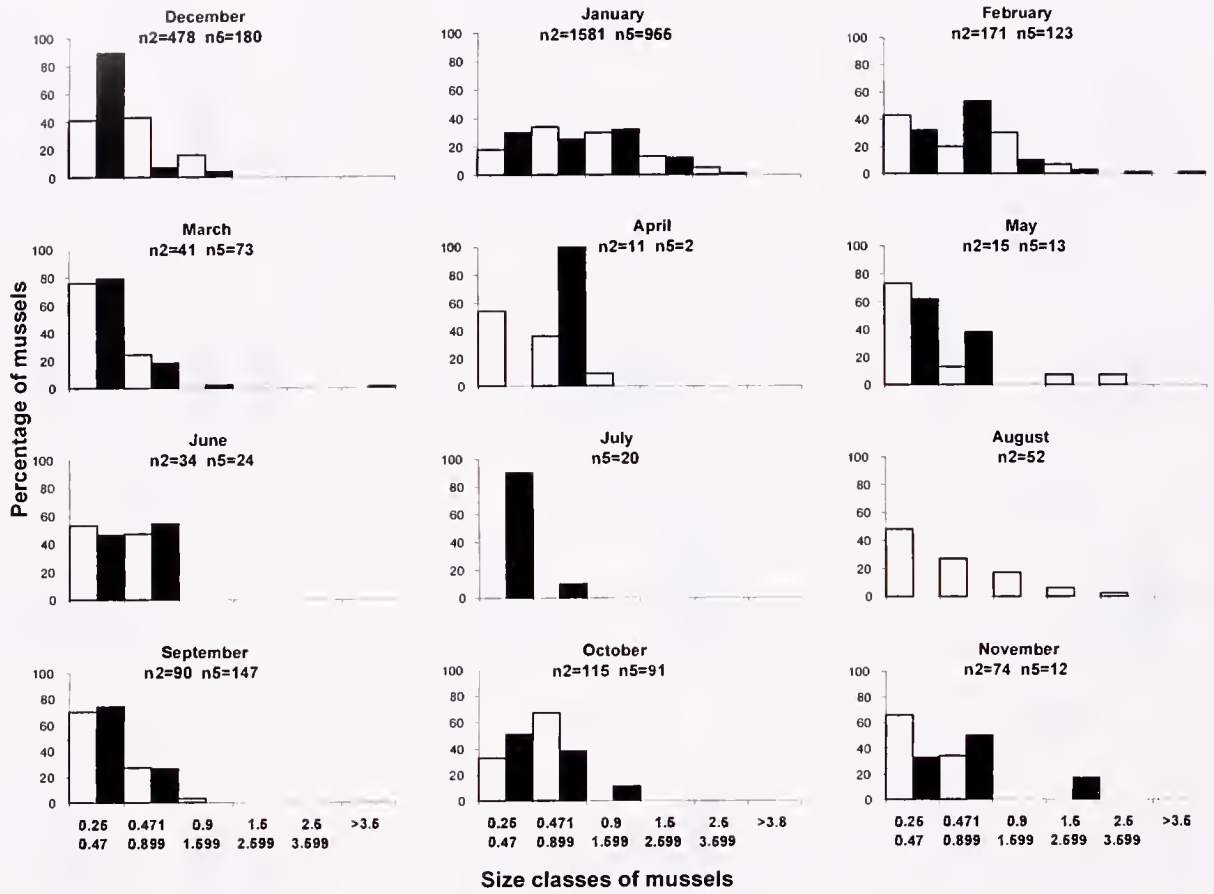


Figure 6. Size distribution in percentage of mussels settled on collectors SF placed at 2- (open bars) and 5-m (filled bars) depth, during a study period in Bahía de Todos Santos, México.

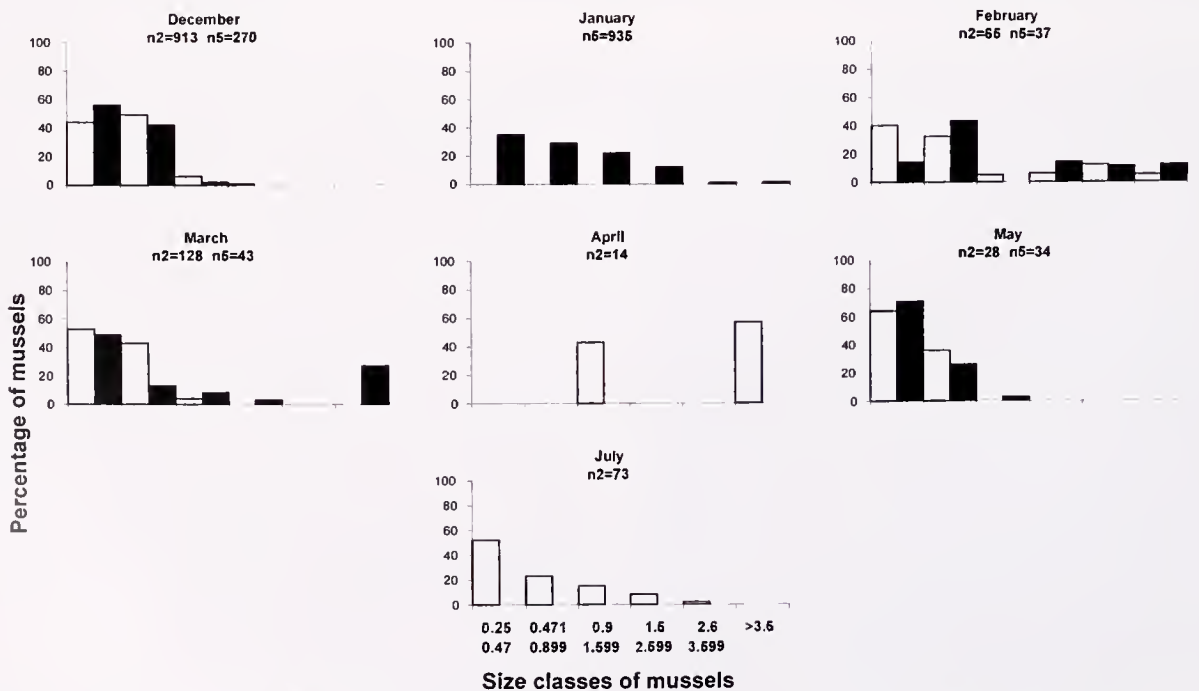


Figure 7. Size distribution in percentage of mussels settled on collectors L placed at 2- (open bars) and 5-m (filled bars) depth, during a study period in Bahía de Todos Santos, México.



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## INDUCTION OF SETTLEMENT AND METAMORPHOSIS OF THE SCALLOP *ARGOPECTEN PURPURATUS* LAMARCK BY EXCESS $K^+$ AND EPINEPHRINE: ENERGETIC COSTS

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**ABSTRACT** Settlement and metamorphosis of marine invertebrate larvae is known to be triggered by specific environmental cues. Neuroactive compounds, particularly some monoamines, have been implicated in this process, and depolarization of receptor cell membranes has been suggested to occur as a response to them. An increase of extracellular  $K^+$  in seawater has been used as an effective inducer of these processes for some species. This study describes work designed to assay effects of epinephrine and excess  $K^+$  as inducers of settlement and metamorphosis of larvae of the scallop *Argopecten purpuratus*. Epinephrine and excess  $K^+$  increased the percentages of settlement, metamorphosis, and survival of these larvae. Responses were dose-dependent, with a maxima under  $10^{-5}$  M (epinephrine) and 10 mM ( $K^+$ ). In the case of epinephrine, the responses did not vary significantly with the time of exposure. An analysis of size and energy content of larvae induced to metamorphosis by the different methods showed that larvae induced with epinephrine produced postlarvae that were significantly smaller in size and energetically weaker than postlarvae produced using excess  $K^+$  or no added exogenous inducer.

**KEY WORDS:** *Argopecten purpuratus* larvae, metamorphosis, scallops, settlement

### INTRODUCTION

The scallop *Argopecten purpuratus*, as do many benthic marine invertebrates, produces pelagic larvae that spend days or weeks in the plankton before settling and metamorphosing into adult forms. Important physiological, morphological, and biochemical changes occur during the transition from pelagic to benthic existence. Settlement and metamorphosis processes are triggered by larval sensory recognition of, and responsiveness to, exogenous chemical and other environmental stimuli (Morse 1990). Various types of settlement-inducing cues have been described, including: (1) *physical* [e.g., illumination, physical texture (Hadfield and Pennington 1990), vibration (Rittschof et al. 1998)]; (2) *biological* [e.g., conspecific individuals, microbial films, prey species (reviewed by Rodriguez et al. 1993)]; and (3) *chemical* [e.g., cues of natural or artificial origin (Yool et al. 1986)]. Many of these chemical cues are compounds whose roles as neurotransmitters are broadly known (Rodriguez et al. 1993) although other chemically similar substances and some fatty acids have also been described to induce settlement and metamorphosis (Pawlik 1988, Kitamura et al. 1993). The ability of epinephrine to induce settlement and metamorphosis has been reported by Coon et al. (1985) (*Crassostrea gigas*); Beiras and Widdows (1995) (*C. gigas*), Kingzett et al. (1990) (*Patinopecten yessoensis*), Tan and Wong (1995) (*C. belcheri*), Chevolut et al. 1991, and Nicolas et al. (1996) (*Pecten maximus*).

It has been assumed that the inducers act on external cellular receptors somewhere on the larva (Hadfield and Pennington 1990). The rapidity and cascade of events in settlement and metamorphic induction suggest the existence of a preformed larval nervous system capable of detecting a specific signal (Hadfield and Pennington 1990, Fenteany and Morse 1993). Although precompetent larvae may have receptors, these may be not sufficient to induce settlement behavior; attainment of competency may be determined by the accumulation of a threshold number of receptors (Barlow 1990).

It is known that the response of receptor cells to an appropriate stimulus is the depolarization of specialized cells. On this basis, Baloun and Morse (1984) have suggested that the perception of inductive cues by larvae may rely on the stimulus-mediated depolarization of cells in a sensory-inductive pathway. Given the knowledge that an increase of extracellular  $K^+$  induces membrane depolarization, several authors have successfully assayed the effects of an excess of this ion in seawater as an inducer of larval settlement and metamorphosis (Baloun and Morse 1984, Yool et al. 1986; Inestrosa et al. 1993a, Campos et al. 1994; Pechenick et al. 1995). The molecular events mediating the inducer-receptor interaction and the hypothesized membrane depolarization are not understood, but it has been suggested that a possible second messenger such as cAMP and  $IP_3$  may participate (Leitz and Müller 1987, Morse 1990; Baxter and Morse 1992; Inestrosa et al. 1993b, Clare et al. 1995). These messengers might regulate ion channel activity (Wickman and Clapham 1995). Besides regulating membrane permeability to ions, these messengers are known to increase the activity of several catabolic enzymes through phosphorylation (Krebs 1985), a fact that must be considered when a compound is chosen for experimental induction of metamorphosis.

Metamorphosis is an energetically costly process (Holland and Spencer 1973, Lucas et al. 1979). Storage reserves of energy-rich organic substrates are usually observed to increase before metamorphosis (Lucas et al. 1986). Studies to this point have focused on variations in the composition of energy reserves and measurements of oxygen consumption during this process (Holland and Spencer 1973, Lucas et al. 1979, Rodríguez et al. 1990, Shilling et al. 1996).

The present study evaluated the effects of excess  $K^+$  and epinephrine as inducers of settlement and metamorphosis in the pectinid *Argopecten purpuratus*. Because epinephrine is a powerful catabolic stimulant, it was important to evaluate the comparative energy costs between individuals stimulated to metamorphosis by epinephrine and those stimulated by excess  $K^+$  (plus nontreated

controls). A null hypothesis was tested that there was no difference in energy cost to the larvae or postlarvae between treatments.

The practical importance of this work is that there has been interest on the part of commercial scallop hatchery managers in obtaining reagents for the massive induction of metamorphosis in cultured scallop larvae destined for aquaculture growout. If a safe and reliable chemical inducer were available, it would significantly reduce production and labor costs and add reliability to this newly evolving industry.

## MATERIAL AND METHODS

### *Assay of Larval Settlement, Metamorphosis, and Survival*

Larvae of *A. purpuratus* were obtained from a commercial scallop hatchery in Tongoy Bay, Chile (30°S). Larvae were mass cultured using methodology similar to that of DiSalvo et al. (1984) and transferred to our laboratory for experimentation at a stage when they were entering competence for metamorphosis (length near 200  $\mu\text{m}$ , with presence of eyespot and foot). Larvae were maintained in 0.45  $\mu\text{m}$  filtered seawater at 20°C and fed daily with mixture of the microalgae *Isochrysis galbana* (T-iso), *Pavlova lutheri*, *Chaetoceros calcitrans*, and *Chaetoceros gracilis*.

Experimental configuration included the use of six 1-L plastic containers, each fitted with rippled plastic plates (ca. 150  $\text{cm}^2$ ) to increase the surface area for larval settlement. Each replica contained 800 mL of test water containing 1,500–1,600 larvae. Test solutions included 0, 5, 10, 20, and 30 mM  $\text{K}^+$  ion, above the normal concentration in local seawater (9 mM); epinephrine concentrations were  $10^{-6}$ ,  $10^{-5}$ , and  $10^{-4}$  M, produced by dilution of a stock solution of the amine diluted in 0.0005 N HCl. Larvae were exposed to test solutions for 48 hours, after which, the free larvae were suspended into fresh, filtered seawater; settled and metamorphosed larvae were quantified in three of the replicates. The experiment was then continued for 96 hours with the remaining replicates, with daily changes of filtered seawater and quantification of settled and metamorphosed larvae at required time intervals. Unattached, living larvae were also included in counts to account for all surviving individuals. Larvae were considered settled when swimming ceased, they had settled on container and plate surfaces and could not be detached by gentle washing with fresh seawater. Larvae were considered metamorphosed if the velum had been resorbed and if they showed development of the tentidium and deposition of dissoconch.

### *Effect of Exposure Time*

A second set of experiments was carried out to determine how the time of exposure to excess  $\text{K}^+$  or epinephrine could affect the response to these signals. Using the same experimental configuration described above, and the optimal inducer concentration then obtained, groups of competent larvae were exposed for periods of up to 96 h (excess  $\text{K}^+$ ) or 48 h (epinephrine). At each time interval (see Fig. 3), larvae were rinsed and placed in fresh, filtered seawater. After 144 h, metamorphosed, attached, and unattached organisms that remained alive were counted to calculate final percentages of survival and metamorphosis.

### *Comparative Energetic Cost Between Inducers*

Energy depletion during metamorphosis induced by the two different treatments was determined by direct calorimetry of the

test organisms. This measurement was carried out in triplicate for each treatment. For each replicate, 80,000 larvae were placed with filtered seawater in 10-L plastic containers, where they were exposed to either 10 mM  $\text{K}^+$  or to  $10^{-5}$  M epinephrine over a period of 24 h. After this, the medium was changed to filtered seawater and maintained with daily changes of water for 6 days, at which time most larvae seemed to have passed metamorphosis. Postlarvae were then removed from the containers with a small paintbrush, and collected on 250- $\mu\text{m}$  mesh plastic screen. These organisms were washed with isotonic ammonium formate, dried to constant weight, made into pellets, and ignited in an OSK calorimeter. Sub samples were used to count the postlarvae and measure their lengths.

Results were analyzed using one-way analysis of variance (ANOVA) and a Tukey test was applied to evaluate probable differences ( $p < .05$ ) between treatments. In the case of percentage values, these were subjected to an arc-sin transformation for calculations.

## RESULTS

### *Effects of $\text{K}^+$ Concentration*

After 48-h exposure to increased external  $\text{K}^+$ , the percentage of larvae settled with 10 mM was statistically higher (Tukey,  $p < .001$ ) than that of larvae incubated under normal  $\text{K}^+$  (Fig. 1.A); however, very few of the larvae had passed metamorphosis (Fig. 1.B), with no statistical difference between percentages of the different experimental groups at this time. At 144 h (96 h after removal of larvae from inductor solutions), almost half of the larvae from the 10 mM treatment had passed metamorphosis, with values for larvae from other concentrations either near or below those obtained with no treatment (Fig. 1.B) The survival of larvae submitted to 10 mM excess  $\text{K}^+$  was significantly higher than that of the other experimental groups (Fig. 1.C).

### *Effects of Epinephrine*

After 48 h in different concentrations of epinephrine, it was shown that the highest percentages of larval settlement were obtained with  $10^{-6}$  and  $10^{-5}$  M (Fig. 2.A).

Although few larvae had metamorphosed after 48 h with epinephrine, significantly higher values were observed at  $10^{-6}$  and  $10^{-5}$  M (Fig. 2.B). On the following days, the number of larvae that metamorphosed increased, and at 144 h (48 h after removal of larvae from the inductor solutions), nearly all the settled larvae had passed metamorphosis, with the most effective concentrations having been  $10^{-6}$  and  $10^{-5}$  M (Fig. 2.B). Survival of larvae submitted to  $10^{-6}$  and  $10^{-5}$  M treatments was significantly higher than that of the other experimental groups (Fig. 2.C).

### *Effect of Exposure Time*

When 10 mM excess  $\text{K}^+$  was used to induce metamorphosis, the percentage of larvae that metamorphosed increased as the time of exposure increased, from 6 to 48 hours. No greater increase was detected when larvae were under this treatment for 96 hours (Fig. 3.A). In the case of larvae exposed to  $10^{-5}$  M epinephrine, from 6 to 48 hours, the time of exposure to this amine did not affect either the percentage of mortality or that of metamorphosis of larvae (Fig. 3.B).

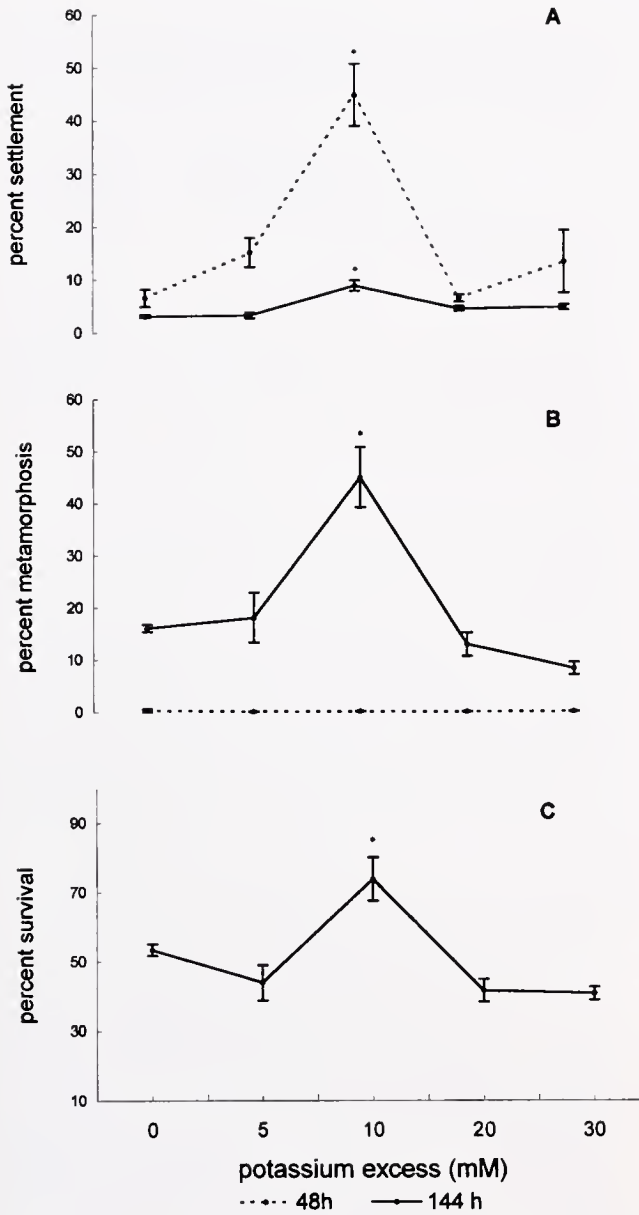


Figure 1. Percentages of *A. purpuratus* larvae that settled (A), metamorphosed (B), and survived (C) in different concentrations of excess K<sup>+</sup>. Values represent the means ± SE of groups of larvae from three replicate treatments; (\*) significantly different from the other values (Tukey's test, p < .05).

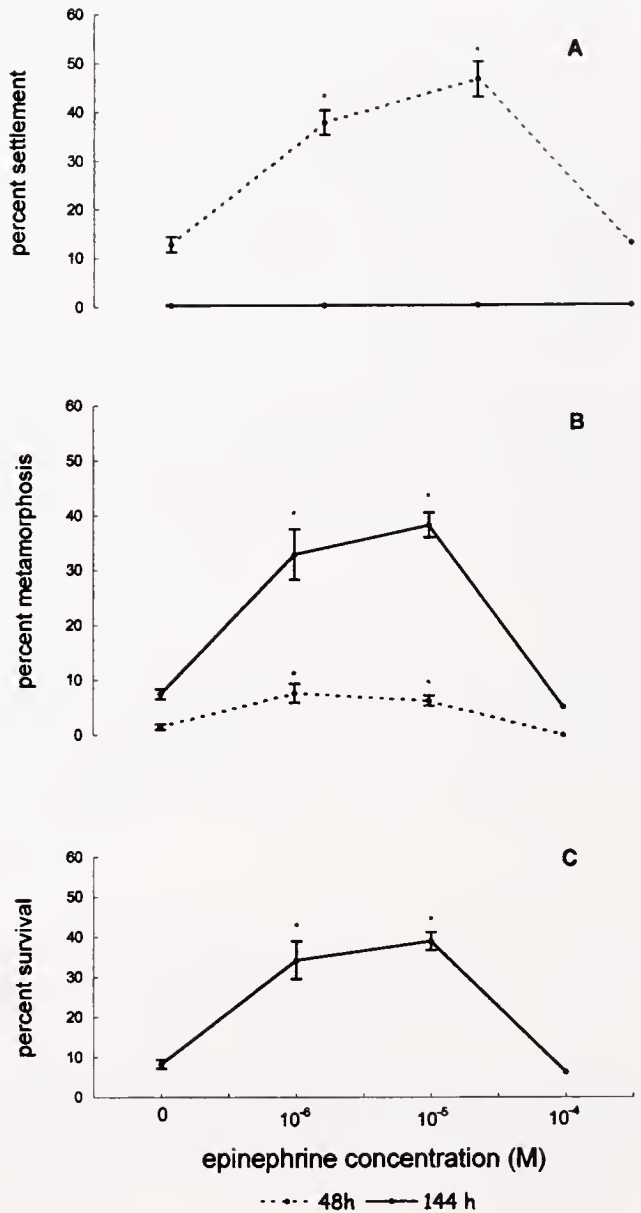


Figure 2. Percentages of *A. purpuratus* larvae that settled (A), metamorphosed (B), and survived (C) in different concentrations of epinephrine. Values represent the means ± SE of groups of larvae from three replicate treatments; (\*) significantly different from the other values (Tukey's test, p < .05).

*Energetic Analysis of Larvae Induced to Metamorphosis*

As it is seen in the Table 1, the energy content of *A. purpuratus* post larvae decreased to less than half the values they had previous to metamorphosis. In the case of larvae induced to metamorphosis by excess K<sup>+</sup>, the decrease in energy content was similar to that of the control group; when epinephrine was used as an inducer, the decrease was considerably greater. Although postlarvae showed considerably increased size over the larvae, no significant difference was found between the groups of larvae induced to metamorphosis by any of the three different treatments.

DISCUSSION

Raised levels of the K<sup>+</sup> ion have not previously been demonstrated as an inducer of metamorphosis in *A. purpuratus* larvae. The present results now add this species to a group of other mollusks that have been shown to be induced to settle and metamorphose by this ion. An early study was that of Baloun and Morse (1984), who showed this effect on larvae of *Haliotis rufescens*. They showed that the larval response to  $\gamma$ -aminobutyric acid (GABA) may be inhibited by a decrease in external K<sup>+</sup>. Yool et al. (1986) showed that an increase in the concentration of this ion induced settlement and metamorphosis in larvae of the mollusks

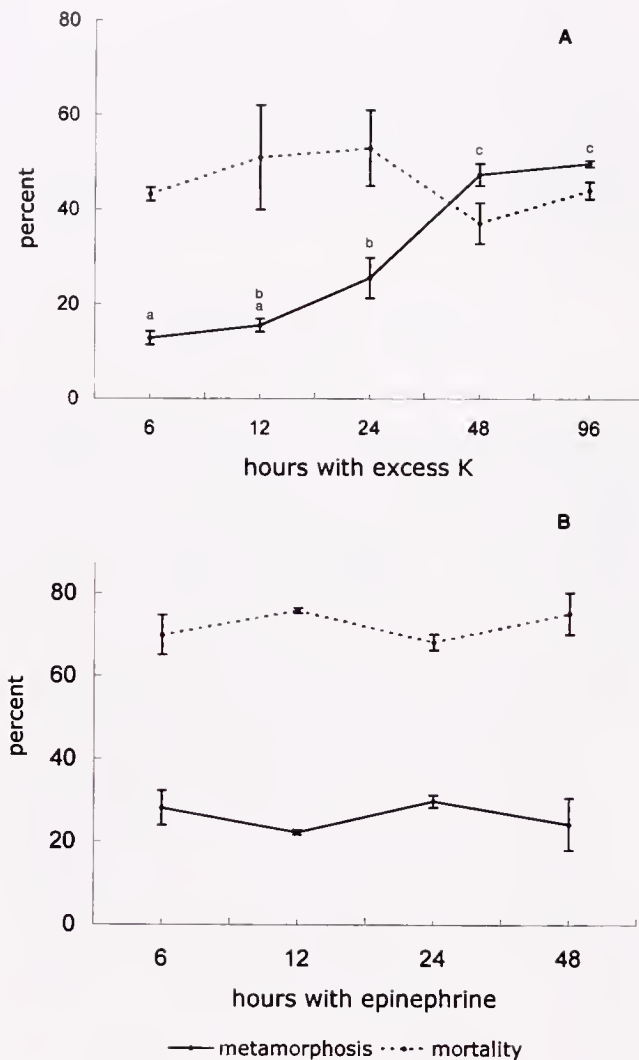


Figure 3. Effect of exposure time to 10 mM excess K<sup>+</sup> (A) and to 10<sup>-5</sup> M epinephrine (B) on metamorphosis and survival of competent larvae of *A. purpuratus*. Values represent means  $\pm$  SE of groups of larvae from three replicate treatments. Means with same letter are not significantly different (Tukey's test,  $p < .05$ ).

*Phestilla sibogae*, *Haliotis rufescens*, and *Astraea undosa* and in larvae of the marine annelid *Phragmatopoma californica*. Positive results were also shown for the mollusk *Concholepas concholepas* by Inestrosa et al. (1993a). In each case (as now with *A. purpuratus*), the effect of potassium has been dose dependent, although optimal inductive dose may vary among species.

Present results also show that, as time of exposure to excess K<sup>+</sup> increases, up to 48 hours, the percentage of *A. purpuratus* larvae that metamorphose also increases. In this respect, our results agree with those of Baloun and Morse (1984), Yool et al. (1986), and Inestrosa et al. (1993a). These authors showed that, not only different doses of excess K<sup>+</sup>, but also cumulative time of exposure, produced a progressive increase in larval metamorphosis in the species cited above.

The present results have shown that 10<sup>-6</sup> and 10<sup>-5</sup> M epinephrine were the optimal concentrations for significantly increasing the percentage settlement and metamorphosis of *A. purpuratus* larvae; a higher dose (10<sup>-4</sup> M) did not show any significant effect.

TABLE 1.

Size and energy content of *Argopecten purpuratus* larvae before and after metamorphosis induced by 10<sup>-5</sup> M epinephrine or by 10 mM excess potassium.

	Energy Content (mJoule/Larva)	Larvae Length ( $\mu$ m)
Before metamorphosis		
Competent larvae	4.0113 $\pm$ 0.2665	216.47 $\pm$ 10.57
After metamorphosis		
Control	1.6983 $\pm$ 0.0516	406.00 $\pm$ 33.68
Induced by epinephrine	0.5168 $\pm$ 0.0368*	364.17 $\pm$ 26.51
Induced by excess K <sup>+</sup>	1.6477 $\pm$ 0.108	399.55 $\pm$ 25.69

Values are mean  $\pm$  SD (n = 3).

\* Significantly different from control ( $p < 0.001$ ).

These values do not agree with those described for other bivalve species, which are also induced by this monoamine. Kingzett et al. (1990) assayed three concentrations of epinephrine (10<sup>-6</sup>, 10<sup>-5</sup>, and 10<sup>-4</sup> M) on larvae of the scallop *Patinopecten yessoensis* and showed similar increases in percentage metamorphosis with each dose. In the case of the scallop *Pecten maximus*, epinephrine exhibited an optimal action between 0.5 to 2.5  $\times$  10<sup>-4</sup> M (Chevolot et al. 1991, Nicolas et al. 1996). Coon et al. 1985, Coon et al. 1986, and Beiras and Widdows (1995) have described maximum inductive activity in the oysters *Crassostrea virginica* and *C. gigas* with 10<sup>-4</sup> M epinephrine. Tan and Wong (1995) described 10<sup>-5</sup> M as the best concentration of epinephrine to induce metamorphosis in the oyster, *C. belcheri*. Larvae of the polychaete *Phragmatopoma lapidosa californica* did not respond to epinephrine assayed at any subtoxic concentration. Thus, sensitivity to inducer compounds may vary from one species to another. Moreover, in the assays described for oysters, most of the metamorphosed individuals induced by epinephrine were "unattached" spat (Coon et al. 1985, Coon et al. 1986, Beiras and Widdows 1995), a fact that was not observed for pectinids. Pawlik (1990) has suggested that different routes to metamorphic activation may be involved in the responses to different cues by the species.

It is apparent in the present data that metamorphosis is more rapidly triggered by the monoamine than by excess K<sup>+</sup>. When epinephrine was used as an inducer cue on *A. purpuratus*, a significant number of individuals metamorphosed within the first 48 hours. On the contrary, in that period, no larvae had metamorphosed using excess K<sup>+</sup>. Pechenik and Gee (1993) and Pechenik et al. (1995) have shown that larvae of the gastropods *Crepidula fornicata* and *Phestilla sibogae* become responsive to excess K<sup>+</sup> at a different time than to "natural cues," suggesting possible different sites of action of the inducers. In the case of *P. sibogae*, larvae become responsive first to the natural cue before they do to excess K<sup>+</sup>; the authors suggested that this ion acted internally rather than directly on surface receptors, showing increasing accessibility to those internal sites as larvae age. The assays of exposure time of *A. purpuratus* larvae to the different cues, agree with the preceding concept. It was shown that, as the time with excess K<sup>+</sup> increases, more larvae pass metamorphosis. Meanwhile, using epinephrine, the percentage of metamorphosis did not increase with exposure time and even was less than the maximum obtained using excess K<sup>+</sup>.

The use of exogenous inducers for metamorphosis has pres-

ently been shown to increase the survival of *A. purpuratus* individuals after metamorphosis. This may be of practical consequence for use in commercial production of hatchery "seed." However, in the case of individuals that had been induced to metamorphose by epinephrine, the energy content of postlarvae was significantly lower than that of individuals that had been treated with excess K<sup>+</sup> or had metamorphosed without any added inducer.

Depolarization of externally accessible, excitable cells has been suggested to be a mechanism common to the induction of settlement and metamorphosis for a number of species (Baloun and Morse 1984, Yool et al. 1986). It is known that the mechanism of action of neurotransmitters, many of which have been reported to induce settlement and metamorphosis of larvae (Rodríguez et al. 1993), is through changing the membrane permeability, and then (Wickman and Clapham 1995) changing the degree of polarization of the cell. The mechanism by which some of these neurotransmitters change this permeability is unclear. These compounds may, through G proteins, increase the concentration of any second messenger (cAMP, IP<sub>3</sub>, Ca<sup>2+</sup>), triggering the phosphorylation of some key proteins (Krebs 1985, Ho 1994). Some of these proteins may be intrinsic to membranes playing a part or a total role as an ion channel, and therefore, its phosphorylation may be changing the corresponding ion conductance of the membrane (Wickman and Clapham 1995). In addition to membrane proteins, there are some enzymes that are phosphorylated, changing their activity (Krebs 1985). Many of the enzymes whose activity is enhanced by phosphorylation are catabolic (e.g., glycogen phosphorylase, lipases). Such a cascade of events, could explain how, when neu-

rotransmitters are used to induce the metamorphosis of invertebrate larvae, the normally high energy cost of the process may be further incremented, perhaps to the point of stress.

Existence of a common mechanism for the inductive effects of potassium and neurotransmitters action is not probable. The effects of K<sup>+</sup> may be explained in terms of their ability to change membrane potential. It is well known that potassium gradients through cell membrane determine the degree of cellular polarization. This type of effect should not mobilize energy reserves of individuals over the degree expected in normal (noninduced) metamorphosis. In conclusion, we reject the hypothesis that there was no difference between treatments, having observed significantly greater energy depletion in organisms treated with epinephrine.

In light of possible anomalous effects of membrane depolarization (K<sup>+</sup>), or possible energy stress associated with neurotransmitters, it is not possible at this time to recommend the practical use of chemical inducers in scallop hatcheries, because the simple measurement of percentage of metamorphosis is not indicative of several other factors that may affect viability of the larvae.

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## EVIDENCE FOR FALL SPAWNING OF NORTHERN BAY SCALLOPS *ARGOPECTEN IRRADIANS IRRADIANS* (LAMARCK 1819) IN NEW YORK

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**ABSTRACT** Spawning of *Argopecten irradians irradians* is generally believed to occur between late May to August; however, some literature reports and anecdotal observations have suggested that ripe individuals may be present well into the fall. This paper reports on evidence for fall spawning of bay scallops that we sampled from different populations in Long Island, New York, waters, in different years. At two sites, a spawning peak in September followed a discrete spawning peak in early summer (late June/early July). Scallops at one or more of four different sites were conclusively shown to spawn well into the fall (late September, October, or early November) during 3 different years: one in which a brown tide (*Aureococcus anophagefferens*) algal bloom occurred (1995) and during nonbrown tide years (1993, 1994). Our work, coupled with reports of other researchers, suggests that fall spawning of *A. i. irradians* in NW Atlantic waters does not seem to be uncommon and may be important in some populations during particular years.

**KEY WORDS:** *Argopecten*, spawning, New York, reproduction, scallop, seasonality, brown tide

### INTRODUCTION

Much of the basic biological knowledge of the northern bay scallop, *Argopecten irradians irradians* (Lamarck 1819), is based on the early work of Risser (1901) and Belding (1910). These authors determined that the typical lifespan of this subspecies is 18–22 months; however, some individuals may reach the age of 3 years (Marshall 1960, S. Tettelbach, pers. obs.). Bay scallops are hermaphroditic and generally are regarded as semelparous, although Belding (1910) estimated that 10–20% of a given year class may survive to spawn in 2 successive years if not removed by the fishery. Bricelj et al. (1987a) reported that perhaps 30% of caged scallops at one site in Long Island, NY survived to spawn during their second year.

Spawning of *Argopecten irradians irradians* over its natural geographic range is generally believed to occur between late May to mid-August (see Table 1), as water temperatures are rising (Sastry 1963). Spawning of northern bay scallops has been described by some authors as a single, catastrophic event; whereas, others report spawning over much of the summer (see Table 1), sometimes with a distinct secondary or tertiary peak later in the period (MacFarlane 1991). A few authors have reported early September spawning in Massachusetts (Kelley and Sisson 1981, Taylor and Capuzzo 1983, Hampson and Capuzzo 1984). Kelley and Sisson (1981) surmised, on the basis of scallop seed sizes, that spawning occurred after September in Nantucket Island, MA. MacFarlane (1991) concluded, on the basis of gross observation, that scallops in Orleans, MA spawned into October during 1980. The belief is still widely held, however, that spawning does not occur past early

September; hence, sampling in many studies has been terminated prior to the fall.

This paper reports on evidence for fall spawning of *Argopecten irradians irradians* that we obtained through the analysis of temporal patterns of bay scallop reproduction in different populations in Long Island, NY waters, during different years. This work was prompted by anecdotal observations made by baymen and our personal observations of visually ripe scallops in the Peconic Bay system during fall months between 1992 to 1996. Histological analyses on archived bay scallop gonadal samples were performed to determine if there was any concrete evidence of spawning between September to November during 1993 to 1995. The 1995 data also provided the opportunity to determine whether bay scallops were able to spawn during a bloom of brown tide, *Aureococcus anophagefferens* (Sieburth et al. 1988).

### MATERIALS AND METHODS

Analyses of reproductive activity primarily focused on three groups of scallops transplanted from natural populations in eastern Long Island, NY during 1994 and 1995. During 1994, scallops were dredged from Northwest Harbor (NWH) on 25 April and 1 May and free-planted directly on the bottom (density = 9.6/m<sup>2</sup>) to the south of Red Cedar Point (RCP) in Flanders Bay (Fig. 1). In 1995, scallops were dredged from Sag Harbor and either deployed in rafts (density = 80.7/m<sup>2</sup>), on 2 May, in East Creek (EC), South Jamesport, NY or free-planted (density = 5/m<sup>2</sup>) on 4 May to the south of RCP (Fig. 1). These three groups of scallops were monitored as part of broader studies (Smith and Tettelbach 1996, Smith

TABLE 1.

Summary of previous studies assessing temporal patterns of reproduction in populations of northern bay scallops (*Argopecten irradians irradians*) in waters of the northeastern United States, in order of decreasing latitude.

Location	Spawning Period	Peak Spawning Period(s)	Depth (m)	Water Temp. (°C)	Year(s)	Means of Assessment	Source
Pleasant Bay, MA	Late May to mid-Oct	June, Aug, Sept	1-3		1980	GO, H	MacFarlane 1991
Buzzards Bay, MA	Early June to early Sept		9		1981,82	H	Hampson & Capuzzo 1984
Buzzards Bay, MA	June to August		2		1981,82	H	Hampson & Capuzzo 1984
Waquoit Bay, MA	Late May to early Sept	June-July	0.3	18-29	1979	H	Taylor & Capuzzo 1983
Massachusetts	Mid-June to mid-Aug	Early to mid-July		≥16.5	<1910	GO, MO	Belding 1910
Woods Hole, MA	Mid-July to late Aug	August			1965	GI, H	Sastry 1970
Narragansett Bay, RI	June to July	Mid-June			<1901	GO	Risser 1901
Nantucket Hbr, MA	Mid-June to early Sept	Late June to early July			1980	GO, S, L, H	Kelley & Sisson 1981
Niantic R., CT	Mid-June to late July	Mid-June to July		19-28	1955	GO, S	Marshall 1960
Poquonock R., CT	Mid-June to August	Mid-June to early July	0.3-0.8	17-24	1983,84	GO, S	Tettelbach 1991
Lake Montauk, NY	Early June to August	Mid-June to early July	0.6-2.6	-17-23	1974	GI, L	Hickey 1977
Accabonac Hbr, NY	?late May to August	Early June	0.6-2.3	-17-23	1974	GI, L	Hickey 1977
Three Mile Hbr, NY	Early June to July	Mid-June	0.6-3.3	-17-23	1974	GI, L	Hickey 1977
Three Mile Hbr, NY	Early June to Aug	Early June	2	≥16-26	1984	GI, GW	Bricelj et al. 1987b
Northwest Hbr, NY	Early June to Aug	Early to mid-June	2	≥16-25	1984	GI, GW	Bricelj et al. 1987b
Northwest Hbr, NY	Early June to Aug	Early to late June	3.5	≥15-24	1984	GI, GW	Bricelj et al. 1987b
Sag Hbr, NY	Late May to Aug	Late May to mid-June	3.5	≥14.5-27	1984	GI, GW	Bricelj et al. 1987b
Flax Pond, NY	Late June to mid-July	Late June to mid-July	2	19-23	1985	GI, H	Epp et al. 1988; Epp 1989

All analyses are based on samples taken from natural populations, except for Flax Pond. At the latter site, scallops were held in cages on the bottom, after being dredged from Northwest Harbor, New York. Means of Assessment: GO = gross observation; MO = microscopic observation; GW = gonad weight; GI = gonad index; H = histology, with oocyte measurement (Oo); L = larval abundance; S = spat abundance (partially adapted from Barber and Blake 1991).

and Tettelbach 1997) intended to evaluate different reseeding techniques (see Tettelbach and Wenzel).

Temporal changes in reproductive condition were evaluated by means of analyses of gonad dry weights (GDW) and gonad indexes (GI) (Barber and Blake 1991), as well as by histological examination (see below). For each individual, shell height was measured to the nearest mm, and then the gonad was dissected proximal to the foot (so the foot remained attached to the excised

gonad). For the GDW and GI analyses, the gonad and remaining tissues were weighed separately for each individual after they were dried to a constant weight ( $\geq 48$  h) at  $\sim 82^\circ\text{C}$ . A GI value was calculated for each individual, as follows:  $\text{GI} = \text{Gonad Wt (g)} \times 100 / [\text{Gonad Wt (g)} + \text{Remaining Tissue Wt (g)}]$  (Barber and Blake 1991). Temporal changes in mean GDW and GI values were examined to determine if spawning had occurred, as inferred from a significant drop in GDW or GI. At the times when scallops were

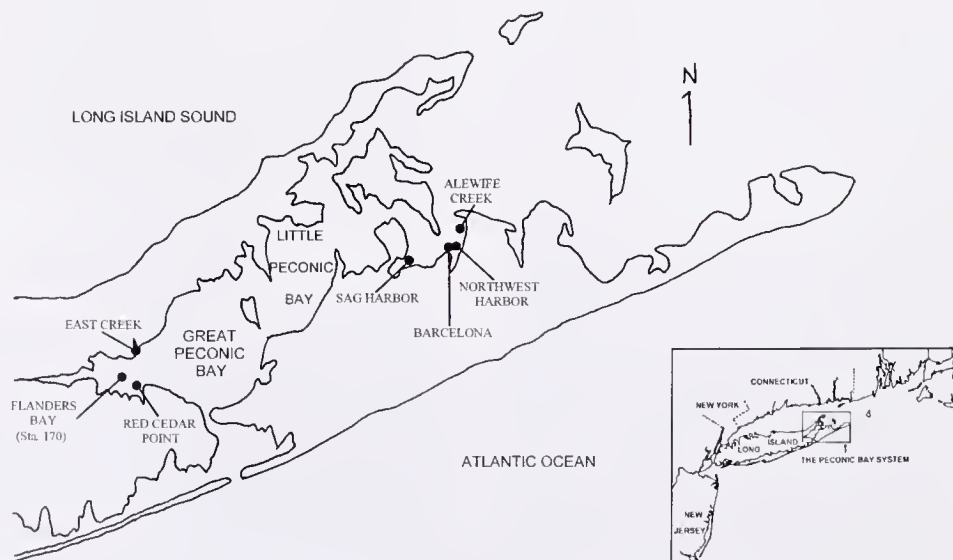
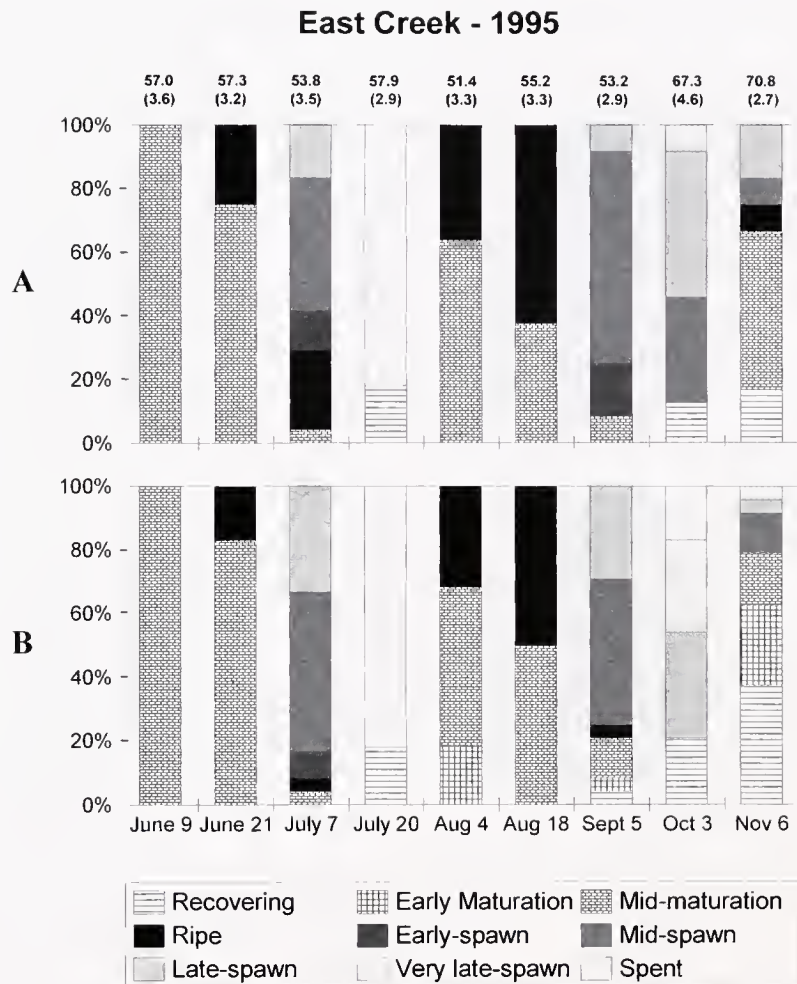


Figure 1. Map of the Peconic Bay system in eastern Long Island, New York, USA, showing the location of bay scallop and *Aureococcus* sampling sites.



**Figure 2.** Temporal change in reproductive condition of bay scallops (male, A, and female, B, gonadal portions) deployed in rafts in East Creek, South Jamesport, New York during early May 1995. Percentage of individuals in a given gonadal stage were determined by histological analysis ( $n = 11\text{--}12$  scallops per date) and scored using a modification of Naidu's (1970) method: stage 4 = recovering from spawning; stage 5 = early maturation of eggs and sperm; stage 6 = midmaturation of eggs and sperm; stage 7 = mature eggs and sperm (ready to spawn); stage 8 = spawning, where: 8e = early-spawn, (<25% of eggs or sperm were spawned); 8m = midspawn, (25–85% of eggs or sperm were spawned); 8l = late spawn, (85–95% of eggs or sperm were spawned); 8vl = very late spawn, (between 96–100% of eggs or sperm were spawned); stage 9 = spent (immediately postspawn). Bay scallop shell height data [mean, (SD)] are given at the top of the figure.

transplanted from Northwest Harbor to RCP (29 April 1994) and from Sag Harbor to EC and RCP (2 May 1995), GI values (13.59 and 15.25, respectively) were marginally different ( $t = 2.14$ ,  $p = .04$ ,  $n = 20$  and 25, respectively, for 1994 and 1995).

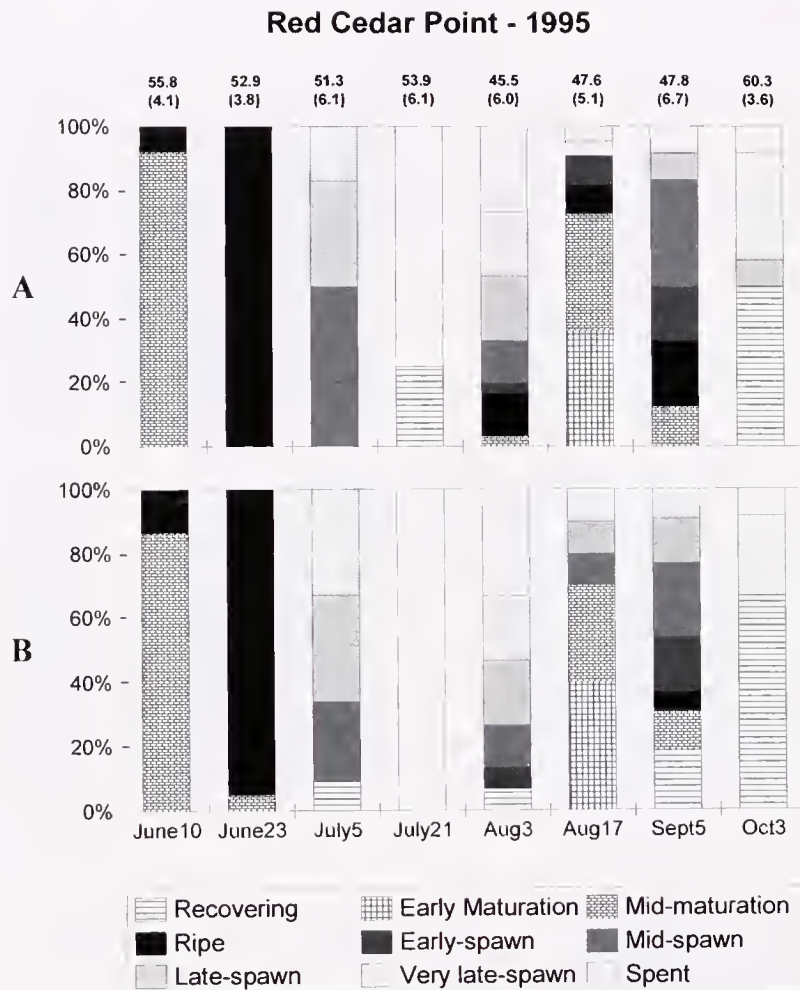
During 1995, GDW and GI data ( $n = 23\text{--}25$  scallops per sample) were collected biweekly between late May to midAugust (RCP) or early September (EC); histological sampling ( $n = 10\text{--}15$  scallops per sample) started in early June, then followed the same schedule. Additional histological samples were collected monthly from early September through early October (at RCP) or November (at EC). (On 21 July 1995, only, it was also necessary to include in the histological analysis scallops that were transplanted to RCP on 25 May.)

During 1994, initial sampling of GDW and GI was done in late April; then GDW, GI, and histology samples were taken biweekly from around late May through late September. GDW/GI sample size was 13–20 scallops, except on 25 May 1994 ( $n = 8$ ). For the 1994 histological analyses, sample size ranged from two (25 May only) to nine scallops, but was usually six.

Eight scallop gonads sampled in 1993 from natural populations in NWH were also examined histologically to provide additional information on the possible occurrence of fall spawning. These samples were taken opportunistically (i.e., tissues were only archived from scallops that visually appeared to be very ripe (ovarian portion of gonad was bright orange with evident veins)) during October and November off Barcelona Neck and south of Alewife Creek, respectively (Fig. 1) and preserved in 70% ethanol.

The methods employed for the fixation and preservation of scallop gonadal tissues for histological analyses (total  $n = 276$ ) followed procedures described by Humason (1979). After fixation, gonadal tissues from each scallop were processed in paraffin. Six micron ( $6\ \mu\text{m}$ ) sections were cut and stained with hematoxylin and eosin using standard histologic methods (Humason 1979). These cross sections (along the dorsoventral axis) were taken  $\sim 1/3$  of the way from the proximal and distal end of each gonad. The proximal and distal end sections contained predominantly male and female tubules, respectively.

Gonadal developmental stages (see Figs. 2–4) were scored via



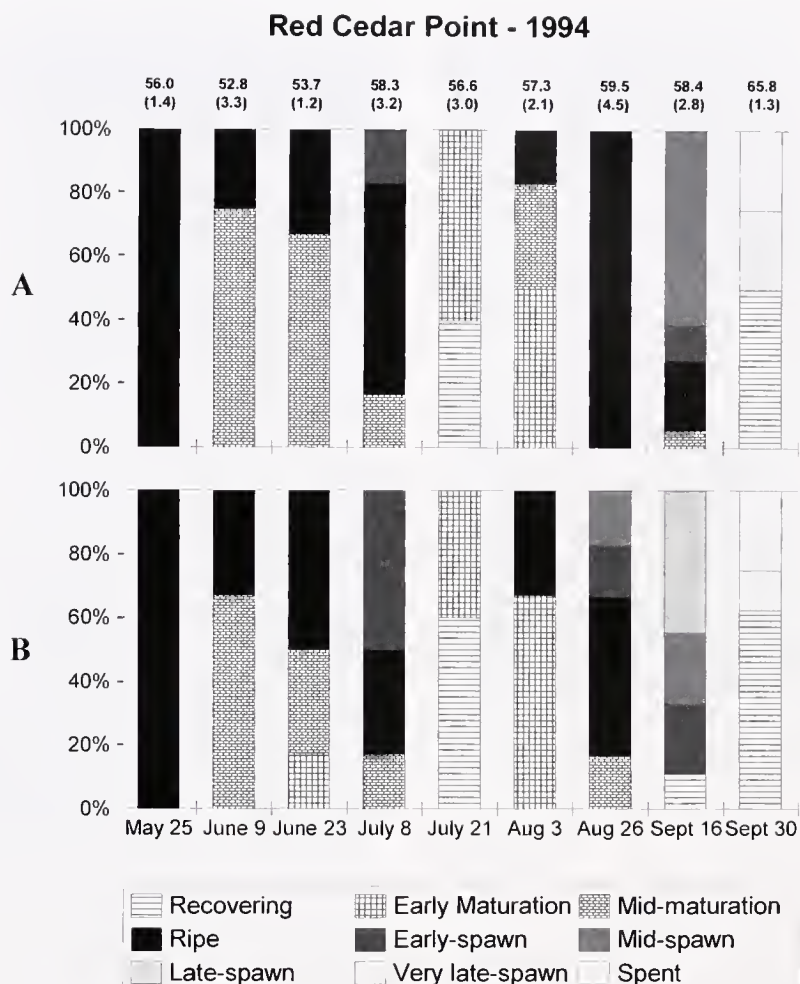
**Figure 3.** Temporal change in reproductive condition of bay scallops (male, A, and female, B, gonadal portions) free-planted to the south of Red Cedar Point in Flanders Bay, New York during early May 1995. Gonadal stages were determined by histological analysis ( $n = 10\text{--}15$  scallops per date) and scored using a modification of Naidu's (1970) method: gonadal stages, scallop shell size data as given in Figure 2.

traditional, subjective tissue evaluation methods performed by a trained pathologist (RS), using a modification of Naidu's (1970) method. In the present study, stage 8 of Naidu (1970) was divided into four substages: 8e (early spawn) in which fewer than 25% of the eggs or sperm were spawned, 8m (midspawn) in which 25 to 85% of the eggs or sperm were spawned, 8l (late spawn) in which 85 to 95% of the eggs or sperm were spawned, and 8vl (very late spawn) in which between 96 to 100% of the eggs or sperm were spawned (see Fig. 5, A to D). The percentage of eggs or sperm spawned was subjectively determined by visual examination of the stained tissue section and was based on the amount of empty space within the mature tubules resulting from the loss of spawned gametes [in stage 7 (= ripe), eggs and sperm are tightly packed within the gonadal tubules]. In cases where more than one stage was clearly evident in the male or female portion of the gonad from a given individual, partial designations were made (e.g., 8l/4).

The degree of reproductive synchrony was assessed histologically at two levels in this study: among scallops from the same sample group (= interanimal) and within the same individuals (= intra-animal). Synchronous interanimal maturation was identified when the male and female gonads of the animals examined varied little from the most common stage within that sample group (Fig.

2: 9 June female and male gonadal portions). Asynchronous inter-animal maturation was identified when numerous developmental stages were present in animals sampled at one given time (Fig. 2: 6 November female and male gonadal portions). Synchronous intra-animal maturation was most obvious in tubules of female gonads that were in stages 6, 7, and 8. For example, normal synchronous maturation in stage 7 (Fig. 6A) was characterized by centrally located mature or submature eggs occupying >85% of the tubule lumen surrounded by lesser numbers of mid- to small-sized eggs lining the tubules (Naidu 1970). Asynchronous intra-animal maturation in stages 6, 7, and 8e female gonads was characterized by variable numbers of mature (70–85%) and submature eggs centrally located in tubules associated with increased numbers of immature eggs ranging from mid- to small sizes in outer layers of the tubules (Fig. 6B). Asynchronous intra-animal developmental maturation in male gonads was defined by alternating areas, within the same tubule or different tubules, of tufts at primarily later stages of sperm development (e.g., stage 8) interspersed with tufts at an earlier stage of development (e.g., stage 6).

Water temperature was monitored through the course of the field sampling during 1993 to 1995. A continuous temperature recorder (Ryan RTM2000), deployed at the bottom [ $\sim 3$  m at mean low water (MLW)], was employed at the RCP site from May to



**Figure 4.** Temporal change in reproductive condition of bay scallops (male, A, and female, B, gonadal portions) free-planted to the south of Red Cedar Point in Flanders Bay, New York during late April 1994. Gonadal stages were determined by histological analysis ( $n = 2$  scallops on 25 May and 4–9 individuals on other dates) and scored using a modification of Naidu's (1970) method: gonadal stages, scallop shell size data as given in Figure 2.

September 1994. In 1995, continuous temperature recorders (Onset Stowaway® Model #1405) were attached to midwater nets (depth = 1–2 m at MLW) at EC and RCP (May–September) and to one of the rafts (depth = 0.15 m at MLW) at EC (May–October). Readings were taken every 0.5 h. Continuous temperature recorders were calibrated against a NITS standardized thermometer to within 0.1°C. Water temperature readings at other times and sites were taken *in situ* with a hand-held thermometer.

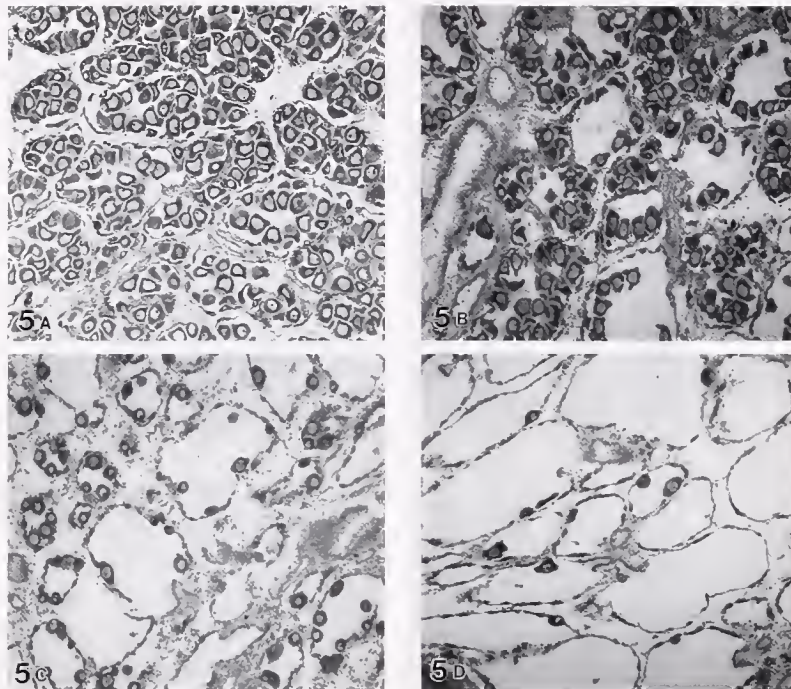
Salinity samples were taken periodically during 1994 and 1995 and analyzed in the laboratory with a Beckman induction salinometer. Between 12 April and 6 October 1994, surface salinity ranged from 24.06–28.79 ppt at a site in central Flanders Bay [Sta. #170 of the Suffolk County Dept. of Health Services (SCDHS)] (see Fig. 1), ~2200 m from our RCP site. During 1995, surface salinity at our EC site ranged from 28.21–29.99 ppt between 9 April and 5 September; surface salinity at our RCP site ranged from 27.20–29.26 ppt between 10 June and 5 September.

Cell concentrations of *Aureococcus anophagefferens* (Sieburth et al. 1988) were monitored throughout 1993 to 1995 by SCDHS. Sampling was done on a biweekly basis at East Creek (Sta. #101),  $\leq 100$  m from our EC site, and weekly at Sta. #170 (see Fig. 1). For Flanders Bay, parameters of the Tetra Tech (1997) hydrodynamic

model of the Peconic Bay system were used to calculate the average transit time for tidal movement of surface waters from SCDHS Sta. #170 to our RCP site. At an averaged residual velocity of 1.14 cm s<sup>-1</sup>, this transit time was calculated to be 69.1 h. (Tettelbach, unpubl. data). Using a maximum doubling time of 0.8 day<sup>-1</sup> (= every 30 h) for *Aureococcus anophagefferens* (based on laboratory studies by Cospser et al. 1989, Gobler 1995), cell concentrations in Sta. #170 and our RCP site would be expected to vary by a factor of no more than 2.3. For 1993, biweekly *Aureococcus* cell counts were available for SCDHS Sta. #118, ~1.5 km from our sampling sites in Northwest Harbor.

## RESULTS

Histological analyses revealed that initial spawning of the RCP scallops in 1994 and of the RCP and EC scallops in 1995 occurred between late June and early July (Figs. 2–4); most scallops were spent (immediate postspawn) by the time of the late (20 and 21) July sampling dates. In 1994, the RCP free-plant group had just begun to spawn by 8 July, at which time 17 and 50% of the male and female gonads, respectively, were in early spawning condition (stage 8e). In 1995, by contrast, 100% of the RCP free-plants were



**Figure 5.** Photomicrographs of four spawning substages of female gonads from the bay scallop (*Argopecten irradians irradians*) (25 $\times$ ); A. early spawning (8e), only a few eggs are missing from the central lumens of the tubules; B. midspawning (8m), large numbers of eggs are missing from the tubules; C. late spawning (8l), most eggs are missing from the tubular lumens; D. very late spawning (8vl), only rare eggs remain within the tubular lumens.

past early spawning condition (stages 8m–9) on 5 July. Fifty-nine and 83% of the male and female gonads, respectively, from EC raft scallops were in mid to late spawning condition (stages 8m–8l) by 7 July 1995 (Figs. 2–4).

Spawning continued after July in all three groups of scallops, but additional peaks of spawning activity occurred at different times. In both the 1994 RCP free-plants and the 1995 EC raft scallops, histological analyses revealed that gonadal maturation proceeded steadily from late July until mid to late August, and then a second spawning peak occurred between late August through late September to early October (Figs. 2, 4). In the 1995 RCP free-planted scallops, however, a second period of spawning was already in progress by early August (Fig. 3). Spawning of these animals continued through early October, although a dramatic spawning peak was not obvious in this group.

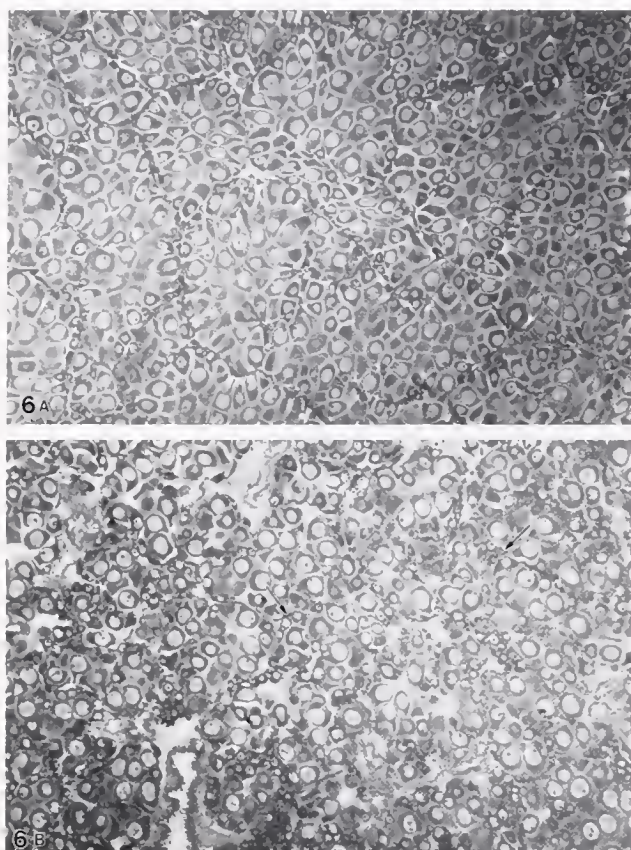
The times of spawning suggested by the GI/GDW analyses, where they overlapped with histological analyses, agreed with the above results, except for the respective periods of spawning initiation in the 1994 and 1995 RCP free-planted scallops. In the 1994 RCP group, GI/GDW trends (see Fig. 14) suggested that spawning commenced between late May and early June, rather than between late June to early July. In the 1995 RCP group, GI/GDW trends (see Fig. 13) showed a more gradual decline from peak values in late May. An analysis of variance (ANOVA) of differences in GI of RCP scallops between late May to early July 1995 ( $F = 104.65$ , 96 df,  $p < .0001$ ) and subsequent Bonferroni multiple comparisons showed that there was no significant difference between GI values on 27 May and 10 June, but these GI values were both significantly greater than those on 23 June ( $p < .001$ ), which were, in turn, significantly greater than those on 5 July ( $p < .001$ ). These GI analyses suggest that spawning of the RCP free-planted scallops in 1995 commenced between 10 and 23 June rather than between 23

June and 5 July, as suggested by the histological analyses. Possible reasons for these apparent disagreements are discussed below.

For the most part, inter- and intra-animal gonadal development stages were fairly comparable among the male and female portions of scallops sampled from a given site at a given time. Developmental stages of male and female gonadal portions from the same animal were almost always within one developmental stage of one another. The interanimal range of designated histological stages never exceeded two to three adjacent developmental stages for a given sample group at a given sampling period.

Interanimal asynchrony was evident in the female and male gonads of scallops at EC and RCP following the end of the first major spawning in late July, as compared to the period between late May to early June. Interanimal asynchrony was more pronounced in 1995 than in 1994, particularly at RCP, where it was first noted in free-planted scallops sampled on 3 August 1995. In East Creek rafts, interanimal asynchrony (first noted on 7 July 1995) was not as severe. Interanimal asynchrony became common in both groups by August and September of 1995 (Figs. 2, 3), then began to decline. Interanimal asynchrony also was observed in the 1994 RCP samples, but was mild as compared to that seen in 1995 samples.

Intra-animal asynchrony was also evident in female gonads sampled from the three groups described above. In 1995, it tended to become more apparent during midsummer to early fall (7 July–5 September) at both sites, but was most pronounced in the East Creek raft scallops. Intra-animal developmental asynchrony was noted in the 1994 samples during the second spawn, but was mild in comparison to that seen in both groups sampled in 1995. Spawning of animals with marked intra-animal developmental asynchrony showed evidence of retention of several undeveloped eggs in the tubules at late (stage 8l) spawning (Fig. 7). Interestingly, in



**Figure 6.** Photomicrograph of a bay scallop's female gonadal tubules (50 $\times$ ). **A.** In this stage (7), gonad tubules are filled with mature eggs. Rarely, eggs of smaller sizes are noted at the edges of the tubules. **B.** These female gonadal tubules are in stage 7/8e, but, in addition to the mature eggs, the tubules also contain many immature eggs (arrows).

samples taken at both sites in September to October 1995, female gonads in stage 8l (late spawning condition) or 8vl (very late spawning condition) also often showed early proliferation of the germinal epithelium with small developing eggs (stage 4) (Fig. 8).

Male gonadal tubules did not exhibit detectable intra-animal asynchrony. Male gonadal tubules from animals sampled in late spring rarely were observed to empty completely, as described by Naidu (1970). However, male gonads of scallops sampled in late summer, which were staged as 8l and 8vl, often showed active, mature, sperm-producing germinal epithelium that was sometimes only a few cells thick. Rarely, intra-animal developmental asynchrony was identified in these animals by the appearance of tufts of less differentiated spermatogenic cells interspersed with spermatocytes and spermatids within the more mature (although greatly thinned) epithelium (Fig. 9).

Postspawn or very late spawn intratubular invasion by hemocytes of the female and male gonadal tubules was rarely noted and was minimal when seen. Intratubular inflammation usually consisted of a few hemocytes (usually between two and 20 cells) associated with degenerating retained eggs (Fig. 10). In cross sections of a single male gonad, approximately five closely associated tubules contained numerous hemocytes, resulting in significant inflammation that filled and slightly extended the walls of the tubules. The cause of inflammation in this male gonad was not apparent.

Interestingly, in one male and one female gonad from two

different animals, rare foci consisting of tumorous proliferations formed cell mounds that projected into the tubular lumen from the germinal epithelium (Fig. 11). No tumorous cells were noted invading through the basement membranes. In both cases, the cells were undifferentiated,  $\sim 10\ \mu\text{m}$  in diameter with a high nuclear/cytoplasmic ratio and mitoses of 1/higher power field.

Water temperatures recorded at the EC rafts were slightly higher than those recorded at the lantern nets at RCP; however, temporal trends were very similar in 1995 (see Figs. 12, 13). At RCP, peak temperatures for the 2 years of study were 29.4°C (on 3 August 1995) and 28.6°C (on 9 July 1994). More significantly, perhaps, there was a sharp drop in water temperature just before spawning seemed to commence during both years (from  $\sim 26$  to 20.5°C between 18–21 June 1995 and from  $\sim 27$  to 23°C between 19–22 June 1994) and then a more or less steady rise in temperature after that (to  $\sim 27.4^\circ\text{C}$  by 14 July 1995 and to  $\sim 28.6^\circ\text{C}$  on 9 July 1994).

Concentrations of *Aureococcus anophagefferens* were  $\geq 10^5$  cells  $\text{mL}^{-1}$  from 20 June through at least 18 July 1995 at East Creek and near Red Cedar Point (Figs. 12, 13), with respective recorded peaks at these two sites of  $9 \times 10^5$  and  $1.2 \times 10^6$  cells  $\text{mL}^{-1}$  on 3 July 1995 (SCDHS 1995). Thus, commencement of spawning at both sites coincided with the time of rising *Aureococcus* concentrations, before peak bloom conditions. The second spawning peak exhibited by EC raft scallops, which started in early September 1995, also coincided with rising *Aureococcus* concentrations before the second brown tide peak ( $3.8 \times 10^5$  cells  $\text{mL}^{-1}$ ) on 12 September 1995. In 1994, *Aureococcus* concentrations at these sites did not exceed  $1.5 \times 10^3$  cells  $\text{mL}^{-1}$  at any time (SCDHS 1994).

At the time the last histological samples were taken in late September to early October or early November, all three of the above groups of scallops included individuals that were still in spawning condition (Figs. 2–4). This was not a rare phenomenon, because about one-third of the EC raft scallops sampled on 6 November 1995 were still in spawning condition (either wholly or in part), while other individuals were ripe or in midmaturation (stage 6). Direct evidence of spawning was obtained on the afternoon of 3 October 1995, when East Creek raft scallops were seen to be spawning *in situ*. Water temperature on this day ranged from 17.0°C (at  $\sim 0530$  h) to 19.9°C (at  $\sim 1300$  h). Gametes collected from a few individuals yielded viable trocophore larvae by the next day.

Additional evidence of fall spawning was provided by means of histological analyses of scallops that had been sampled from two different natural populations in NWH during 1993; GI/GDW data were not collected at that time. Of the six individuals sampled off Barcelona Neck on 14 October, all of the female gonadal portions and five of six male portions were in early to late spawning condition (stages 8e–8l). The sixth male gonad was ripe (stage 7). One of two individuals sampled from south of Alewife Creek on 7 November was in midspawning condition (stage 8m), with the female gonadal portion also showing some sections that were recovering from spawning (stage 4). The second individual was ripe (stage 7), but had not begun spawning. Only these eight individuals had been selectively archived out of larger samples of scallops, because they visually appeared to be very ripe (ovarian portion of gonad was bright orange with evident veins). Thus, we can conclude that the minimum proportions of scallops in the process of spawning at NWH and Barcelona Neck on 14 October and 7 November 1993 were 6/49 (12.2%) and 1/88 (1.1%), respectively.

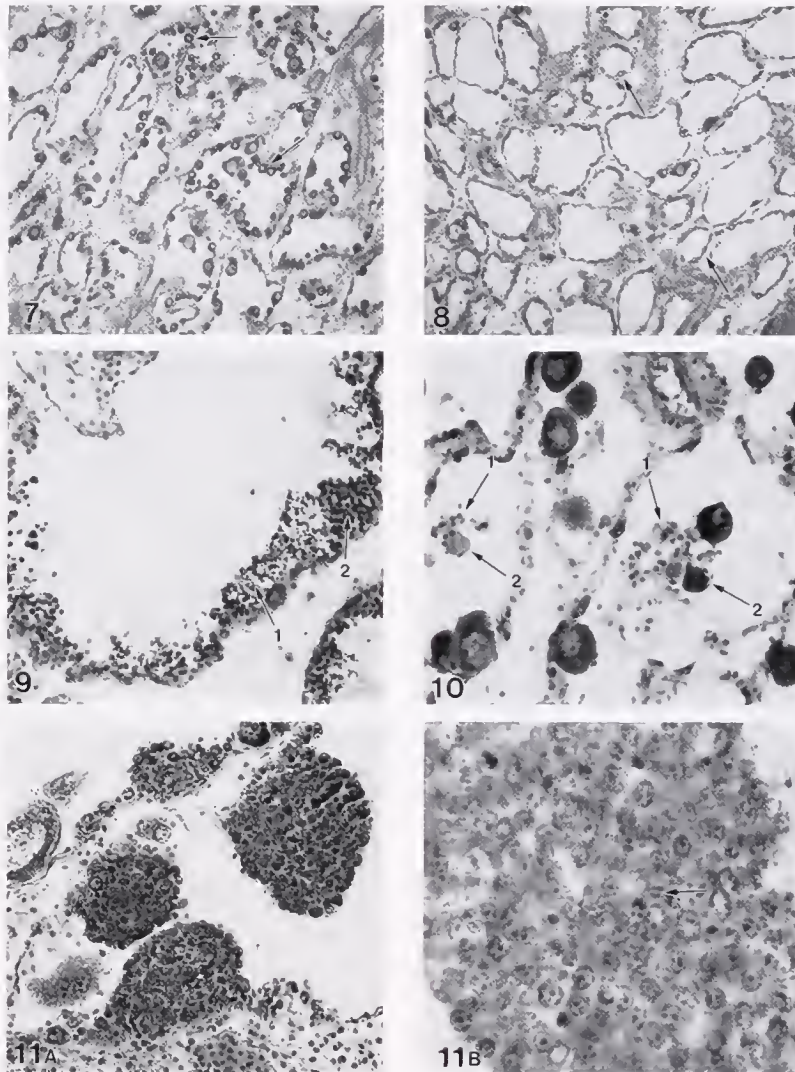


Figure 7. Photomicrographs of a bay scallop's female gonad in stage 8m/l sampled in the late summer. Most mature eggs have been spawned; however, numerous immature eggs of various sizes remain within the tubules (arrows) (25 $\times$ ) (5 September 1995, East Creek rafts).

Figure 8. Photomicrographs of a bay scallop's female gonad in stage 8vl. Although only few mature eggs remain in the tubules, early regeneration of the tubule epithelium has already begun (arrows) (25 $\times$ ) (3 October 1995, East Creek rafts).

Figure 9. Photomicrograph of a bay scallop's male gonad in stage 8vl. (1) Foci of maturing spermatids alternate with (2) some foci of spermatocyte proliferation (25 $\times$ ) (7 July 1995, East Creek rafts).

Figure 10. Photomicrograph of a bay scallop's female gonad in stage 8l. Hemocytes surround and engulf portions of eggs remaining within the tubular lumens (1: hemocytes; 2: eggs) (100 $\times$ ) (7 July 1995, East Creek rafts).

Figure 11. Photomicrographs of a bay scallop's female gonad. A. Nodules of tumor cells project into the tubular lumens (100 $\times$ ). B. Mitotic figures are identified within the mass (arrow) (250 $\times$ ) (14 October 1993, Barcelona Neck).

Water temperature on the latter date was 10.0°C. During 1993, *Aureococcus* concentrations never exceeded  $1.1 \times 10^3$  cells mL<sup>-1</sup> in Northwest Harbor (SCDHS 1993).

#### DISCUSSION

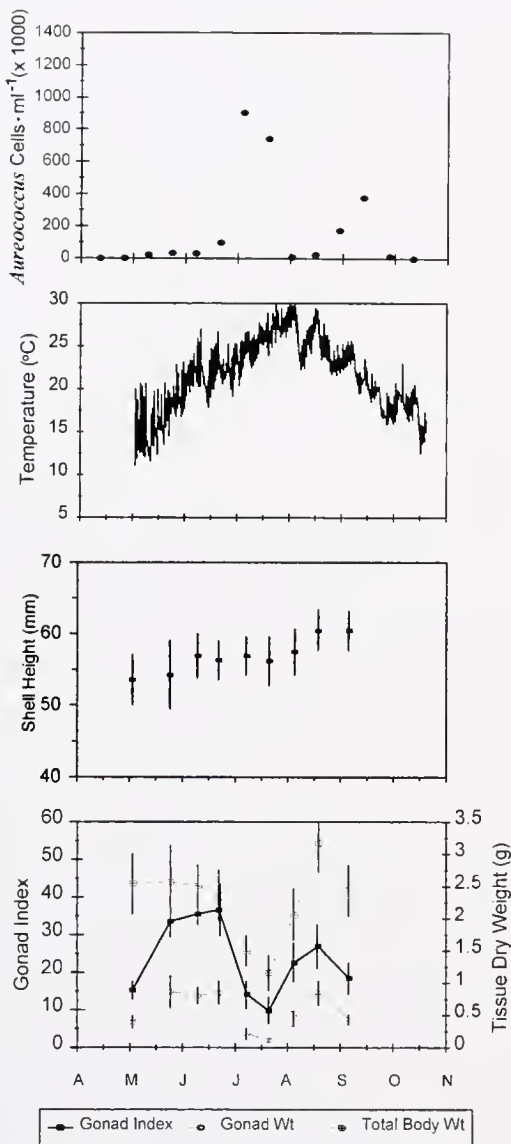
Temporal patterns of bay scallop reproductive condition revealed by our histological analyses and GI/GDW monitoring agreed in many, but not all, instances in this study. Histological analyses may simply have missed the late May to early June 1994 spawning that was suggested by the GI/GDW analyses of the RCP free-plants because of the small sample size ( $n = 2$ ) on 25 May. However, we might then have expected that some evidence of postspawning recovery would show up in the next group of

samples ( $n = 6$ ) on 9 June 1994. It did not. Further work is needed to elucidate the apparent disagreement between the two methods; however, because histology is considered the most definitive way to assess reproductive condition in scallops (Barber and Blake 1991), we base the ensuing discussion on these results.

Two clear spawning peaks (in late June to July and late August to September) were evident in the 1994 RCP free-plants and the 1995 EC raft scallops; whereas, spawning of the 1995 RCP free-plants showed one distinct peak (late June to early July) followed by a prolonged and less dramatic period of spawning. The latter group also showed the least synchronous pattern of reproductive development following the end of the first spawn in late July. The mean shell size of the 1995 RCP free-planted scallops was considerably smaller, several weeks after deployment, than the 1994



EAST CREEK - 1995



RED CEDAR POINT - 1995

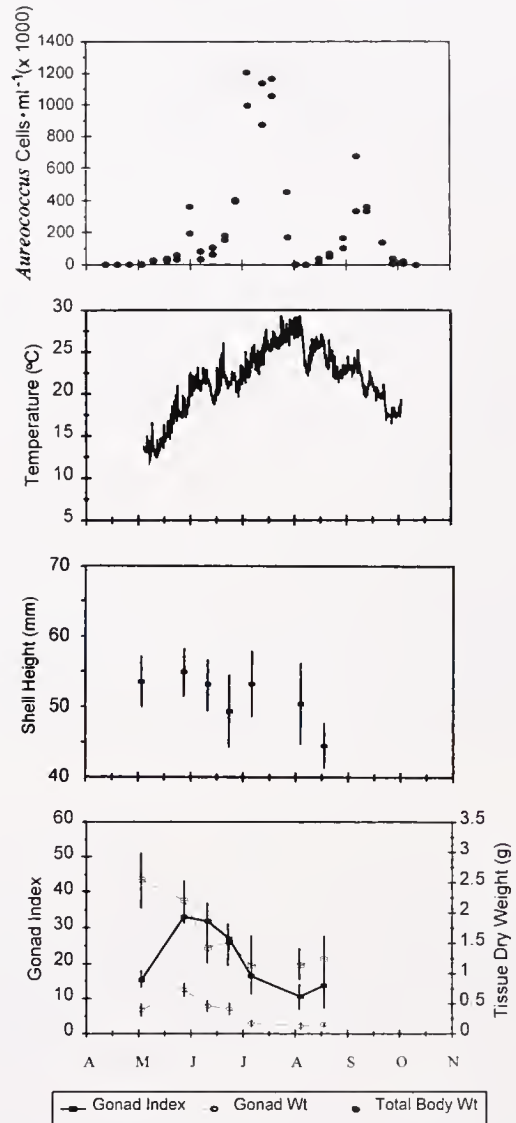


Figure 12. Temporal changes in water quality parameters (*Aureococcus* cell concentrations, water temperature), and size (shell height) and reproductive condition (gonad index, gonad dry weight, total body weight) of bay scallops deployed in floating rafts at East Creek, South Jamesport, New York in early May 1995. Initial values were obtained from a sample of scallops collected from Sag Harbor at the time when transplants were initiated. Datapoints for scallops are mean values  $\pm$  1 SD; n = 24–25 scallops per sampling date.

Figure 13. Temporal changes in water quality parameters (*Aureococcus* cell concentrations, water temperature), and reproductive condition (gonad index, gonad dry weight, total body weight) of bay scallops free-planted south of Red Cedar Point in Flanders Bay, New York in early May 1995. Initial values were obtained from a sample of scallops collected from Sag Harbor at the time when transplants were initiated. Datapoints for scallops are mean values  $\pm$  1 SD; n = 23–25 scallops per sampling date.

RCP free-plants and the 1995 EC raft scallops (see Figs. 2–4, 12–14) (this may have been a sampling artifact, but the reason for the apparent decline in size of the surviving 1995 RCP scallops is unclear). Dry tissue weights are highly correlated with shell size in *A. i. irradians* (Epp et al. 1988), however, the exhibited magnitude of size differences for this group is not expected to have affected temporal spawning patterns: bay scallops of three groups (“large” natural, “small” natural, and hatchery-reared animals with respective mean sizes of 53.4, 37.6, and 32.6 mm at the time of deployment in pearl nets at the same site in Hallock Bay, NY during

spring 1994) showed nearly identical temporal patterns of reproduction as shown by changes in GI and GDW (Smith and Tettelbach 1996).

Several authors have suggested that when environmental conditions are less than favorable for synchronous spawning of scallops, “dribble” spawning may help to ensure that some larvae are able to survive (Langton et al. 1987, Paulet et al. 1988). The different patterns of inter- and intra-animal developmental asynchrony within scallop groups we observed in this study may simply fall within the range of normal variability between different populations (see Bricej et al. 1987b) and different years. However,

## RED CEDAR POINT - 1994

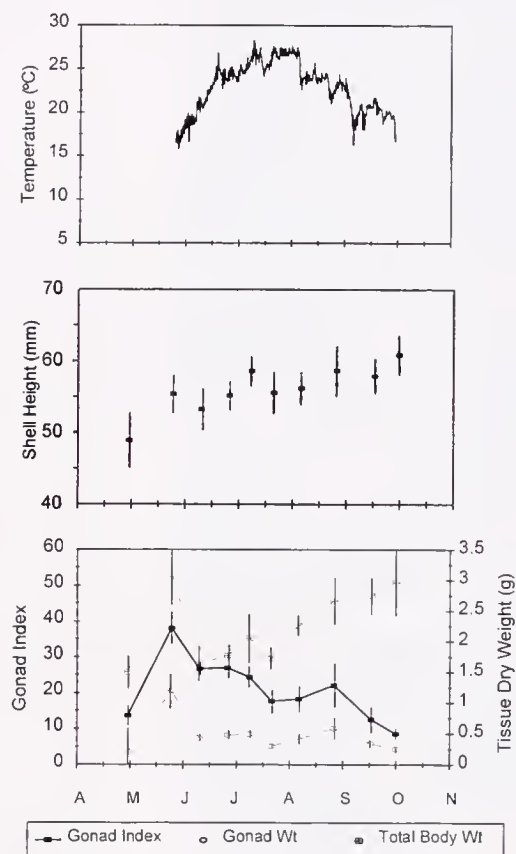


Figure 14. Temporal changes in water temperature, and size (shell height) and reproductive condition (gonad index, gonad dry weight, total body weight) of bay scallops free-planted south of Red Cedar Point in Flanders Bay, New York in late April 1994. Initial values were obtained from a sample of scallops collected from Northwest Harbor at the time when transplants were initiated. Datapoints are mean values  $\pm$  1 SD;  $n = 8$  scallops on 25 May and 13–20 individuals on other sampling dates.

the differences in reproductive patterns exhibited by the 1995 RCP free-planted scallops warrant further examination of the possible effects of the brown tide (vs. nonbrown tide years) and depth in the water column (i.e., surface rafts vs. on-bottom).

Our finding that scallops at EC and RCP first spawned during the period of rising *Aureococcus* concentrations, before the peak of the brown tide bloom in 1995, is of particular interest because: (1) it demonstrates, for the first time, that bay scallops definitely spawned during a brown tide algal bloom (this seems to be coincidental rather than to have been a causative factor in spawning); and (2) it helps to answer the question posed by Bricelj et al. (1987b) and Bricelj and Kuenstner (1989) as to whether a temporal decline in scallop gonad weights or gonad indexes during a brown tide bloom represents actual spawning or gamete resorption. If gamete resorption had occurred, it is expected that extensive infiltration of the gonadal tubules by hemocytes and the presence of numerous degenerative eggs within the tubules would have been apparent. However, only mild inflammation was noted in tubules and then only in association with a few degenerative eggs. This low level of inflammation was noted in scallops sampled during

both 1994 and 1995 and thus probably represents a low level of atresia of unripened, but normal, eggs, as occurs in most other types of animals (Coe and Turner 1938, Van der Kraak et al. 1998).

The occurrence of tumorous proliferations of cells of gonadal epithelial origin in gonads from animals that have actively produced gametes for 3 to 4 months may parallel the phenomena of hormonally stimulated tumors as seen in other animals (Jubb et al. 1985) and at least partially result from repetitive gonadal cycling.

Laboratory studies have shown that 3- to 10-day old bay scallop larvae experienced reduced growth and elevated mortality when exposed to *Aureococcus* concentrations of  $\geq 1.8 \times 10^5$  cells  $\text{mL}^{-1}$  (Gallager et al. 1989); thus, it seems unlikely that spawning of scallops during brown tide bloom conditions in 1995 resulted in successful recruitment of scallop larvae. This was borne out by the virtual absence of scallop seed in any part of the Peconic Bays during the fall of that year; the commercial harvest of adult (1+ y) bay scallops in 1996 was among the poorest in New York over the last 50 years.

Histological analyses conducted in the present study conclusively demonstrated that spawning occurred at least through late September to early October in the 1994 and 1995 RCP free-plants and into November during 1993 and 1995 for the NWH and EC scallops, respectively. These are the latest spawning dates yet reported for *Argopecten irradians irradians* at this latitude.

Based on the data we have presented here and prior reports from other locations (see Kelley and Sisson 1981, MacFarlane 1991) we believe that fall spawning of northern bay scallops is not an unusual phenomenon. Fall spawning probably has been missed in other studies, because reproductive sampling is usually terminated before the end of the summer, although Bricelj et al. (1987b) did conduct GI analyses through October 1984 in Northwest Harbor, NY and found no evidence of a fall spawn. Research on other pectinid species has demonstrated the occurrence of "late" spawning; for example, *Placopecten magellanicus* (MacDonald and Thompson 1988) and *Argopecten irradians concentricus* (Bologna 1998). In the latter species, reproduction may occur throughout the year in St. Joseph Bay, FL (Bologna 1998).

We do not yet know the full significance of fall spawning in *Argopecten irradians irradians*, especially given the reduced fertilization success and recruitment that may result when broodstock densities are low and/or when spawning individuals are separated by some critical distance (Levitan et al. 1992, Peterson and Summerson 1992). Virtually nothing is known about the latter phenomena in *Argopecten irradians irradians*. The problem of ensuring successful fertilization later in the fall is also likely to be exacerbated because of a reduced proportion of spawning individuals in the population. Even if bay scallop spawning occurred throughout the Peconic Bay system after the brown tide subsided in 1995, the very low adult population size relative to 1994 and other years (based on reported commercial bay scallop landings by the NY State Dept. of Environmental Conservation) suggests that potential recruitment resulting from the 1995 fall spawn was probably unimportant.

Nevertheless, the occurrence of unusually small *Argopecten irradians irradians* seed at the end of some fall growing seasons or of adults with growth rings very close to the hinge (Kelley and Sisson 1981, MacFarlane 1991, Tettelbach et al. 1994) suggests that "late" spawning may be important to some bay scallop populations in certain years. Tettelbach et al. (1994) found that during the winter of 1990 to 1991, following a nonbrown tide year, the

percentage of "small" ( $\leq 20$  mm) seed ranged from 0–9% in eight different bay scallop populations in eastern Long Island. In 1992, 1 year after a brown tide bloom, 100% ( $n = 268$ ) of the adult scallops sampled at one of the same sites (south of Alewife Creek in NWH) had growth rings that were 2–7 mm from the hinge, indicating that the adults had only reached this size, as seed, at the end of their first growing season. These small seed may have resulted from late spawning, an extended larval period and/or slow growth following larval settlement, but our present findings lend further credence to the possibility that these small seed resulted from a fall spawn. In that case, fall spawning clearly may be essential to the persistence of certain populations during some years. Larvae of *A. i. irradians* have been shown to exhibit >90% survival, 8 days after fertilization in the laboratory, at a temperature of 10°C and salinity of 25–30 ppt (Tettelbach and Rhodes 1981); thus, larval survival is probable well into November in local waters. However, although larval growth also occurred in these laboratory conditions, it was an order of magnitude slower than at 25°C (Tettelbach and Rhodes 1981).

Our histological results suggest that eggs were not routinely resorbed during the spring to fall months when samples were taken, but continued to mature until they were spawned. The environmental stimuli that induce spawning of *Argopecten irradians* in the fall, however, are presently unknown. Barber and Blake (1991) have suggested that there does not seem to be any critical temperature, *per se*, at which scallop reproduction occurs and, furthermore, that a rapid temperature change ( $\Delta T$ ) is probably a more important spawning trigger than an absolute temperature or the direction of change. Direct disturbance of the raft during the

process of sampling on 3 October 1995 may possibly have triggered spawning, but the fact that water temperature at the EC rafts did spike to over 22°C at the end of September 1995 and rose from 17 to 19.9°C on 3 October, just before the time when spawning was observed *in situ* in East Creek rafts suggests that these conditions may have provided the appropriate stimuli for spawning. Further work is necessary to elucidate the mechanisms that trigger fall spawning of northern bay scallops and the importance of this phenomenon in bay scallop populations.

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## SOME METHODS FOR QUANTIFYING QUALITY IN THE SCALLOP *PECTEN MAXIMUS* (L.)

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**ABSTRACT** Because biological systems do not work in isolation, behavioral, biochemical, and physiological tests can give an overview of an individual's vital processes and reaction to stress. Two stress gradients were applied in this study, a short acute desiccation stress and a long-term density stress. These stress gradients were used to assess the usefulness of various techniques for quality assessment: namely, a standard salinity stress test, condition index, reccessing speed of the scallop, adenylic energetic charge (AEC), and percentage carbohydrate content of the striated muscle. The results showed that AEC could be used effectively to measure the effect of a short-term stress. In the striated muscle, AEC levels were useful in discriminating between good and poor quality scallops. The total carbohydrate content in the striated adductor muscle and condition index were useful in assessing the effect of long-term stress on scallop quality. The most promising results arose from the reccessing trials, because this nondestructive test successfully discriminated the different qualities of scallops arising from both long- and short-term stress.

**KEY WORDS:** *Pecten maximus*, stress, quality, desiccation, density

### INTRODUCTION

Juvenile scallops are either collected by natural settlement onto artificial collectors or produced in a hatchery. Intermediate culture of spat then takes place in suspended culture or cages on the sea bottom until the scallops reach a size (35–50 mm) that offers some protection from predation. Final outgrowth can take place in suspended cage culture or by ranching them on the seabed. Large variations in the survival and performance of spat and juveniles during transport, nursery, and outgrowth have demonstrated the need for research into the effect of stress on the quality of the scallop *Pecten maximus* (Maguire 1998). Stress has been defined as "the effect of any environmental alteration or force that extends homeostatic or stabilizing processes beyond their normal limits at any level of organization." (Esch and Hazen 1978).

Chronic sublethal stress, such as pollution from heavy metals or stocking at high densities, can cause an even or negative scope for growth (Thompson and MacDonald, 1991) and can occur over months or even years. Short acute stresses can occur over hours or days for example, desiccation, thermal shock, and salinity, but both types of stress can eventually result in mortality. The stress effect of various husbandry practices on the physiology of bivalve mollusks is virtually unknown but is believed to be significant.

Dhert et al. (1992a), Dhert et al. (1992b) considered stress tests to be invaluable in testing the nutritional requirements of aquaculture species at various stages of their development and established a standard stress test to determine the quality of shrimp and fish fry, in which they used elevated salinity as a stressor. Duran-Gomez et al. (1991) developed a test to be performed on postlarval prawns *Penaeus japonicus* (Bate) using salinity and pH shocks as stressors. Likewise, Ashraf et al. (1992) employed a standard salinity stress test to detect differences in nutritional studies when no

differences existed in survival and growth using larval striped bass *Morone saxatilis* (Walbaum) and the silverside *Menidia beryllina* (Cope) as the experimental organisms.

Because biological systems do not work in isolation, a combination of physiological, biochemical, and behavioral tests can give a more complete picture of an individual organism's reaction to stress. Examples of some techniques used for assessing quality in bivalve molluscs are listed in Table 1.

Scallops have some unique behavioral traits among bivalves in that they have the ability to swim relatively long distances in an oriented way. They can also recess into the sediment, first described by Baird and Gibson (1956). Therefore, potential behavioral tests could include reccessing and righting behavior (turnover after being placed flat side down), which would affect their ability to withstand predation. Reccessing requires a large energetic cost, and scallops that are already weakened by the stress of handling or exposure to air during transport would be less able to escape from predators by reccessing or swimming when returned to the sea. Fleury et al. (1997) completed a study of the reccessing behavioral of three sizes of ranched scallops during three seasons and three sizes and used adenylic energetic charge as an index. They discovered that the best seeding time was in the spring and summer and that within this period, medium sized scallops (30 mm) reccessed more effectively than the small (15 mm) or larger (42 mm) sized scallops. In our study, reccessing speed was used as a method for stress assessment.

The effect of a short-term stress on the biochemistry of the animal can be measured by its level of adenylic energetic charge (AEC). AEC is defined by the ratio:  $AEC = (ATP + 0.5 ADP) \div (ATP + ADP + AMP)$  where (ATP = adenosine triphosphate, ADP = adenosine diphosphate, AMP = adenosine monophosphate). The triphosphate bond of the ATP molecule has maximum energy, the diphosphate bond of ADP is half as rich, and the monophosphate bond (AMP) lacks energy. The AEC ratio ranges from 0 to 1; that is, (when 0, all nucleotides are AMP, and when 1, all nucleotides are ATP). Therefore, the relative level of these bonds can be used as a measure of the energy directly available to

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TABLE 1.  
A review of techniques used for quality assessment.

General Category	Technique Used	Species	Stress	Reference
Standard stress test	<sup>a</sup> Aerial exposure	<i>Mytilus edulis</i> (L.)	Chronic Acute	Veldhuizentsoerkan et al. (1991) Viarengo et al. (1995)
Biometrics	<sup>a</sup> Condition index	<i>Ostrea edulis</i> (L.) <i>M. edulis</i> <i>Pinctada fucata martensii</i> (Dunker) <i>Crassostrea virginica</i> (Gmelin) <i>Ruditapes philippinarum</i> (Adams and Reeve) <i>Argopecten irradians irradians</i> (Lamarck)	Chronic	Rogan et al. (1991) Lundebye et al. (1997) Numaguchi (1995) Fisher et al. (1996) Isono et al. (1998) Rheault and Rice (1996)
	Flesh condition	<i>M. edulis</i>		Agirregoikoa et al. (1991)
Behavior	<sup>a</sup> Recessing	<i>Pecten maximus</i>	?	Fleury et al. (1997)
Biochemical	<sup>a</sup> Adenylic energetic charge	Bivalve mollusks	Acute	Moal et al. (1989a)
	<sup>a</sup> Carbohydrate content	<i>C. gigas</i> (Thunberg) <i>Dreissena polymorpha</i> (Pall.)	Chronic Short	Kaufmann et al. (1994) Sprung and Borcharding (1991)
	Lipid content			
	Total oxyradical scavenging capacity	<i>M. edulis</i>	Chronic	Regoli et al. (1998)
	RNA:DNA	<i>Euvola ziczac</i> (L.) <i>P. maximus</i> <i>Placopecten magellanicus</i> (Gmelin)		Lodeiros et al. (1996) Robbins et al. (1990) Kenchington (1994)
Cytochemical	Lysosomal membrane fragility	<i>M. edulis</i> <i>Mya arenaria</i> (L.)	Chronic	Pelletier et al. (1991) Tremblay et al. (1997)
	Digestive tubule and vesicular connective tissue condition	<i>C. virginica</i>		Fisher et al. (1996)
Physiological	Scope for growth	<i>O. edulis</i>	Chronic and acute	Hutchinson and Hawkins (1992)
	Oxygen consumption: ammonia excretion	<i>Perna viridis</i> (L.) <i>M. edulis</i> <i>Amblema plicata</i> (Say)	Chronic  Acute	Cheung and Cheung (1995)  Hatcher et al. (1997) Barker and Horbach (1997)
	Lipofuscin accumulation	<i>P. viridis</i> <i>Sumetta scripta</i> (L.)		Mathew and Damodaran (1997)

<sup>a</sup> Techniques used to measure stress in this study.

the cells at that particular time. For example, empirical studies have shown that a very weak, stressed scallop would have an AEC level (measured from the striated muscle) of 0.3 to 0.5 (Fleury, pers. comm.). Such a scallop would have a negative scope for growth and would have a poor chance of recovery. A scallop recording a level of 0.5 to 0.7 would have reduced growth, would not reproduce but could recover to its original quality. A healthy scallop would have an AEC level of 0.8 to 1. Adenylic energetic charge was first proposed as a stress index by Atkinson (1968), who suggested that modulations in the levels of adenyolphosphate reflected variations of enzyme activity at key points in metabolic pathways that yield energy in the form of high energy adenine-phosphate bonds. These variations are a result of external stress. In other words, the more stressed an animal becomes, the more energy it uses to counteract the stress, thus lowering its AEC level.

Many studies have been carried out using AEC as a stress index

or in nutritional studies on different marine animals; for example, the marine isopod *Cirrolana borealis* (Lijborg) (Skjoldal and Bakke 1978), the European sea bass *Dicentrarchus labrax* (L.) (Reali et al. 1987), the oyster *Crassostrea gigas* (Moal et al. 1989b), the spider crab *Hyas araneus* (L.) (Harms 1992), the sturgeon *Acipenser bearii* (Brandt) (Salin 1992), the oyster *C. angulata* (Lamarck) (Madureira et al. 1993) and the scallop *P. maximus* (Fleury et al. 1997).

In juvenile scallops, the level of AEC varies between tissues. Le Coz (1989), in a comparative study of different tissues in the juvenile scallop *P. maximus*, found the highest AEC ratios in the adductor muscle. Within the muscle, the highest level was found in the striated part (mean = 0.93), which is concerned with the fast repetitive opening and closing of the valves of the scallop, thus enabling the animal to swim, to escape from predators, and to recess. In the smooth part of the muscle, the AEC results were

more variable. The smooth muscle has slower contractions and is capable of keeping the scallop shell closed for long periods, with little energy expenditure (Chantler 1991).

Energy is transported from the muscle to the various organs via the haemolymph. The haemolymph of bivalves is also concerned with a variety of physiological functions; that is, transport of nutrients and wastes, gas exchange, osmoregulation, and defence (Benniger and Le Pennec 1991). Therefore, in this study, we looked at the effect of a desiccation stress on AEC levels in the smooth and striated part of the adductor muscle and in the haemolymph of *P. maximus* juveniles.

The effect of a long-term stress on the biochemistry of an animal can be measured by the carbohydrate content of the smooth and striated adductor muscle, respectively. The adductor muscle is the main storage area for energy reserves. Many studies have concentrated on the seasonal partitioning of energy reserves in bivalves: for example, Epp et al. (1988) studied energy partitioning in the bay scallop *A. irradians*. Walne (1970) assessed the seasonal variation of the glycogen content of seven populations of the oyster *O. edulis*. De Zwaan and Zandee (1972) studied the utilization of glycogen and accumulation of some intermediates during anaerobiosis in *M. edulis*. In this study, the effect of high stocking density on the carbohydrate content of cultured scallop spat was assessed.

The criteria for a useful "stress detector" are that it should be reliable and significant; that is with little individual variation within the populations and significant differences between populations. Quality in this study was defined by the degree of acute (emersion) or chronic (density) stress endured by the scallops during these trials. Therefore, the objectives of this study were divided into two parts. First, to create different juvenile scallop qualities using a desiccation stress gradient and to use these reference animals to compare different laboratory techniques, (standard salinity stress test, reccessing behavior and level of adenylic energy charge) for quality assessment. Second, to use the same laboratory techniques, (including total carbohydrate content instead of level of AEC) to measure quality in a case study where scallops were cultured at three different densities.

## MATERIALS AND METHODS

The scallop spat (30 mm) used in this experiment were collected from the Rade de Brest, France. Shell length, height, depth, and total wet weight measurements were taken from a subsample of 100 spat used in each experiment, and a condition index was compiled; condition index =  $[\text{Weight}/(\text{Height} \times \text{Length} \times \text{Depth})] \times 10,000$  (Fleury, pers. comm.).

The scallops were acclimated in tanks and were maintained at a temperature of 15°C and a salinity of 35‰ and fed an equal mixture ( $1 \times 10^7$  mL<sup>-1</sup>) of batch cultured algae *Pavlova lutheri* (Droop), *Isochrysis galbana*, and *Chaetoceros calcitrans* (Paulsen) in volumes sufficient to give a tank concentration of 30–50 cells  $\mu\text{L}^{-1}$ . The scallop were scrubbed clean of epibiota and used in experiments within 2 weeks.

### *Creation of a Gradient of Scallop Qualities Using a Desiccation Stress (Short-Term Stress)*

Four batches (A–D) containing three replicates each of healthy scallop spat ( $n = 30$ ) were used for each of three experiments (Expt. 1–3). The spat were individually weighed, labeled with a

permanent marker, and placed out of water in a constant temperature room for 0, 3, 6, and 12 h (=A, B, C and D, respectively). The air temperature used to stress the scallops was 19°C for the first experiment, 15°C for the second, and 17°C for the third. The stress detector tests (standard stress test, reccessing ability, and level of adenylic energetic charge) were carried out on each batch (A–D) to determine whether the tests could discriminate among the batches.

### *Determination of Various Scallop Qualities Using Scallop Spat Cultured at Different Densities (Long-Term Stress)*

The scallop postlarvae (2 mm), were taken from Tinduff Hatchery/Nursery in April 1995. They were transferred to the Bay of St. Brieuc. Three months later (July 5), the scallops were removed from the cages and graded by shell size (mean  $\pm$  SD height  $12 \pm 2$  mm). They were placed in new cages (0.75 m<sup>2</sup>) with a larger mesh size (5  $\times$  5 mm). A range of stocking densities from 700 to 900 to 1,250 scallops per tier was set up and was referred to as density 1, 2, and 3. Nine replicates of each experimental density were used.

After a 3-month period (October 5), the scallops were retrieved by SCUBA diving from the cages at each density. During transport (4 h), the spat were wrapped in towels soaked with seawater. The juveniles were then stored in aerated seawater tanks at 16°C overnight. Over the next 2 weeks, various stress tests were carried out. These were a standard salinity stress test (2-wk duration), reccessing ability (2-wk duration), and total carbohydrate content fixed immediately. A description of these tests follows.

### *Standard Stress Test*

A useful stress test will pick up differences induced by a stress gradient. The ultimate reaction to stress is mortality, so this was used as a standard assessment. Shell height, length, depth, and various wet weight measurements were taken before and after the standard stress was completed to enable condition indices to be computed.

The standard stress tests were performed in a cubic recirculating tank (1.5  $\times$  1.5 m). The experimental salinity was 25‰, temperature  $15 \pm 1^\circ\text{C}$  for experiments 1 and 2, this was made up using seawater and distilled water. This acted as a semi-severe stress to the already stressed spat to hasten mortality. The experiments took 2 weeks to complete. In experiment 3, the salinity stress test was carried out using freshwater (temperature  $14 \pm 1^\circ\text{C}$ ) to achieve a quicker result.

The spat were given food daily at the same rate with the same species of algae used during their acclimation period. However, even in experiments 1 and 2 (25‰) the scallops were so stressed that they did not seem to feed. Survival was monitored twice per day over a 2-wk period in experiments 1 and 2 and every 15 min over a 2 h period for the freshwater test (experiment 3). The criterion for death was open valves with a lack of valve contraction when touched by a glass rod. All scallops were then reweighed and the shell length, height, and depth were recorded.

### *Reccessing Behavior*

Twenty scallops each from the different groups of spat were quickly measured for shell length, height, depth, and total wet weight. The spat were color labeled and placed in a tank (length 2m, width 0.5 m) with recirculating seawater (salinity 35‰, temperature 15°C). The bottom of the tank was covered with 10 cm of

sediment (collected from a scallop bed) with a predetermined granulometry of 5% > 5 mm particle size, 58% 2 to 5-mm, 35% 1 to 2-mm, and 3% < 1-mm particle size.

The juveniles were fed a mixture of batch-cultured algae, at the same volume used during their acclimation period. Recessing time was monitored every 4 h, and scallops were recorded as recessed (completely covered by substrate), semi-recessed (half covered by sediment), or not recessed.

#### Extraction and Analysis of Nucleotides

Scallop parameters (shell length, height, depth, and total wet weight) were quickly measured for each batch of spat. The scallop was rapidly dissected and the striated and smooth muscle separately removed and frozen in liquid nitrogen. There it was stored until analysis (within a few days). Moal et al. (1989a) found that a better nucleotide extraction was obtained when the required tissue, rather than the whole animal, was frozen.

At the time of the analysis, the striated and the smooth part of the muscle were withdrawn from the liquid nitrogen. One mL of 0.5M ice-cold TCA was then added immediately to each sample, as better recovery of ATP was observed using TCA as compared to other acids: for example perchloric acid (PCA) (Moal et al. 1989a). Preliminary crushing of the extracts increases the stability of the neutralized extracts. The tissue (still frozen) was instantaneously homogenized at 25,000 rpm for 10 s. The homogenate was centrifuged for 10 min at 4,500 rpm, and the supernatant was neutralized with 0.5 M fresh amine freeon solution. The neutralized sample was either stored at  $-18^{\circ}\text{C}$  or immediately analyzed by high-performance liquid chromatography (HPLC).

#### Analysis

The HPLC apparatus was composed of a pump (Waters model 510), an automatic injector (Kontron 460), and a spectrophotometer (Merck L4250). The separation took place in a C18 column of length 150-mm, diameter 4.6-mm (model SFCC/Shandon Spherisorb 3 $\mu$ -OD52), and ultraviolet light (254 nm) was used for the detection of the nucleotides. An isocratic  $\text{NaH}_2\text{PO}_4$  (0.15 M) buffer (pH 6) containing an ion-pairing agent (0.005 M tetrabutylammonium) and 5% methanol was used to elute the nucleotides. All chemicals were of analytical grade and supplied by Sigma. Separation took approximately 30 min at a flow rate of 1 mL/min.

#### Carbohydrate Content

Biometric measurements were taken for each scallop from the different spat groups. The animals were rapidly dissected, and the striated muscle was removed and immediately placed in liquid nitrogen. At the time of analysis, the samples were withdrawn and freeze dried using a HETOSICC CD 53-1 freeze dryer. The carbohydrate content was analyzed using a miniaturization of the Dubois et al. (1956) method. Twenty  $\mu\text{g}$  of the muscle sample were crushed and resuspended in 1 mL of distilled water. Fifty  $\mu\text{L}$  of the mixture was placed in an epindorff tube, 50  $\mu\text{L}$  of 5% phenol was added, and the resultant solution was allowed to stand for 20 min at  $15^{\circ}\text{C}$ . Five hundred  $\mu\text{L}$  of 98%  $\text{H}_2\text{SO}_4$  was added, and the tube was placed on ice. After centrifugation, the absorbance of the supernatant was read at 492 and 620 m $\mu$  using a spectrophotometer model SLT Spectra. A glucose standard was used at concentrations of 0, 50, 100, 150, and 200  $\mu\text{g}$  of glucose per mL of distilled water, and blanks were made using distilled water.

#### Statistical Analyses

Nonparametric data were normalized by log transformation or arcsine square root transformation for percentage data. One-way analyses of variance (ANOVAs) were used to test significant differences among treatments, and *a posteriori* Tukey test was used to contrast treatments. The level of significance was set at 0.05.

## RESULTS

#### Standard Stress Test

Figure 1 shows the mean survival times (over 2-week test period) of each population for each test (desiccation temperature  $19^{\circ}$  and  $15^{\circ}\text{C}$ ). It showed that the degree of desiccation endured (0–12 h) by each group was directly proportional to the mortality rate of each group. However, the desiccation temperature of  $19^{\circ}\text{C}$  was too high, because all the spat from group D (12-h emersion) died either during the last hour of desiccation or immediately after reimmersion. Despite this, a significant difference was found between the spat groups created by using the higher desiccation temperature. The data for test 2 (desiccation temperature  $15^{\circ}\text{C}$ ) showed a significant difference in the mean survival times between groups A/B, C, and D (0, 3, 6, and 12-h desiccation) with similar mortalities occurring between groups A and B (0- and 3-h desiccation).

Figure 2 shows the survival of the four populations (A–D) in test 3, using a freshwater standard stress (temperature  $14^{\circ}\text{C}$ ). The data showed no significant difference between the populations ( $p > 0.05$ ). The stress used in this test was too severe to pick up the subtle differences in quality between the populations.

The standard salinity stress test (water temperature  $15^{\circ}\text{C}$ , salinity 25‰) was carried out on the groups 1–3 of the spat density experiment, and no significant difference was found between the survival of the different density treatments. Only 10% mortality was recorded in the test.

#### Recessing Behavior

Table 2 shows the recessing time of the four scallop groups in the desiccation experiment and the three groups in the density experiment. Recessing speed was directly proportional to the desiccation endured (0–12 h) by the spat and the density (700–1,250 spat per tray). A significant difference was found among the treat-

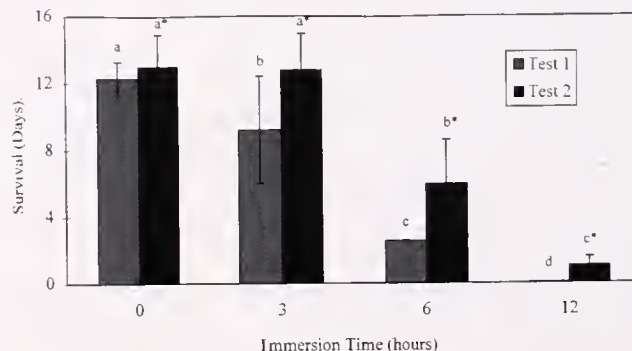
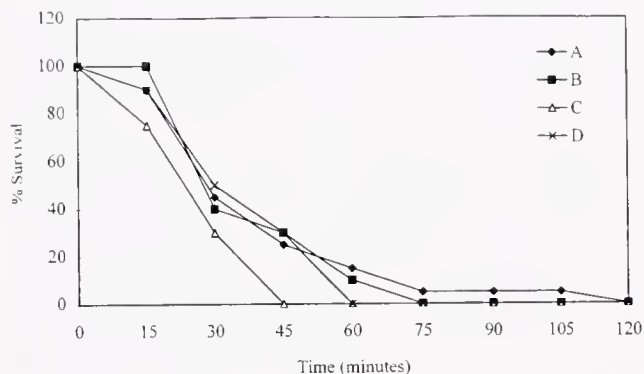


Figure 1. The mean survival times over a 2-week period of four different qualities (desiccation times: A = 0 h, B = 3 h, C = 6 h, and D = 12 h) of juvenile scallops to a standard salinity stress of reduced salinity (S = 25‰, T =  $15^{\circ}\text{C}$  and  $19^{\circ}\text{C}$ ).





**Figure 2.** The survival of four different qualities (desiccation times: A = 0 h, B = 3 h, C = 6 h, and D = 12 h) of juvenile scallops to a standard salinity stress of freshwater (14°C).

ments in the desiccation experiment ( $F_{76,3} = 74.2, p < 0.01$ ) and the density experiment ( $F_{117,2} = 13.67, p < 0.01$ ).

**Adenylic Energetic Charge**

Table 3 shows the relationship between the striated and smooth adductor muscle of the four populations of spat. The highest levels of AEC for all groups was found in the striated adductor muscle. The AEC level in the striated muscle was significantly higher than the AEC level in the smooth muscle for each population (group A  $t_{3,4} = 19.56, p < 0.01$ ; group B  $t_{3,6} = 12.12, p < 0.01$ ; group C  $t_{3,5} = 3.34, p < 0.01$ ; and group D  $t_{3,4} = 7.32, p < 0.01$ ).

The AEC levels in the striated adductor muscle clearly showed two significant groups ( $F_{72,3} = 24.15, p < 0.01$ ). Scallops from group A/B had higher AEC levels ( $> 0.85$ ) than the scallops from group C/D ( $< 0.75$ ). In the smooth muscle, the highest levels of AEC were found in population B, and again levels significantly decreased from this in group C and D ( $F_{65,3} = 4.53, p < 0.01$ ). Hemolymph was also extracted, but the AEC results were deemed to be unreliable because of the difficulty of extracting the hemolymph.

**Carbohydrate Content**

Table 4 shows percentage carbohydrate in dry weight of the striated adductor muscle and the condition index of scallops cul-

**TABLE 2.**

Mean ± SD reccessing time of different spat qualities in the short- (desiccation) and long-term (density) experiments.

Spat Group Experiment 1	Average Recessing Time (Days)
A (0-h desiccation)	1.6 ± 0.4 <sup>a</sup>
B (3-h desiccation)	2.4 ± 0.6 <sup>b</sup>
C (6-h desiccation)	3.5 ± 0.7 <sup>c</sup>
D (12-h desiccation)	5.7 ± 1.6 <sup>d</sup>
Spat Group Experiment 2	Average Recessing Time (Days)
Group 1 (density 700 per tray)	1.73 ± 0.93 <sup>a</sup>
Group 2 (density 900 per tray)	2.28 ± 1.24 <sup>a</sup>
Group 3 (density 1250 per tray)	3.13 ± 1.38 <sup>b</sup>

Any two means sharing a common letter in each column are not significantly different at  $p < 0.05$  (Tukey test).

**TABLE 3.**

Levels of AEC (mean ± SD) in the adductor muscle of four different groups of scallop spat.

Group	Striated Muscle	Smooth Muscle
A (0-h desiccation)	0.89 ± 0.04 <sup>d</sup>	0.55 ± 0.07 <sup>ab</sup>
B (3-h desiccation)	0.87 ± 0.11 <sup>a</sup>	0.65 ± 0.11 <sup>a</sup>
C (6-h desiccation)	0.71 ± 0.1 <sup>b</sup>	0.61 ± 0.08 <sup>ab</sup>
D (12-h desiccation)	0.72 ± 0.08 <sup>b</sup>	0.53 ± 0.08 <sup>b</sup>

Any two means sharing a common letter in each column are not significantly different at  $p < 0.05$  (Tukey test).

tured at three different densities. The carbohydrate content increased significantly from spat cultured at a density of 700 and 900 per tray to those cultured at 1,250 per tray ( $F_{56,2} = 5.25, p < 0.01$ ).

**Biometrics**

Shell length, height, depth, and total wet weight measurements were taken from all spat held at each stocking density, and a condition index was calculated: condition index = [Weight / (Height × Length × Depth)] × 10,000.

Table 4 represents the average value calculated per scallop at each density. Spat cultured at a density of 700 and 900 per tray had a similar condition index. The condition index decreased significantly for those cultured at a density of 1,250 per tray ( $F_{117,2} = 4.188, p < 0.05$ ).

**DISCUSSION**

**Standard Stress Test**

In our study, salinity was reduced to 25 and 0‰, respectively, with the aim of inducing stress and, hence, mortality, which could be used to quantify the quality of the spat. Quality in this study was defined by the degree of acute (emersion) or chronic (high-density) stress endured by the scallops during these trials. Similarly Viarengo et al. (1995) reported that a simple secondary stress response in mussels showed a sensitivity in the same range as other commonly used general stress indices at the cellular level. The results showed that short-term exposure of mussels to sublethal concentrations of pollutants significantly reduced mussel survival in air. Dredge (1997) suggested that saucer-shaped scallops *Amusium japonicum balloti* (Bernardi) can withstand exposure to air for up to 2 h before suffering significant mortality.

**TABLE 4.**

Mean ± SD percentage carbohydrate content of dry weight in the striated adductor muscle and the mean condition index of scallops cultured at three different densities.

Scallop Density per Tray	% Carbohydrate Content	Condition Index Value
700	8.9 ± 2.4 <sup>a</sup>	5.81 ± 0.42 <sup>a</sup>
900	8.8 ± 2.5 <sup>a</sup>	5.81 ± 0.46 <sup>a</sup>
1,250	11.5 ± 3.8 <sup>b</sup>	5.55 ± 0.48 <sup>b</sup>

Any two means sharing a common letter in each column are not significantly different at  $p < 0.05$  (Tukey test).

Although different spat qualities were obtained when the spat were removed from water for 0, 3, 6, and 12 h (groups A–D) at a desiccation temperature of 19°C, this temperature was considered too high, particularly for the group D scallops, because some of the spat from this group died during the 12-h desiccation period. Therefore, an air temperature of 15°C is recommended to give a wider range of spat quality. Similarly, Hutchinson and Hawkins (1992) measured stress in the oyster *O. edulis* using scope for growth as an index. A severe reduction in scope for growth was observed when oysters were placed in conditions where high temperatures were combined with low salinity. The third test using freshwater as the stress test was found to be too severe, and no difference was found among the treatments because of the rapid mortality (within 120 min) of all groups. This is contrary to a study by Dhert et al. (1992b), who worked on the use of stress evaluation as a tool for the quality control of hatchery-produced shrimp and fish fry. In their experiments, the best results were achieved with stress tests performed within a 60 to 90 min period containing 15 to 30 evaluation points.

A standard salinity stress test (water temperature 15°C, salinity 25‰) was carried out on the groups 1 to 3 of the density experiment, and no difference was found among the populations. Very few mortalities were recorded in the test. Dhert et al. (1992b) emphasized the importance of using the appropriate salinity level for each species and for each larval stage. Apparently, in our test, the salinity level was not severe enough to differentiate the different densities, or there was no difference in the quality of spat.

#### Recessing Behavior

It is not surprising that the best quality scallop recessed into the sediment quickest (Table 2). Dao et al. (1985) found that when seeding scallop spat on the seabed, success seemed to depend upon three factors; namely, the quality, the size of the scallop, and the time of year that seeding takes place. By removing seasonal and size variables, we were able to demonstrate a relationship between quality and behavior in juvenile scallops. This is believed to be the first time this has been demonstrated experimentally. Tyurin (1991) worked on the behavioral reactions of the scallop *Mizuhopecten yessoensis* (Jay) to reduced salinity and oxygen exposure to synthetic detergents. Under unfavorable conditions, the test scallops were stressed, could not recess, and elicited an escape response instead. In this study, the recessing speed of scallops deteriorated significantly as the stocking density increased. The recessing test is, therefore, not only sensitive to subtle changes in the spat quality, but is also a very quick and simple test to perform.

#### Adenylic Energetic Charge

In general, the results indicated that as the stress level increased, the AEC level decreased in the striated muscle, to a certain point where the AEC level did not decrease any more. This seems to be the threshold level for this test. Similar results have been shown by Madureira et al. (1993), who looked at the effect of polychlorinated biphenyl (PCB) on adenylic energetic charge in the oyster *C. angulata*, which was fed a PCB-contaminated algal cocktail. They found that the level of PCB increased with time within the animal and that this sublethal stress resulted in the reduction of AEC levels as PCB concentration increased.

In our study, the striated muscle was found to be the best tissue to use when measuring AEC levels, because there was little indi-

vidual variation within the groups and a large difference between stressed (group C and D) and unstressed (group A and B) treatments. Similarly, Le Coz (1989) reported that highest levels of AEC were found in the striated adductor muscle of *P. maximus* and that AEC results were more variable in the smooth muscle. In prior studies, (Fleury et al. 1997) the level of AEC in the striated muscle seemed to be a better measure of stress and quality, than the smooth muscle, because the decrease of AEC in this part of the muscle attributed to stress was more pronounced. In our study, as well, a similar AEC decrease was found in the striated muscle. The hemolymph of bivalves is concerned with a variety of physiological functions, but also the transport of ATP from the striated muscle to various organs. We expected similar results as those found in the muscle. Our results, however, were unreliable because of the difficulty of the hemolymph extraction procedure.

The results of our study were consistent with those found by Moal (1989a, 1989b, 1991), who showed that the effect of short-term desiccation on the oyster *C. gigas* was dependent upon season. AEC levels remained high after 3 h of desiccation in January, but decreased after 3 h of desiccation in May and July. Therefore, there is a negative correlation between AEC and season. Our study was only carried out in May, and the results showed a decrease in AEC levels after a 3-h desiccation period. Further experiments would have to be carried out to determine whether AEC levels could be used to quantify stress in *P. maximus* at other times of the year.

Overall, the recessing test was just as reliable as the level of AEC in measuring the effect of stress on scallops. The recessing test is nondestructive; therefore it can be used for continuous monitoring of the same scallop and is more cost effective than the biochemical test. However, to monitor stress, the testing of the shellfish must take place immediately after sampling so that the condition of the scallop will not be altered by handling. Therefore, because a sample can easily be frozen for biochemical analysis later, it may be more convenient to use AEC rather than have to set up a recessing trial immediately after sampling.

#### Carbohydrate Content

The main energy reserve in scallops is glycogen, which is stored in the adductor muscle. It is mobilized and converted into usable energy (ATP) when needed. In general, scallops contain relatively low levels of glycogen in the adductor muscle attaining maximum levels of up to 24% in *P. maximus* (Ansell 1978), 23–25% of dry muscle weight in *A. irradians* (Epp et al. 1988) and 18% in *Chlamys islandica* (Muller) (Vahl 1981); whereas, the mussle *M. edulis* attains glycogen levels of 42–53% in the mantle (Gabbott 1983). The percentage carbohydrate content in this study measured in October was quite low, ranging from 8.8–11% dry weight of the adductor muscle. This is the period when maximum levels of carbohydrate should be found in the adductor muscle after the summer period. Ansell (1978) suggested that the carbohydrate content varies among sites and among years in the maximum levels found but generally varies from a minimum in March (2.5%) to a maximum in September (24%).

Table 4 showed a surprising result, with the highest level of carbohydrate found in scallops cultured at the highest density (1,250 juveniles per tray). However, the microanalytical technique used for carbohydrate analysis was precise, and the coefficient of variation between subsamples of a single sample was 2.0. This result was contrary to a study by Kaufmann et al. (1994), who reported that the glycogen content of the Pacific oyster *C. gigas*

decreased by 90% after 5 weeks during a growth trial in Maderia Island. This decrease was attributed to a combination of stress factors.

#### Biometrics

The use of condition indices are the traditional method for measuring quality. In this study, the condition index used was sensitive enough to pick up a significant difference in quality between the scallops held at the lower density treatments (700 and 900 scallops per tray) and the high-density treatment (1,250 scallops per tray). Similarly, Rheault and Rice (1996) found that doubling the stocking density of the eastern oyster *C. virginica* resulted in a 20% reduction in the condition of the bivalve.

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## CLONING AND CHARACTERIZATION OF TROPOMYOSIN cDNAs FROM THE SEA SCALLOP *PLACOPECTEN MAGELLANICUS* (GMELIN, 1791)

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**ABSTRACT** Two different complimentary DNAs (cDNAs) encoding tropomyosin have been characterized from adductor muscle of the sea scallop *Placopecten magellanicus*. These cDNAs fall into two size classes of approximately 2,540 and 2,030 base pairs with the larger clones containing a longer 3' untranslated region. This difference apparently arises from the utilization of two different polyadenylation signals. All clones are identical in both coding and noncoding regions, indicating that they represent the same gene. Northern analysis indicates that this gene is expressed highly in adductor muscle and at a much lower level in several other tissues. Southern blots indicate a small (1–3) number of tropomyosin genes in the sea scallop, and population studies detect a high degree of individual polymorphism at this locus.

**KEY WORDS:** *Placopecten magellanicus*, sea scallop, cDNA, tropomyosin

### INTRODUCTION

Tropomyosins are highly conserved, actin-binding proteins present in virtually all eukaryotic cells (see Lees-Miller and Helfman 1991 for review). Different tropomyosin isoforms are expressed in developmental and tissue-specific patterns and are broadly categorized into three major classes: nonmuscle (cytoplasmic), smooth muscle, and striated muscle specific. Tropomyosin mediates Ca<sup>2+</sup>-dependent actomyosin contraction through its interaction with troponin in striated muscle or caldesmon in smooth muscle and nonmuscle cells. In addition to this importance as an essential structural and functional component of the actin microfilament system of the cell, tropomyosins have also been identified as the major protein causing allergic reactions to shrimp (Shanti et al. 1993, Daul et al. 1994, Leung et al. 1994, Witteman et al. 1994).

Like many cytoskeletal proteins, the diversity of tropomyosin isoforms is generated from a few genes through alternative RNA processing or expression from alternate promoters rather than through individual genes for each isoform (Pittenger et al. 1994). In the rat, at least 16 different tropomyosin isoforms have been identified that are encoded by only four genes (Balvay and Fiszman 1994). The four genes are the  $\alpha$ -gene,  $\beta$ -gene, TM-4 gene, and hTMnm gene, each named after a protein they encode (striated muscle  $\alpha$ - and  $\beta$ -TM, fibroblast TM-4, and human nonmuscular TM-30, respectively). The  $\alpha$ -gene encodes at least nine different isoforms that are generated from two promoters (which results in the use of two different initial exons) and alternative splicing of exons 2, 6, and 9 (two alternate exons are encoded for exons 2 and 6, and four different exons are available for exon 9). The alternate exons have been shown to encode tropomyosin sequences essential for critical interactions with other proteins. For example, the 9a exon of the  $\alpha$ -gene, which is only expressed in striated muscle, is required for troponin to mediate high-affinity actin bind-

ing (Hammell and Hitchcock-DeGregori 1996). The use of alternate promoters and splicing to generate multiple tropomyosin isoforms has been found in all vertebrates investigated as well as *Drosophila* (Hanke and Storti 1988).

In this communication, we describe the isolation and characterization of cDNA clones encoding tropomyosin from sea scallop adductor muscle. This paper contributes to a better understanding of the structure–function relationship of the tropomyosin gene in bivalves, because there have been no detailed studies previously. We demonstrate that the region surrounding the tropomyosin gene is highly polymorphic in individual scallops and may prove to be an excellent marker for genetic studies of sea scallop. The molecular characterization of tropomyosin cDNA will also be useful to future studies defining the physiological and molecular basis of allergic sensitivity.

### MATERIALS AND METHODS

Sea scallops were obtained from commercial beds near Yarmouth and Sable Island, Nova Scotia and from St. Pierre Bank near Newfoundland, Canada. DNA extraction, cDNA library construction and screening, probe preparation for southern blot hybridization, and the preparation of genomic blots were as described previously (Patwary et al. 1996).

Using a commercial RNA isolation kit (Stratagene), total RNA was extracted from pooled sea scallop adductor muscle, gill, gonad, heart, liver, and mantle tissues from several individuals that had been snap-frozen in liquid nitrogen immediately after collection and stored at  $-70^{\circ}\text{C}$ . All RNAs were further extracted twice with phenolchloroform and once with chloroformisoamyl alcohol (24:1), precipitated with 7.5 M NH<sub>4</sub>Cl (DEPC-treated) and 2.5 volume ethanol and dissolved in DEPC-treated water. For northern blots, 15  $\mu\text{g}$  of each RNA were electrophoresed on a 0.8% agarose-formaldehyde gel according to standard methods (Sambrook

1	ccctttcgagtctctgaggagcccggtgtgtgtaggaataaggcagaagtcaggaggtctctcgtctgcagttgttcagca	80
81	tttcccttctgcttcacacttcttcttcatctttctatttagataccgttaattctcaacaacaaa	156
1		M D A
157	ATC AAG AAG AAG ATG CAG GCC ATG AAG GTC GAC AGG GAG AAT GCC CAG GAC ATG GCC GAA	216
4	I K K K M Q A M K V D R E N A Q D M A E	23
217	CAG ATG GAG CAG AAA TTG AAG GAC ACC GAG ACA GCC AAG GCA AAG TTG GAG GAA GAT TTC	276
24	Q M E Q K L K D T E T A K A K L E E D F	43
277	AAC GAA CTC CAG AAG AAG CTC GGC ACC ACC GAA AAC AAC TTT GAT ATA GCC AAC GAA CAA	336
44	N E L Q K K L G T T E N N F D I A N E Q	63
337	TTG CAG GAA GCT AAT ACC AAG CTC GAA AAC TCA GAC AAA CAG ATC ACC CAG CTA GAA AGT	396
64	L Q E A N T K L E N S D K Q I T Q L E S	83
397	GAT GTT GCT GGA CTC CAG AGG AGG CTC CAA CTG CTG GAA GAC GAT TAT GAG CGA TCT GAA	456
84	D V A G L Q R R L Q L L E D D Y E R S E	103
457	GAG AAG CTT AAC ACA ACA GCA GAG AAA TTG GAA GAG GCA TCC AAA GCT GCA GAT GAG AGT	516
104	E K L N T T A E K L E E A S K A A D E S	123
517	GAG AGA AAT CGC AAG GTG TAT GAA GGC AGG AGT AAC ACT TGT GAG GAG AGG ATT GAT GAG	576
124	E R N R K V Y E G R S N T C E E R I D E	143
577	CTA GAA AAA CAG TTG GAT ACT GCT AAA ACC ATT GCA ACA GAT GCT GAC TCT AAG TTT GAT	636
144	L E K Q L D T A K T I A T D A D S K F D	163
637	GAG GCC GCC CGT AAG CTT GCT ATT ACA GAA GTG GAC CTT GAG CGC GCC GAG ACT AGG CTG	696
164	E A A R K L A I T E V D L E R A E T R L	183
697	GAG GCC GCT GAC GCC AAA GTA CAC GAA CTC GAA GAA GAG CTC ACT GTT GTT GGT TCA AAT	756
184	E A A D A K V H E L E E E L T V V G S N	203
757	ATC AAA ACC CTT CAG GTG CAA AAC GAT CAG GCA TCA CAG AGA GAG GAT AGC TAC GAG GAA	816
204	I K T L Q V Q N D Q A S Q R E D S Y E E	223
817	ACC ATT AGA GAT CTC ACC AAA AGC CTG AAG GAT GCT GAG AAC AGG GCC ACA GAA GCT GAT	876
224	T I R D L T K S L K D A E N R A T E A E	243
877	AGA CAA GTA GTC AAA CTC CAG AAA GAG GTG GAC AGA CTC GAA GAT GAG CTG CTT GCG GAG	936
244	R Q V V K L Q K E V D R L E D E L L A E	263
937	AAG GAA AGA TAC AAG GCA ATC AGT GAC GAA CTG GAC CAG ACC TTT GCC GAG ATT GCT GGT	996
264	K E R Y K A I S D E L D Q T F A E I A G	283
997	TAC TAA tgtgtccagcaaggacatattccctcaccaaatttatatcataaattacgaggaatgacagacaagaaaa	1074
284	Y *	285
1075	<u>gactgtcaattggaaaaataatattacatcagctttgtacagctatacaaatcgctcgtgcaagaattcgaaacagagaac</u>	1154
1155	<u>ctgccataaaggatccaacatttcttctcaatgtgtgtaatggtacagacgatgaaattccaatttataaattattat</u>	1234
1235	<u>taagaaatgctaatactctatgtgaccttgcgcgcatattgcgacccagcgcgqqgagtgaqagactagagggcaaggacgg</u>	1314
1315	<u>gtgqqgtcgaccgqgtccgctttttaacctctctactcttctggtattttgtatgtatggtacttttgtgaacgqgttctt</u>	1394
1395	<u>ttcgaacacctgtcagacttctgcaaaagctactacttctgqqggqtaaaataatggttcatttctatatttatatacaatg</u>	1474
1475	<u>tatatataactgatacatatcatgattcatatttttctgtattttatttatatcatggttatgacatcggaaacacgacacag</u>	1554
1555	<u>aaqatttgtgttatccatgcctggcgcattctgtacttgaggctcgggaagqcaatgctagcggcacatggttaccattta</u>	1634
1635	<u>agttagtactacgcagagqgttggaccagcgtacaaaaaccaatcggggqtaaatattgcgagatacgcacagtgtgaa</u>	1714
1715	<u>atgtgacagcttttagacatgtagagtcgtttatagatacaagttagttgtagaggtgtagagagactgtaagtagtcat</u>	1794
1795	<u>cctgtatgcctagagagcggctcacatctgcccttaccattcaaaaagcagcaccacaagaaattccaqcattcattttaacc</u>	1874
1875	<u>accacatgactatattttttctatttttqttttatataaaaaagcttaatggcaatatccaqacaacgccattgtgat</u>	1954
1955	<u>gatttttttcaaaqaaaaaactaaaaagctttaatttccacgqcttctggtcctgccctattttaaataaaqaaaggtgt</u>	2034
	*	
2035	<u>atgtactatgtctttggaatgatttattttgcatgtgtttgtgtatagaagaatgtgtgtatggactacaacaaaac</u>	2114
2115	<u>gtagctggctatattattaattgaaaaacaaatttatacatttcttccacagattatttacccttatattatactttt</u>	2194
2195	<u>gattgagaattgatttttctcatctataaaatcttattatggtacgaaatttttactatacatatattgtgtaga</u>	2274
2275	<u>cacagataaagagaactttttaggtgtaactaatttctggtgacattgttcatgttacatttgccatgtgaccaagactag</u>	2354
2355	<u>ttagctgtacaggagggaaaagcgagaactattttgtcatgtgacaactgtagacggaagactctagtgtttgtcatgtg</u>	2434
2435	<u>actgtcatgtgacaactgtagacggaagactcctagtattcctcacaattcagcttccattgcatttccctggatattgc</u>	2514
	*	
2515	caatttgttttaaccaacaataaacttgtattgcttcaaaaaaaaaaaaaaaaaaaaaaaaaaaaa	2580

Figure 1. Nucleotide sequence and the derived amino acid sequence for sea scallop tropomyosin (nucleotides and amino acids follow standard abbreviations). The nucleotide residues are numbered from the 5' end of clone PmC 128. The amino acid residues are numbered from first in-frame methionine (M). The polyadenylation signals are in bold. The stars indicate the end of transcripts attributable to polyadenylation at different sites. Sequence for the 3' UTR probe is underlined.

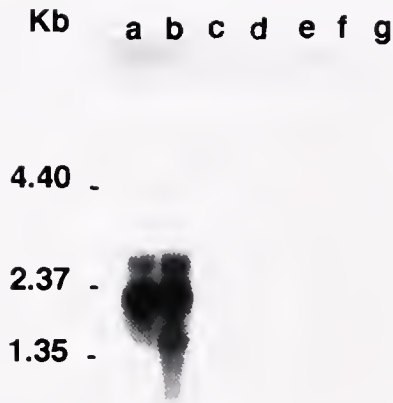


Figure 2. Northern analysis of sea scallop tropomyosin cDNA. Fifteen  $\mu$ g of total RNA in each lane was electrophoresed on a formaldehyde agarose gel, blotted to a nylon membrane, and probed with a  $^{32}$ P-labeled 3'UTR probe. Source of RNAs are from adductor muscle (lanes a & b), gonad (lane c), heart (lane d), liver (lane e), mantle (lane f), and gill (lane g). The membrane was exposed for one hour at  $-70^{\circ}\text{C}$  with an intensifying screen.

et al. 1989) and transferred to a nylon membrane (Boehringer Mannheim) with a Pharmacia Vacu-Genie XL unit following the manufacturer's protocol No 4.

Tropomyosin cDNA probes were amplified from a plasmid clone by the polymerase chain reaction (PCR) using two nested primers. Amplification products were visualized on agarose gels, and the PCR products were excised and purified using a QIAquick Spin PCR Purification Kit (Qiagen Inc). PCR products (25–50 ng) were labeled by random priming with  $^{32}$ P-dCTP(3000 Ci/mmol) using a Ready-to-go labeling kit (Pharmacia Biotech), and the

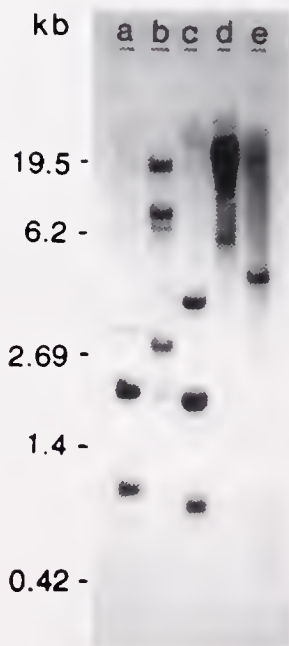


Figure 3. Southern hybridization of the sea scallop tropomyosin coding region to genomic DNA. The blot contains DNA from a single animal digested with EcoR I (lane a), EcoR V (lane b), Hind III (lane c), Sal I (lane d), and Xba I (lane e).

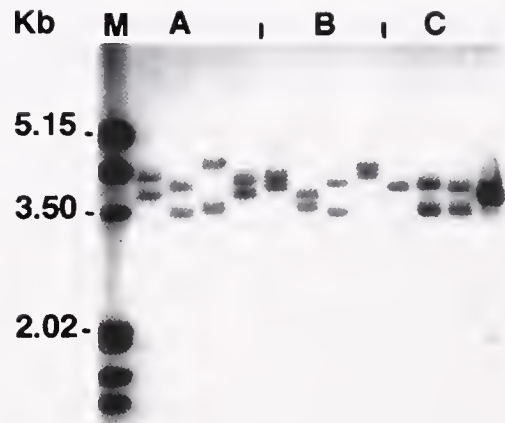


Figure 4. Polymorphisms in the region surrounding the sea scallop tropomyosin gene. Each lane contains Hae III-digested DNA (10  $\mu$ g) from a different animal hybridized with a tropomyosin cDNA coding region probe. DNAs in panel A lanes are from Yarmouth, Nova Scotia, panel B lanes are from Sable Island, Nova Scotia, and panel C lanes are from Newfoundland, Canada. M is digoxigenin-labeled DNA molecular-weight marker (Boehringer Mannheim).

unincorporated nucleotides were removed using Nick columns (Pharmacia Biotech).

Prehybridization and hybridization of northern blots were carried out in a hybridization oven at either 55 or 65 $^{\circ}\text{C}$  in 15 mL of hybridization buffer (0.25 M  $\text{Na}_2\text{HPO}_4$ , pH 7.2, 7% SDS, 50 mg/mL sheared, denatured salmon sperm DNA). Hybridization was for 24–36 h with  $1-2 \times 10^6$  cpm denatured probe. The membranes were then washed twice for 40–50 min each in 20 mM  $\text{Na}_2\text{HPO}_4$ , pH 7.2, 5% SDS and twice for 35–45 min each in 20 mM  $\text{Na}_2\text{HPO}_4$ , pH 7.2, 1% SDS at the same temperature used for hybridization.

To characterize the major transcripts of scallop adductor muscle, 130 plaques from an adductor muscle cDNA library were randomly selected and sequenced on each end. About 4% of these clones were identified as encoding tropomyosin. Inserts from five clones (PmC 60, PmC 92, PmC 104, PmC 118, PmC 128) were subcloned and sequenced completely in both directions.

## RESULTS AND DISCUSSION

The five DNAs characterized by sequencing (PmC 60, PmC 92, PmC 104, PmC 118, PmC 128) can be divided into two groups on the basis of size. PmC 60, PmC 104, and PmC 118 are approximately 2,030 base pairs (bp) in length (excluding the polyA tail), and PmC 90 and PmC 128 are 2,531 and 2,546 bp, respectively. The slight differences in length within each group are caused by incomplete first strand cDNA synthesis; whereas, the difference between the two groups is caused by different lengths of the 3' untranslated region (3'-UTR). The two larger clones have a 1,550 nucleotide 3'-UTR, and that of the shorter clones is 1,036 nucleotides. The difference in the 3'-UTR region seems to arise from polyadenylation at two different sites, with the shorter clones resulting from the recognition of a polyadenylation signal (AATAAA) at position 2020, whereas, the longer clones result from utilization of the polyadenylation signal at 2533 (Fig. 1). The use of alternative polyadenylation sites has been found previously in tropomyosins from a wide range of organisms (Balvay and Fisman 1994).

All five cDNA clones represent transcripts from the same tro-

pomyosin gene, because they are identical in both their coding and noncoding nucleotide sequences. The cDNAs encode an open reading frame of 284 amino acid residues, with a predicted molecular mass of 30,280 d. Sea scallop tropomyosin is approximately 70% identical to other molluscan tropomyosins, 60% identical to those of flukes, 55% identical to those of insects and worms, and 52% identical to vertebrate tropomyosins; the tropomyosin cDNA described herein may be useful as a heterologous probe. The observation that all five cDNA clones encode the same protein suggests that in adductor tissue, which contains both striated and smooth muscle (Chantler 1991), both muscle types express this tropomyosin isoform.

Northern hybridization with a 3'-noncoding region probe to total RNA from several sea scallop tissues revealed intense signals only in the adductor muscle lanes (Fig. 2). Two bands consistent with the sizes of the two cDNAs that were isolated were seen, with the smaller, approximately 2.1 kilobase (kb) band present in greater abundance. Upon longer exposure, faint signals were also detected in lanes from other tissues (results not shown), indicating that the gene represented by this cDNA is also expressed in many scallop tissues. The cDNA we report here seems to represent the principal tropomyosin gene expressed in sea scallop adductor muscle.

The number of genes encoding tropomyosin in sea scallop was estimated by southern hybridization with a probe covering most of

the coding region (Fig. 3). The results indicate that there are only a few (1–3) tropomyosin genes in sea scallop, and similar results were obtained with a shorter probe from the 5' end of the coding region (not shown).

As a part of an effort to develop DNA-based genetic markers to conduct population genetic studies on sea scallop (Patwary et al. 1994a, Patwary et al. 1994b, Patwary et al. 1996), we examined the utility of tropomyosin cDNA as a probe to reveal polymorphisms. A genomic blot containing Hae III-digested DNAs from 12 sea scallops from three distant locations was probed with a tropomyosin coding region probe. The probe revealed a highly polymorphic locus with a total of six alleles (Fig. 4). Although heterozygote deficiency has been reported to be common in sea scallop population (Foltz and Zouros 1984, Beaumont and Zouros 1991), this locus is highly heterozygous. Although the polymorphisms revealed here are limited by a small sample size, the tropomyosin probe seems to be a useful marker for various genetic studies in sea scallop.

#### ACKNOWLEDGMENTS

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## GROWTH CHARACTERISTICS OF *CHLAMYS FARRERI* AND ITS RELATION WITH ENVIRONMENTAL FACTORS IN INTENSIVE RAFT-CULTURE AREAS OF SISHILIWAN BAY, YANTAI

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**ABSTRACT** The growth characteristics of the scallop *Chlamys farreri* under intensive raft-culture and its relationships with major environmental factors were studied. The experiment was conducted from May 1997 to April 1998 in four farming areas of Sishiliwan Bay, Yantai, China. The instantaneous growth rates of shell height, wet weight, fresh and dried soft tissue of scallops were measured and calculated during the course of this study. The results showed obvious seasonal variation in the growth in Sishiliwan Bay. The main factors affecting the growth rate of the scallops were water temperature and food supply in the farming areas. The growth rates of the scallops cultured in Jinggouwan and Yueliangwan areas were faster than that in Kongtongdao and Qingqiangzhai areas. The relationships between the instantaneous growth rates in dried tissue weight of *C. farreri* with water temperature, and particulate organic matter (POM) in Jinggouwan area and Yueliangwan area were simulated. Growth rate declined when water temperature was below 5°C. Between 5 and 23°C, growth rate increased with the increasing of water temperature. Growth rate sharply declined when water temperature was above 23°C. The scallops stopped growing when POM was less than 0.90 mg/L and grew rapidly with increasing POM. When POM was above 3.67 mg/L, the growth rate of the scallops decreased again.

**KEY WORDS:** Sishiliwan Bay, *C. farreri*, instantaneous growth rate, intensive raft-culture, temperature, particulate organic matter, scallop, mariculture

### INTRODUCTION

The scallop *C. farreri* is the main cultured species over the coastal farming areas in the northern China Sea. In the recent decade, the growth rate of *C. farreri* sharply declined because of high culture density and exhaustion of food supply. Furthermore, spats used for farming were mostly collected from the recruitment reproduced by the cultured and possibly inbred stock in recent years. Mass mortality of this species occurred in most of the farming areas in the northern China Sea, especially during the summer and the autumn 1997, and the mortality rate was above 60.0%. The exact cause for the mortality remains unknown. Therefore, it is necessary to study the growth characteristics of cultured scallops systematically among the different farming areas, and its relationship with the major environmental factors, such as water temperature and availability of natural food supply.

The main factors influencing growth are water temperature and the amount of food ingested. From feeding experiments carried out by Winter and Langton (1976) with *Mytilus edulis*, it is obvious that with increasing amounts of food available, there is an increase in growth rate. Since 1970s, the growth and its relationship with the main environmental factors have been studied in detail, in many pectinids, including *Amusium japonicum balloti* (Williams and Dredge 1981), *Argopecten irradians* (Briceli et al. 1987, Cahalan et al. 1989, Duggan 1972, Kirby-Smith and Barber 1974, Rhodes and Widman 1984, Zhang et al. 1987, Zhang et al. 1991a, Zhang et al. 1991b), *Chlamys islandica* (Vahl 1980), *Chlamys opercularis* (Broom and Mason 1978, Taylor and Venn 1978), *Chlamys varia* (Conan and Shafee 1978, Shafee 1980), *Pecten alba* (Gwyther and Meshane 1988), *Patinopecten caurinus* (Haynes and Hitz 1971), *Placopecten magellanicus* (Macdonald 1986, Shumway et al. 1987), and *Pecten maximus* (Mason 1970).

### MATERIALS AND METHODS

Sishiliwan Bay, Yantai, and its near sea areas, located on near Yantai city (121°20'–40'E, 37°25'–40'N) including Zhifuwan Bay, Jinggouwan Bay, and Sishiliwan Bay, is 26 km in width, and 13 km farthest from the shore. It is ear-shaped and half enclosed. The mouth of the bay faces eastward and is divided into two parts by Kongtongdao Island. The smaller one is between Zhifudao Island and Kongtongdao Island, and the larger one between Kongtongdao Island and Yangmadao Island (See Fig. 1). The bay has a muddy and sandy bottom.

The total area is about 13,000 ha, and the depth is about 9–15 meters. Sishiliwan Bay is one of the earliest farming areas, where the kelp *Laminaria japonica* raft-culture was developed in 1949. At the end of 1960s, researchers from the Institute of Oceanology, Chinese Academy of Sciences, and other institutions had studied the artificial collection of mussel *M. edulis* spats and tested raft-culture techniques. The mariculture farms of Yantai had also collected *C. farreri* spats in Jinggouwan area in the middle of 1970s. Between the end of 1970s and the early 1980s, hatchery production of *C. farreri* seed was successfully developed and used, and the scallop was cultured in large scale. The northern bay scallop *Argopecten irradians* has been one of the main species cultured in the bay since 1986. Now, *M. edulis*, *C. farreri*, *A. irradians*, and the kelp *Laminaria japonica* are the primary species under raft-culture in the bay. The culture areas include 830 ha (1 ha = 6,000 cages, the same as for mussels) for scallops, 460 ha for mussels, 250 ha (1 ha = 6000 strings) for kelps. Mussel and scallop seeds are mainly collected from the wild, and the spats of the bay scallop and the seedling of the kelp are hatchery-produced.

#### *Set-Up of Research Stations*

Four farming areas, Jinggouwan farming area, Yueliangwan farming area, Kongtongdao farming area, and Qingquanzhai

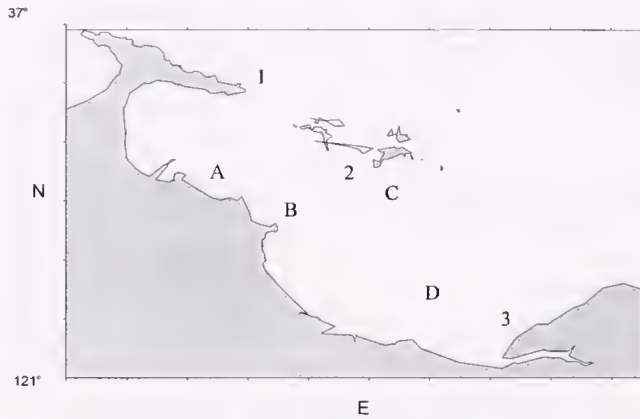


Figure 1. Main farming areas in the Sishiliwan Bay, Yantai. A: Jinggouwan, B: Yueliangwan, C: Kongtongdao, and D: Qingquanzhai; 1: Zhifudao Island, 2: Kongtongdao Island, 3: Yangmadao Island.

farming area in Sishiliwan Bay were chosen in this study. In each farming area, three stations were set, totaling 12 stations.

#### Sampling

The specimens sampled in May to September 1997, were 1-year scallops, which were collected in the spring of 1996, and those sampled in October 1997 to April, 1998 were 1-year scallops collected in the spring of 1997. Fifty scallops were collected monthly at each station. After being taken back to the laboratory, the specimens were cleaned (the epibionts were cut off) and boiled, the adductor muscle and the viscera were divided, and the shell height, wet weight, shell weight, wet and dried weight of soft tissue (65°C, for 48 h) were measured. Water temperature, salinity, the biomass of seston and particulate organic matter (POM) were determined at the same time. The seston was filtered by GF/C and dried at 65°C for 48 h and weighed, and then washed at 450°C for 6 h and weighed again. The biomass of POM equals the difference between the weight of dried sestons minus that of ash.

#### Calculation

The instantaneous growth rates of the shell height, wet weight, the fresh and dried weight of the soft tissues were calculated using the following equation  $I\text{GR} = ((\ln s_2 - \ln s_1)/t) \times 100$ . IGR stands for instantaneous growth rate,  $s_1$  stands for initial shell height, wet weight, the fresh or dried weight of the soft tissues measured first

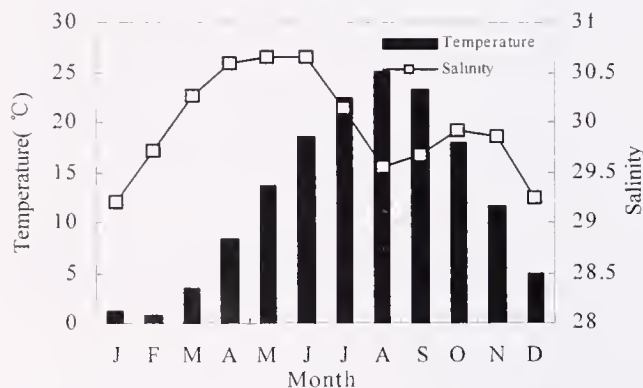


Figure 2. Annual variation of water temperature and salinity in the Sishiliwan Bay, Yantai.

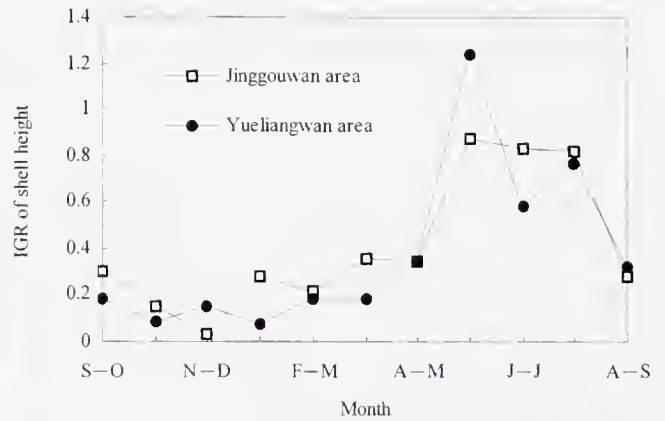


Figure 3. Annual variations of instantaneous growth rate in shell height of *C. farreri* in Jinggouwan area and Yueliangwan area.

time,  $s_2$  for ending shell height, wet weight, the fresh or dried weight of the soft tissues, and  $t$  for the interval days between initial and ending measurement.

## RESULTS AND ANALYSIS

#### Annual Variation of the Growth of *C. farreri* in Relation to Water Temperature

The surface water temperature and salinity variations of Sishiliwan Bay, Yantai are illustrated in Figure 2. The lowest and highest water temperatures are observed in February and August, respectively. The annual fluctuation of salinity is small, around  $29.95 \pm 0.51$ . The annual variations of instantaneous growth rates of shell height, wet weight with shell, fresh and dried weight of soft tissue in Jinggouwan and Yueliangwan farming area are shown in Figures 3, 4, 5, and 6. It is obvious that the growth of *C. farreri* in two areas varies with the season. The growth rate of the scallops is fastest from May to July. The relationships between the instantaneous growth rate of soft tissue and water temperature are similar at these two areas (Fig. 7). The regression equations are as follows.

Jinggouwan farming area

$$I\text{GR} = -0.0031T^3 + 0.0911T^2 - 0.4568T + 0.6218, r^2 = 0.9788$$

Yueliangwan farming area

$$I\text{GR} = -0.0027T^3 + 0.0780T^2 - 0.3801T + 0.4635, r^2 = 0.9916$$

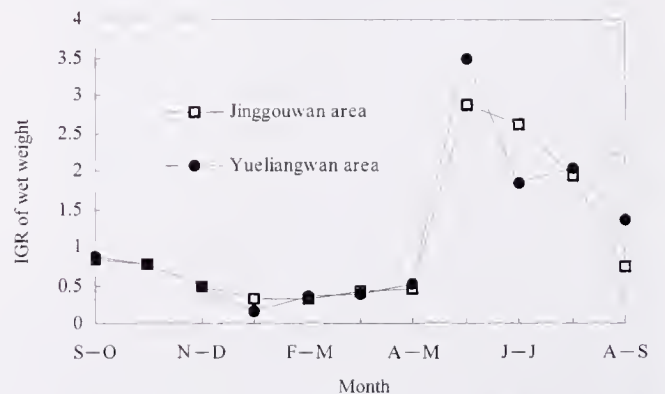


Figure 4. Annual variations of instantaneous growth rate in wet weight of *C. farreri* in Jinggouwan area and Yueliangwan area.

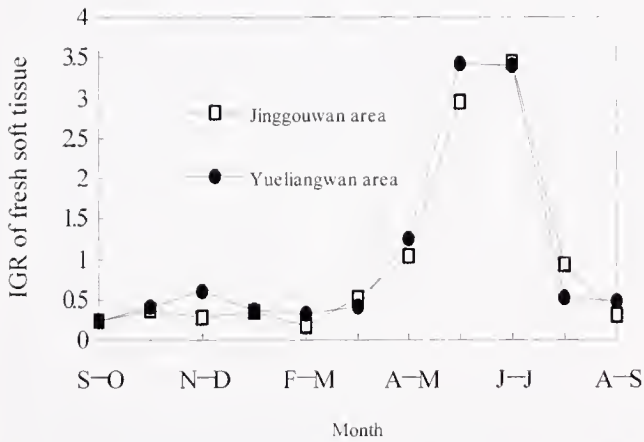


Figure 5. Annual variations of instantaneous growth rate in fresh soft weight of *C. farreri* in Jinggouwan area and Yueliangwan area.

IGR stands for the instantaneous growth rate of soft tissue and T for water temperature (°C)

These equations clearly show that there is a strong correlation between the growth of *C. farreri* and water temperature. The growth rate is slow when water temperature is below 5°C, and then increases sharply with increasing temperature. The fastest growth rate is at 16–18°C. The growth rate sharply declined when water temperature was over 23°C.

Variation Among Different Culture Areas

Results from the main growth period, May to September, show that there are differences in instantaneous growth rate of shell height, wet weight, fresh and dried weight of soft tissue in the four culture areas (Figs. 8, 9, 10, 11), especially from May to August. The dried weight of soft tissue in Yueliangwan and Jinggouwan areas increases fastest from May to July, and the growth rate in these two areas is faster than those in Kontongdao and Qingquanzhai areas. The growth rate of *C. farreri* in Kontongdao and Qingquanzhai areas is slow, and their instantaneous growth rate varies little. That is quite different from the scallops cultured in Yueliangwan and Jinggouwan areas, where the growth rate is relatively faster in May to July, and gradually declines after that period. It is necessary to note that scallops in these four farming areas mostly died by September 18, 1997, up to 80.0%. The instanta-

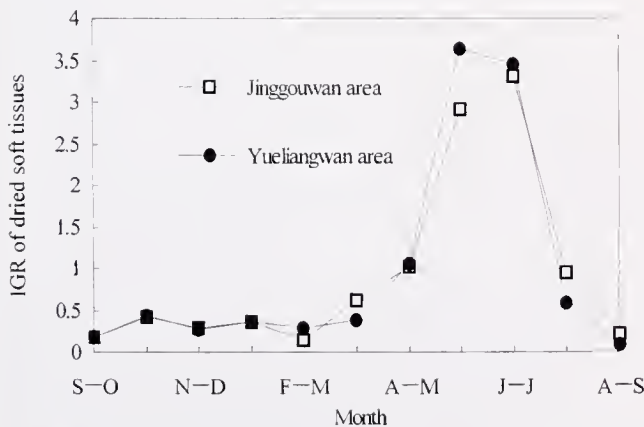


Figure 6. Annual variations of instantaneous growth rate in dried soft weight of *C. farreri* in Jinggouwan area and Yueliangwan area.

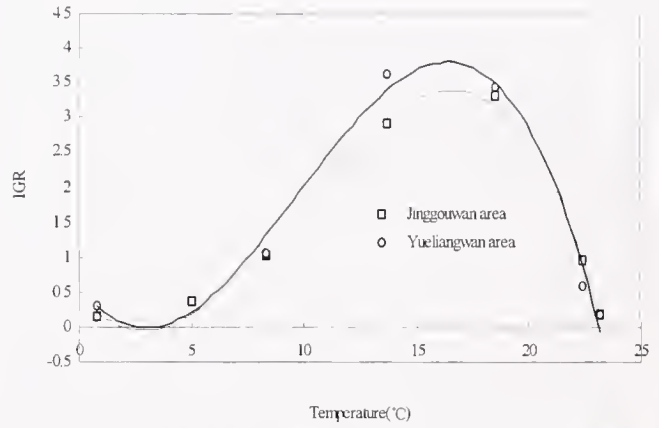


Figure 7. The relationship between water temperature and the instantaneous growth rate of dried soft tissue of *C. farreri* in Jinggouwan and Yueliangwan areas.

neous growth rate of the survivors decreased considerably, especially the instantaneous growth rate of dried soft tissue.

Relationship Between the Growth and POM Biomass

The measurements of the biomass of seston and POM are listed in Table 1. The differences in the biomass of seston and POM are obvious among different culture areas. The relationship between the instantaneous growth rate of dried soft tissue and the biomass of POM can be formulated as  $IGR = -0.5695[POM]^2 + 4.1780[POM] - 3.4031$ ,  $r^2 = 0.9338$  (Fig. 12).

It shows that the instantaneous growth rate of dried soft tissue of *C. farreri* tends to be zero when the biomass of POM is less than 0.90 mg/L and increases with the increasing POM biomass. The scallop growth rate declines when the POM is more than 3.67mg/L. It is clear that the growth of *C. farreri* is limited in some degree by the abundance of natural foods in the farming area.

DISCUSSION

Pectinids can be partitioned into four broad groups according to their patterns of life history (Orensanz et al. 1991): (1) long-lived, iteroparous species; (2) short-lived, iteroparous species; (3) short-lived, semelparous or quasi-semelparous species; and (4) small-sized, presumably short-lived, brooding species. The first group

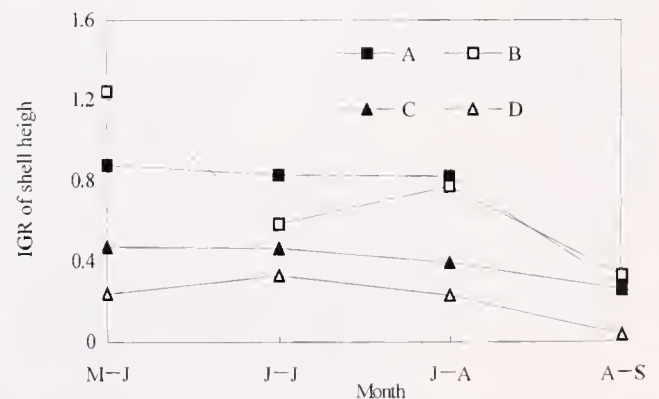


Figure 8. The instantaneous growth rate in shell height of *C. farreri* in the culture areas. A: Jinggouwan, B: Yueliangwan, C: Kontongdao, and D: Qingquanzhai.

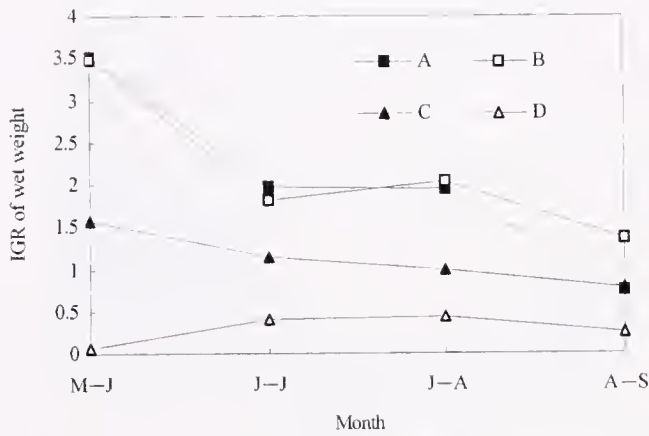


Figure 9. The instantaneous growth rate in wet weight of *C. farreri* in the culture areas. A: Jinggouwan, B: Yueliangwan, C: Kongtongdao, and D: Qingquanzhai.

can be further divided into two types: large-sized (above 100 mm), relatively long-lived (maximum longevity usually above 12 years) species, and medium-sized (60–100 mm) species with maximum longevity usually less than 10 years. The scallop *C. farreri* belongs to the second type of the first groups. There are a lot of environmental factors influencing the growth of scallops, mainly being water temperature, water current, the biomass of natural food, culture density, and the amount of other filter-feeding animals within the farming area (Zhang et al. 1987, Zhang et al. 1991a, Zhang et al. 1991b). Water temperature and the biomass of natural food in culture area might be the most important factors affecting the growth of *C. farreri*.

In most previous studies (Lou 1991, Wang et al. 1993, Zhang et al. 1956), the shell height of *C. farreri* was used to measure the growth of the scallops. Their results reflected that, *C. farreri* grow fast in the months in which water temperature is high, and vice versa in normal culture conditions. In the winter, *C. farreri* totally stop growing. From March in each year, the growth of *C. farreri* increases gradually with the increasing water temperature and reaches its peak in July. When water temperature is above 25°C, the growth obviously decreases. From January to March, the water

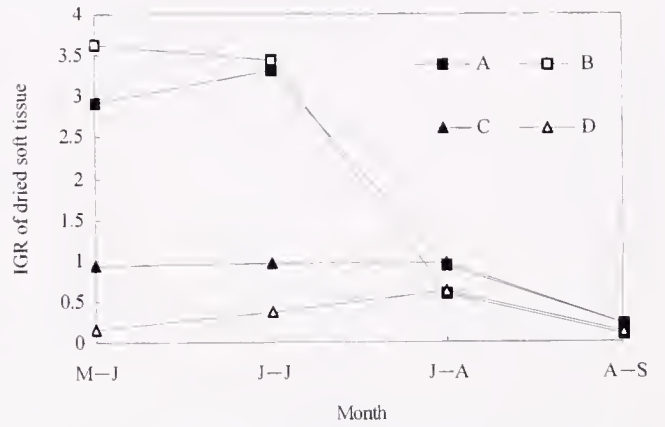


Figure 11. The instantaneous growth rate in dried soft weight of *C. farreri* in the culture areas. A: Jinggouwan, B: Yueliangwan, C: Kongtongdao, and D: Qingquanzhai.

temperature is lower than 5°C, the growth of shell is nearly zero. In this paper, the instantaneous growth rate was used for the first time to describe the growth of this species, and the resulting model with the instantaneous growth rate and water temperature support the viewpoints above. A combination of many environmental factors might have strongly affected the growth of *C. farreri*, leading to the death of most *C. farreri* in this area in 1997. The results of this study differ from previous studies on the influence of high temperature on the growth of *C. farreri*: when temperature is over 23°C, the instantaneous growth rate of dried weight of soft tissue of *C. farreri* sharply declines.

Our findings indicate that the growth of *C. farreri* varies differently with different areas in the same season. The environmental differences of farming areas include the difference in water current, concentration of nutrients, primary production, and stocking density. The variation in water quality and food supply, especially the food supply, has an obvious influence on the growth of *C. farreri*. The biomass of POM is higher in the Yueliangwan and Jinggouwan areas than that in the Kongtongdao and Qingquanzhai farming areas. The Yueliangwan area lies upstream to the Sishiliwan Bay (following the direction of water current), and it is the

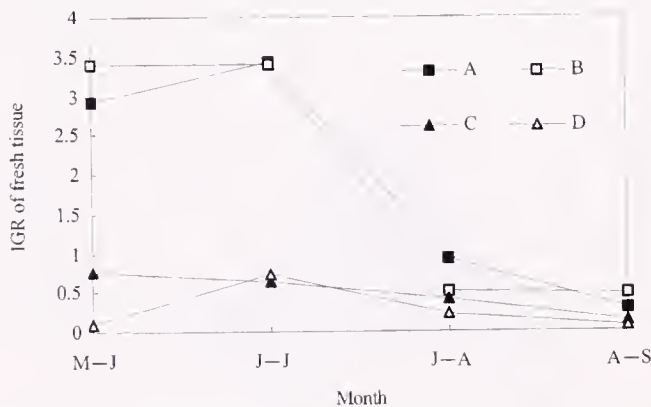


Figure 10. The instantaneous growth rate in fresh soft weight of *C. farreri* in the culture areas. A: Jinggouwan, B: Yueliangwan, C: Kongtongdao, and D: Qingquanzhai.

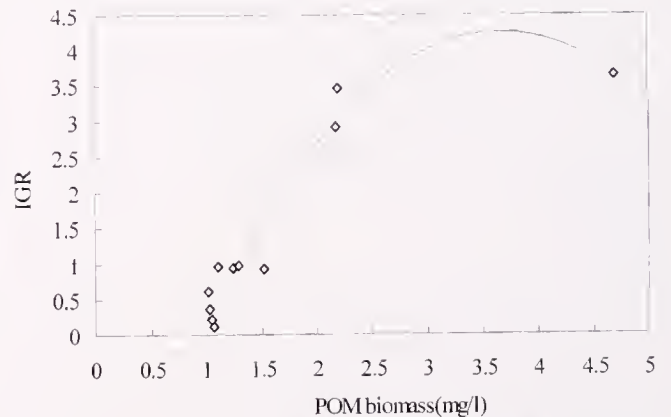


Figure 12. The relationship between the instantaneous growth rate in dried soft weight of *C. farreri* and the biomass of POM.

TABLE I.

Biomass of seston (S, mg/L) and particulate organic matter (POM, mg/L) in the farming areas of Sishiliwan Bay.

Sampling Date	Jinggouwan		Yueliangwan		Kongtongdao		Qingqianzhai	
	S	POM	S	POM	S	POM	S	POM
May 15	11.28	2.70	8.55	4.72	4.47	0.99	13.80	2.28
June 18	3.66	2.17	6.26	4.69	3.11	1.52	3.03	1.70
July 15	2.07	1.15	3.46	2.19	2.54	1.29	2.30	1.03
Aug. 20	2.12	1.24	2.84	1.57	1.97	1.11	2.18	1.02
Sept. 18	3.48	1.05	3.64	1.53	4.37	2.03	3.93	1.07

front part of the whole culture area (Yantai Port is above it). The Jinggouwan farming area is downstream of it, and the Qingqianzhai farming area is at the bottom of the bay, and water current in the Kongtongdao farming area is slow, and the culture density is highest.

The modeling results of this study suggest that, when the biomass of POM is lower than 0.90 mg/L, the instantaneous growth rate of dried soft tissue tends to be zero; the growth rate increases with the increasing biomass of POM, and then the growth rate declines as the biomass of POM is above 3.67 mg/L. Roland and Brown (1990) established the relationships between POM and the growth of the *Crassostrea gigas*, which is similar to ours, except that the upper limit of POM that restricts the growth of *C. farreri* is 5.00 mg/L. The metabolism of *C. farreri* increases with the increasing temperature from 10°C to 23°C (Yang et al. 1998). The depletion of natural foods and the food selection of *C. farreri*

(Wang et al. 1989) make the energy consumed by the scallops for metabolism, rather than for growth. The overabundance of natural foods limits the growth of filtering-food bivalves, because of the increased mucus excretion and the pseudofeces production of scallops, which consume large amounts of energy and lead to the discharge of carbon and nitrogen of the scallops.

## ACKNOWLEDGMENTS

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## CULTURE OF *MERCENARIA MERCENARIA* (LINNAEUS): EFFECTS OF DENSITY, PREDATOR EXCLUSION DEVICE, AND BAG INVERSION

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**ABSTRACT** Growth, survival, and condition index (CI) of the northern quahog, *Mercenaria mercenaria* (Linnaeus, 1758), cultured in nylon mesh bags (1.2 × 1.2 m) were assessed against density and predator exclusion device (PED, Vexar net with 2.5-cm openings) in the northern Indian River Lagoon at Oak Hill, Florida. Nursery seed [mean ± SD: 6.0 ± 0.8 mm shell length (SL)] were stocked in February 1997 at densities of 7,500 (5,210), 10,000 (6,944), and 12,500 (8,680) clams/bag (clams/m<sup>2</sup>) (n = 4) and monitored until the end of May 1997. Two replicates of each treatment were inverted 5 weeks before harvesting to smother fouling organisms and examine their influence on growth. Growout seed (mean ± SD: 21.1 ± 1.7 mm SL) were stocked in October 1996 at densities of 750 (521), 1,000 (694), and 1,250 (868) clams/bag (clams/m<sup>2</sup>) (n = 4) and monitored until early June 1997. At the end of the nursery seed experiment, the average final SL of clams was significantly different among the density treatments (p = .03) and not significantly different between the PED (p = .31) treatments. Nursery seed in the inverted bags were significantly larger (p = .03), and a higher percentage of them reached growout seed size (12 mm in SL). Density (p = .60) did not have a significant effect on survival; whereas, the bags with PED had significantly (p = .005) lower survivorship than that of the bags without PED. Density (p = .15) and PED (p = .79) did not significantly affect mean CI at the end of the study, but inversion significantly (p = .002) increased CI. At the end of the growout seed experiment, SL was not significantly different among the treatments (density, p = .40; PED, p = .17). There was a significant (p = .04) effect of density on percentage of the seed that reached legal harvest size (16 mm in shell thickness). In general, percentage of seed that reached harvest size decreased with increasing density. The effects of density (p = .04) and PED (p = .0009) on survival were significant, but there was no apparent pattern. Density (p = .29) and PED (p = .88) did not affect mean final CI. Chlorophyll *a* concentration and water current speed measured in April and May, 1997 indicated that food was not a limiting factor on growth of the northern quahog at the study site. Our recommendations for northern quahog culture in the Oak Hill area are: (1) use a planting density of 7,500 clams/bag for nursery seed and 750 clams/bag for growout seed; (2) could use PED to reduce fouling on the culture bags, although PED may not improve clam survivorship; and (3) invert culture bags periodically.

**KEY WORDS:** *Mercenaria mercenaria*, density, Florida, fouling

### INTRODUCTION

The northern quahog, *Mercenaria mercenaria* (Linnaeus, 1758), is a commercially important shallow-water species that has been harvested by subsistence fishermen since pre-Columbian times and today supports an important commercial and recreational fishery (Crawford 1992). Since the early 1950s, the feasibility of artificially enhancing the commercial crop of cultured northern quahog has been studied intensively (MacKenzie 1979). Some of the factors affecting the growth and survival of commercial northern quahog are planting density and predation (Flagg and Malouf 1983). Most studies dealing with densities found that high planting density resulted in slower growth of clams (Eldridge et al. 1976, Eldridge et al. 1979, Hadley and Manzi 1984, Walker 1984), although others indicated no significant difference in final size (Godwin 1968, Summerson et al. 1995).

A major inhibition to commercially viable northern quahog culture has been predation (Carriker 1959, Menzel and Sims 1964, Haven and Loesch 1973, Menzel et al. 1976, MacKenzie 1977, Whetstone and Eversole 1978, Steinberg 1980, Castagna and Kraeuter 1981, McHugh 1981, Peterson et al. 1995), with crabs representing the most serious problem from Massachusetts to Texas (MacKenzie 1977, Virnstein 1977, Boulding and Hay 1984, Jory et al. 1984, Hines et al. 1990, Eggleston et al. 1992, Summerson et al. 1995, Kraeuter et al. 1998). Several techniques have been developed to minimize predation, including rafts, trays, cages, nets (Manzi et al. 1980), addition of gravel to inhibit foraging effectiveness (Castagna and Kraeuter 1977), introduction of organisms that consume clam predators (Castagna and Kraeuter

1981, Castagna 1984, Jory et al. 1984, Bisker and Castagna 1989) and combinations of the various methods (Kraeuter and Castagna 1985). Despite the partial effectiveness of these control measures, predation remains a critical factor in northern quahog aquaculture.

The field culture of bivalve mollusks is also dependent on the production and supply of phytoplankton and other food sources. Seston depletion is a major influence on cultured suspension feeders whose growth can be limited by both food quality and quantity (Fegley et al. 1992).

It has proved most economical to grow quahogs in the natural environment at controlled densities, because space and food requirements increase exponentially as clams grow (Castagna and Kraeuter 1977). Although field nursery and growout offer a low-cost production system for the shellfish, one of the disadvantages is fouling. Fouling can affect production in various ways. The most obvious is a reduction of water flow through the enclosure, which, in turn, decreases food availability (Paul and Davies 1986, Wildish and Kristmanson 1984). In addition, fouling organisms are often themselves filter feeders, so they compete with the cultured species for food resources. Finally, fouling may reduce oxygen supply (Wallace and Reinsnes 1985). Several solutions have been used to remove fouling: addition of animals that prey upon the biofoulers (Flimlin and Mathis 1993), cleaning and changing structures often (Claereboudt et al. 1994), and inversion of bags (Mojica and Nelson 1993).

In Florida, growth of northern quahogs is more rapid than that observed for northern populations (Jones et al. 1990, Arnold et al. 1991) because of the warmer temperature and longer growing

season. The best growth occurs in fall and spring (Eldridge et al. 1976, Eldridge et al. 1979, Menzel 1961). Fouling of culture bags and heavy predation force modifications in culture techniques, such as different mesh size of bags and predator excluding devices (Vaughan et al. 1988), as well as maintenance and cleaning of the system. In Florida, cultured clams reach legal harvest size (16 mm in ST) in approximately 9 to 10 months, and the commercial "littleneck" size (25.4 mm in ST, legal harvest size of wild clams) in approximately 12 to 18 months (Vaughan et al. 1988).

Clam farming is an important industry in the Indian River Lagoon (IRL) of east central Florida. During the early to middle 1980s, a successful fishery for naturally occurring northern quahogs developed in the IRL. Landings from this fishery peaked in 1985 at more than 1.5 million pounds with an estimated value of more than US \$8 million (Florida Department of Natural Resources 1986). Landings decreased substantially since then. The magnitude and success of this fishery influenced the development of a culture-based fishery in the IRL. The IRL possesses the necessary components of a suitable growout site for northern quahog aquaculture (Arnold et al. 1990): area of the lagoon is considerable, water quality is generally good, and extensive shoreline is available from which to monitor and maintain aquaculture operations. The present study was designed to determine how clam growth, survival, and CI was affected by planting density, predator exclusion device (in addition to the culture bag), and bag inversion at Oak Hill, Florida.

## MATERIALS AND METHODS

### Experimental Design

This study took place in the IRL at Oak Hill, Florida (Fig. 1). In the IRL, water depth generally does not exceed 1–1.5 m, except near the Atlantic Intra-coastal Waterway, and tidal range does not exceed 0.5 m (Sheng et al. 1990).

Hatchery-reared northern quahog seed used in this study were produced by Harbor Branch Oceanographic Institution, Inc. in Fort Pierce, Florida. The seed were stocked in bags made of a flexible nylon mesh material. The experiment consisted of two different growing periods. Nursery seed clams were planted on February 27, 1997 and monitored until May 29, 1997 (13 weeks), and growout seed clams were planted on October 10, 1996 and monitored until June 6, 1997 (34 weeks). The experimental densities utilized for the nursery seed were 7,500/bag (5,210/m<sup>2</sup>), 10,000/bag (6,944/m<sup>2</sup>), and 12,500/bag (8,680/m<sup>2</sup>), with four replicates for each density. Growout seed densities were 750/bag (521/m<sup>2</sup>), 1,000/bag (694/m<sup>2</sup>), and 1,250/bag (868/m<sup>2</sup>), with four replicates for each density. For both nursery and growout seed experiments, an additional four replicates were planted and covered with PED for each density treatment. The PED was a 1.6 × 1.5 m Vexar cover net of 2.5-cm mesh size laid over the bags to exclude predators. Water temperature (to the nearest 0.1°C with a thermometer), salinity (to the nearest 1 ppt with a hand-held temperature-compensated refractometer (Atago S/Mill)), dissolved oxygen (to the nearest 0.01 ppm with a temperature-compensated dissolved oxygen meter (YSI Model 57)), current speed (to the nearest 0.01 cm/second) and direction (with a mechanical flowmeter (Model 2030R, General Oceanics Inc.)), and Secchi disc depth (to the nearest 1 cm with a 15-cm diameter Secchi disc) were measured weekly.

### Nursery Seed

Nursery seed [mean ± SD shell length (SL): 6.0 ± 0.8 mm; n = 100] were stocked in 24 3-mm mesh size bags (1.2 × 1.2 m) on

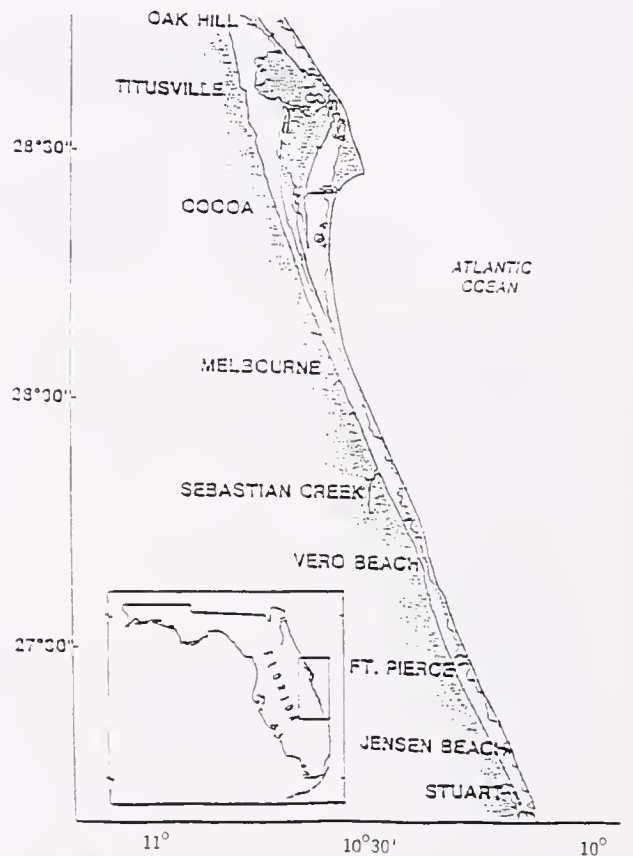


Figure 1. Map of the Indian River Lagoon, including the study area (Oak Hill).

February 27, 1997. The number of seed placed in each bag was determined volumetrically. The bags and cover nets were kept in place on the bottom with metal stakes. PVC pipes were placed underneath the PED at the corners to maintain tension and prevent predators from entering the bags.

Growth was assessed by measuring 100 clams per bag for SL, shell height (SH), and ST to the nearest 0.01 mm with Vernier calipers every 4 weeks. Only SL was used in further analysis because of the high correlations between the measurements ( $r^2 = 0.97$  for SL and SH;  $r^2 = 0.80$  for SL and ST). The measured clams were then returned to the bags. At the beginning of the study, 100 clams were sacrificed and dried in an oven at 65°C for 48 hours to determine shell and soft tissue dry weight (Walne and Mann 1975). At the end of the 13-week trial, another sample of 100 animals per bag was similarly sacrificed and measured. During weekly monitoring, any dead clams found were removed and recorded, but not replaced. Bags and cover nets were inspected weekly and cleaned biweekly to assure proper water flow. Cleaning consisted of manually removing fouling organisms that grew on the bags. Two replicates from each density and PED treatment combination were inverted on April 27, 1997, 5 weeks before harvesting to smother fouling organisms further and to examine their influence on growth. The 24 bags containing nursery seed clams were harvested on May 29, 1997. Surviving clams were counted, and the percentage of seed reaching the growout size was determined by sieving (10 to 11-mm mesh screen) clams from each bag. Clams that were retained on the screen were large enough for the growout phase, whereas, seed that passed through



the sieve were not. The percentage of growout seed from each bag was calculated based on the total number of seed harvested.

#### *Growout Seed*

Growout seed (mean  $\pm$  SD SL: 21.1  $\pm$  1.7 mm;  $n = 100$ ) were stocked in 10.5-mm mesh size bags (1.2  $\times$  1.2 m,  $n = 24$ ) and planted on October 10, 1996 following the same method described for nursery seed. One hundred clams per bag were sampled every 4 to 5 weeks. Growth was assessed by measuring SL, SH, and ST, but only analysis on SL was conducted, because that shell measurement was highly correlated ( $r^2 = 0.94$  for SL and SH;  $r^2 = 0.83$  for SL and ST). One hundred clams were sacrificed to measure shell and tissue dry weight, as described earlier, at the beginning and again at the end of the study. Inversion of bags to smother fouling organisms was not performed on the growout seed. The 24 bags containing growout seed clams were harvested on June 5, 1997. Surviving clams were counted and the percentage of seed reaching legal harvest size was determined by grading (16 mm width on bar grader) clams from each bag. Clams that were retained on the bar grader had reached legal harvest size for cultured clams in Florida. The percentage of harvestable clams from each bag was calculated based on the total number of clams harvested.

#### *Food Availability*

Chlorophyll *a* concentration and current speed were estimated at five locations in the study site to assess food availability along the prevailing flow gradient. Over a period of several days, floating objects were placed in the water during incoming and outgoing tides and followed to establish the prevailing current pattern in the area. Once the prevailing current pattern was established, five locations (two before, one inside, and two after the clam bed) were chosen to estimate food availability. Three 1-liter water samples were taken at each location, on 3 days during incoming tide (April 10, May 8, and May 22, 1997) and 3 days during outgoing tide (April 18, May 15, and May 29, 1997). Water samples were taken to the laboratory and kept cool and dark until analyzed within a few hours. An appropriate volume (500 to 1000 mL) of seawater was vacuum filtered onto a synthetic filter (Millipore AA 47-cm diameter). Chlorophyll *a* was measured by spectrophotometric analysis (Strickland and Parsons 1976). Pigments were extracted from the filter with 90% acetone, and pigment absorbance was estimated spectrophotometrically at 750, 660, 647, and 630 nm wavelengths. A standard concentration curve was produced using a commercial chlorophyll *a* extract (SIGMA Chemical Company, St. Louis, MO).

#### *Data Analysis*

All data were examined for variance heteroscedasticity using F max test (Sokal and Rohlf 1995), and no data transformation was necessary. Final SL of the clams was analyzed by a two-way (density and PED) ANOVA for growout seed and a three-way ANOVA (density, PED, and inversion) for nursery seed. Bonferroni's multiple comparison test was used to compare the means if there was a significant difference in the ANOVA (Sokal and Rohlf 1995). Survival of nursery seed was analyzed by a three-way ANOVA (density, PED, and inversion), and survival of growout seed was analyzed by a two-way (density and PED) ANOVA. Condition index was calculated using the formula: dry soft tissue wt. (g)  $\times$  1000/ dry shell wt. (g) (Walne and Mann 1975) and was analyzed by a three-way ANOVA (density, PED, and inversion)

for the nursery seed and a two-way (density and PED) ANOVA for the growout seed. Percentage of clams that reached growout size was analyzed by a three-way (density, PED, and inversion) ANOVA, and percentage of clams that reached legal harvest size was analyzed by a two-way (density and PED) ANOVA. Pearson product moment correlation was used to correlate growth with temperature, salinity, and Secchi disc depth (Sokal and Rohlf 1995). The significance level ( $\alpha$ ) for all statistical tests was 0.05.

## RESULTS

#### *Environmental Parameters*

Water temperature ranged from 8.8 to 28.8°C during the study period, with a mean ( $\pm$  SD) of 21.7 ( $\pm$ 4.1)°C ( $n = 32$ ). Mean ( $\pm$  SD) salinity was 32 ( $\pm$ 1.7) ppt ( $n = 32$ ) with a range of 29 to 34 ppt. Mean ( $\pm$  SD) dissolved oxygen concentration (D.O.) was 11.6 ( $\pm$ 4.7) ppm ( $n = 32$ ) at the surface (range: 4.8–20.0 ppm) and 11.3 ( $\pm$ 4.8) ppm ( $n = 32$ ) at the bottom (range: 5.0–19.0 ppm). Water depth was between 1 and 1.5 m. Mean ( $\pm$  SD) Secchi disc depth was 0.86 ( $\pm$ 0.14) m (range: 0.57–1.00 m,  $n = 32$ ). Mean ( $\pm$  SD) current speed was 6.8 ( $\pm$ 4.2,  $n = 32$ ) cm/s at the surface (range: 2.0–15.0 cm/s,  $n = 32$ ) and 6.6 ( $\pm$ 3.3,  $n = 32$ ) cm/s at the bottom (range: 3.0–13.8 cm/s). Water temperature, salinity, D.O., Secchi disc depth, and current speed did not show significant ( $p > .05$ ) correlation with SL.

#### *Nursery Seed*

Nursery seed clams of all treatments grew at almost perfect linear rates over time, from an average initial SL of 6.0 mm to a mean final SL of 14.6 mm in the 13-week study. Density ( $p = .03$ ) and inversion ( $p = .03$ ) had a significant effect on the final SL; whereas, PED did not ( $p = .31$ ). Low density clams tended to be larger than those of medium and high density treatments; and clams from inverted bags tended to be larger than those from the noninverted bags (Table 1). Mean percentage of nursery seed that reached growout size ranged from 25.5 to 91.8% (Table 1). There was a significant effect of density ( $p = .02$ ) and inversion ( $p < .001$ ) on percentage of clams attaining growout size. A higher percentage of clams in the low-density treatment reached growout size than that of clams in the medium- and high-density treatments (Table 1); inversion resulted in a higher percentage of nursery seed that reached the growout size (Table 1).

Survival at the end of the study ranged from 59.0 to 94.5% (Table 1). PED ( $p = .005$ ), and inversion ( $p = .002$ ) effects were significant, but density effect was not ( $p = .60$ ). Surprisingly, survival of clams in bags with PED was lower than that of clams in bags without PED ( $p < .05$ , Bonferroni's test) (Table 1); inversion resulted in higher survivorship ( $p < .05$ , Bonferroni's test) (Table 1).

Mean ( $\pm$  SD) initial condition index (CI) of nursery seed was 49.6 ( $\pm$ 28.3) ( $n = 100$ ), and it changed little after the 13-week trial period (Table 1). The density ( $p = .15$ ) or PED ( $p = .79$ ) had no significant effect; whereas, the inversion ( $p = .002$ ) did. The CI of the clams in the inverted bags was larger than that of the clams in the noninverted bags ( $p < .05$ , Bonferroni's test) (Table 1).

#### *Growout Seed*

Growout seed clams grew from an initial SL of 21.1 mm to a mean final SL of 33.3 mm. There was no significant difference (density:  $p = .40$ , PED:  $p = .17$ ) in the final SL among the

TABLE 1.

Mean ( $\pm$  SD) of shell length, percentage reached growout size, survival, and CI of nursery seed grown for 13 weeks.

Inversion (I, NI)	Density (Clams/Bag)	PED (N, C)	Shell Length (mm)	% Reached Growout Size	Survival (%)	Condition Index
I	7,500	N	17.1 $\pm$ 0.0 <sup>a</sup>	91.8 $\pm$ 9.8 <sup>a</sup>	94.5 $\pm$ 6.4 <sup>d</sup>	55.2 $\pm$ 0.1 <sup>abc</sup>
I	10,000	N	14.1 $\pm$ 0.5 <sup>ab</sup>	62.5 $\pm$ 13.4 <sup>abc</sup>	76.0 $\pm$ 11.3 <sup>abc</sup>	65.3 $\pm$ 3.2 <sup>a</sup>
I	12,500	N	14.2 $\pm$ 0.8 <sup>ab</sup>	45.5 $\pm$ 6.4 <sup>cd</sup>	81.0 $\pm$ 5.7 <sup>ab</sup>	52.9 $\pm$ 6.1 <sup>abc</sup>
I	7,500	C	15.1 $\pm$ 0.2 <sup>ab</sup>	67.5 $\pm$ 7.8 <sup>ab</sup>	82.0 $\pm$ 5.7 <sup>ab</sup>	56.5 $\pm$ 2.6 <sup>ab</sup>
I	10,000	C	14.4 $\pm$ 0.3 <sup>ab</sup>	56.0 $\pm$ 7.1 <sup>bcd</sup>	83.5 $\pm$ 7.8 <sup>ab</sup>	52.3 $\pm$ 4.1 <sup>abc</sup>
I	12,500	C	13.7 $\pm$ 0.7 <sup>b</sup>	57.5 $\pm$ 0.7 <sup>abcd</sup>	75.5 $\pm$ 3.5 <sup>abc</sup>	42.9 $\pm$ 2.0 <sup>bc</sup>
NI	7,500	N	14.2 $\pm$ 2.0 <sup>ab</sup>	40.5 $\pm$ 3.5 <sup>bcd</sup>	78.0 $\pm$ 2.8 <sup>abc</sup>	45.9 $\pm$ 9.0 <sup>bc</sup>
NI	10,000	N	13.1 $\pm$ 1.0 <sup>b</sup>	29.5 $\pm$ 6.4 <sup>cd</sup>	77.0 $\pm$ 1.4 <sup>abc</sup>	36.5 $\pm$ 8.0 <sup>f</sup>
NI	12,500	N	14.3 $\pm$ 1.1 <sup>ab</sup>	37.0 $\pm$ 1.4 <sup>bcd</sup>	81.0 $\pm$ 1.4 <sup>ab</sup>	43.2 $\pm$ 4.5 <sup>bc</sup>
NI	7,500	C	14.1 $\pm$ 1.4 <sup>ab</sup>	35.5 $\pm$ 28.9 <sup>bcd</sup>	59.0 $\pm$ 8.5 <sup>e</sup>	52.8 $\pm$ 10.0 <sup>abc</sup>
NI	10,000	C	13.3 $\pm$ 0.8 <sup>b</sup>	29.5 $\pm$ 10.6 <sup>cd</sup>	69.5 $\pm$ 9.2 <sup>e</sup>	43.5 $\pm$ 5.7 <sup>bc</sup>
NI	12,500	C	13.9 $\pm$ 0.6 <sup>b</sup>	25.5 $\pm$ 4.9 <sup>d</sup>	62.5 $\pm$ 6.4 <sup>e</sup>	47.1 $\pm$ 4.9 <sup>abc</sup>

Values within a column with different superscripts were significantly different.

Under inversion, "I" means inverted, "NI" means noninverted; under PED, "N" means not cover, and "C" means cover.

treatments (Table 2, Fig. 2). Mean percentage of growout seed that reached 16 mm in ST (legal harvest size for cultured northern quahog) at the end of the study ranged from 30.1 to 66.8% (Table 2). The density effect was significant ( $p = .04$ ); whereas, the PED effect was not ( $p = .25$ ). In general, the percentage decreased with increasing density (Table 2).

Survival at the end of the study ranged from 75.0 to 87.0% (Table 2). The effects of density ( $p = .04$ ) and PED ( $p = .0009$ ) were significant, but there was no apparent pattern (Table 2).

Mean ( $\pm$  SD) initial CI of growout northern quahog seed was 65.4 ( $\pm$ 28.3). The CI decreased after the 34-week trial period to an average of 36.3, with no significant difference among the treatments (Table 2).

#### Food Availability

Generally, chlorophyll *a* concentration was similar among the stations and between incoming and outgoing tides at a given date. Average chlorophyll *a* concentration in the April 1997 samples was 0.0642  $\mu$ g/L. Mean surface current during the April sample days was 11.3 cm/s, and mean bottom current was 9.5 cm/s. May 8 and May 15 samples showed an order of magnitude increase in chlorophyll *a* concentration to 0.96  $\mu$ g/L. Average late-May measurements of chlorophyll *a* was 0.81  $\mu$ g/L. In May, mean surface

current speed was 6.7 cm/s and mean bottom current speed was 6.1 cm/s.

#### DISCUSSION

Ansell (1968) reviewed the growth of northern quahog in various locations along the eastern coast of the United States and concluded that the optimum temperature for growth was approximately 20°C and that shell growth ceased below 9°C or above 31°C. In the present study, the mean water temperature over the 34-week period ranged from 15.1 to 26.3°C. Small changes in salinity do not have a major influence on growth rates, unless the salinity goes below 20 ppt (Castagna and Kraeuter 1981). The optimal salinity for the growth of northern quahog is reported to be about 26 and 27 ppt (Rice and Pechenik 1992). In the present study, salinity ranged from 29 to 36 ppt. In the Oak Hill area, D.O. was high (mean = 13 ppm) during the 34-week period. High D.O. has been found to be common in the southern IRL as well (Arnold et al. 1990, Dierberg et al. 1986).

Manzi et al. (1981) recommended that intensive field culture is best initiated with seed size larger than 10 mm in SL. However, larger seed are more expensive, and their cost may be >60% of the total cost of producing the final product in northern quahog aquaculture (Adams et al. 1991). In a study conducted in New Jersey

TABLE 2.

Mean ( $\pm$  SD) of shell length, percentage reached legal harvest size, survival and CI of growout seed grown for 34 weeks.

Density (Clams/Bag)	PED (N, C)	Shell Length (mm)	% Reached Legal Harvest Size	Survival (%)	Condition Index
750	N	33.5 $\pm$ 2.1	53.7 $\pm$ 4.3 <sup>d</sup>	75.0 $\pm$ 3.4 <sup>b</sup>	37.2 $\pm$ 6.0
1,000	N	32.9 $\pm$ 2.1	49.1 $\pm$ 11.2 <sup>ab</sup>	87.0 $\pm$ 1.3 <sup>a</sup>	34.8 $\pm$ 4.9
1,250	N	32.8 $\pm$ 2.5	30.1 $\pm$ 4.8 <sup>b</sup>	75.0 $\pm$ 3.1 <sup>b</sup>	35.5 $\pm$ 5.7
750	C	34.0 $\pm$ 2.2	66.8 $\pm$ 8.2 <sup>a</sup>	81.0 $\pm$ 3.4 <sup>ab</sup>	35.3 $\pm$ 5.8
1,000	C	33.0 $\pm$ 2.2	38.9 $\pm$ 10.0 <sup>b</sup>	81.0 $\pm$ 2.3 <sup>ab</sup>	34.1 $\pm$ 5.5
1,250	C	33.8 $\pm$ 2.5	49.5 $\pm$ 3.8 <sup>ab</sup>	82.0 $\pm$ 1.8 <sup>ab</sup>	38.6 $\pm$ 6.4

Values within a column with different superscripts were significantly different.

Under PED, "N" means not cover and "C" means cover.

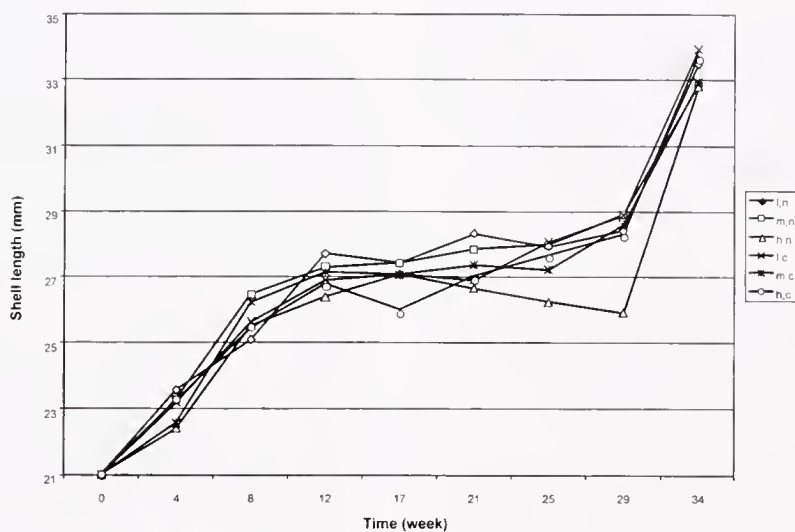


Figure 2. Mean ( $n = 4$ ) shell length of growout seed over time at the different density and PED treatment combinations. (l,n: low-density hags without PED; m,n: medium density without PED; h,n: high density without PED; l,c: low with PED; m,c: medium with PED; h,c: high with PED).

(Kraeuter et al. 1998) and the present study, successful culture was achieved with nursery seed of 5 and 6 mm SL, respectively. Our nursery seed grew on average 2.76 mm/month in SL, similar to that found by Kraeuter et al. (1998) in New Jersey in the summer and by Sturmer et al. (1995) on the west coast of Florida, and higher than that found by Summerson et al. (1995) in North Carolina. The growth rates of nursery seed were similar among the months. The winter months had much smaller effects in reducing growth rates on the nursery seed, as it did on the growout seed (Fig. 2).

Growout clams in high density bags showed retarded growth during the winter months (Fig. 2), when environmental conditions were not optimal for clam growth. As soon as environmental conditions became optimal, clams in the high-density bags grew rapidly to reach similar SL as the clams of the other treatments (Fig. 2). However, a lower percentage of clams in high-density bags reached the legal harvest size (Table 2).

Menzel et al. (1976) and Kraeuter et al. (1998) suggested that survival of planted clams should be more than 40 to 50% for the commercial culture to be profitable. In the present study, average survival of nursery clams ranged from 59.0 to 94.5%. This survival is similar to the 87% found by Summerson et al. (1995) for the nursery seed grown in raceways in North Carolina and to the 58 to 88% found by Sturmer et al. (1995) in a 3-month field study on the west coast of Florida.

Densely packed seed clams without PED in southeastern states have demonstrated massive losses to predation in very short periods of time (e.g., Menzel et al. 1976, Gibbons and Castagna 1985, Peterson 1990, Kraeuter et al. 1998). Predators did not seem to be a significant problem in the present study. Although crabs and sheepshead were observed in the area, and sometimes small crabs were found inside the bags. Also, some of the dead clams found showed signs of crab predation: chipped margins or crushed shells (Vaughan et al. 1988). The PED used in the present study did not improve clam survival, and the maintenance of the PED was time consuming. However, biofouling on the culture bags without the PED was heavier than those with the PED. Some PEDs were found floating or folded over the bags during the study and were re-installed.

The use of PED may affect such biological processes as growth

(Virnstein 1977, Dayton and Oliver 1980, Riese 1985). In the present study, survival was slightly higher in bags with PED in the growout period, and clams in bags with PED were found to grow slightly, but not significantly, faster than those in the bags without PED. Water flow in bags without PED seemed to be retarded because of fouling (by drift algae and sea squirts) of the bags. Fouling was found to be higher in bags without PED than in bags with PED where fouling was mainly observed on the PED itself. Fouling organisms diminish the water flow that passes through a bag, preventing clams from getting food necessary for optimal growth (Flimlin and Mathis 1993, Mojica and Nelson 1993). Drift algae (*Gracilaria* sp.) and tunicates were the most abundant fouling organisms in the present study. They grew rapidly if left unchecked. Inversion of the bags to smother fouling organisms was found to increase the growth of clams in the area. Inversion increased the percentage of nursery clams that reached the growout size and resulted in higher CI. Inversion also increased survival of the clams.

A large increase in chlorophyll *a* concentration was observed from late April to late May. This increase in food supply in conjunction with increasing temperature may explain the rapid growth of the clams during this period. Since the pioneering work of Kellog (1903), it has been recognized that current speed has a major effect on the growth of northern quahog (Kerswill 1949, Haskins 1952, Hadley and Manzi 1984, Manzi et al. 1986). Grizzle and Morin (1989) and Grizzle and Lutz (1989) suggest that northern quahog growth is primarily determined by horizontal seston flux past the animals and that intermediate seston flux rates produce the highest growth rates in *Mercenaria mercenaria* in sandy sediments. In the present study, mean current velocity was 6.7 cm/s for the 34-week period, and mean chlorophyll *a* concentration was 0.61  $\mu\text{g/L}$  in April and May. Cahalan et al. (1989) indicated that growth rate of scallops peaked at 6.5 cm/s at 6,000 algal cells/mL.

In conclusion, a planting density of 7,500 and 750 clams/bag for nursery seed and growout seed, respectively, should be used in the Oak Hill, Florida area, because the highest percentage of seed that reached growout seed or legal harvest size, respectively, was

found at these low densities. The PED used in this study did not improve survival, and its maintenance is time consuming. However, it could be used to reduce biofouling on the culture bags. It is much easier to clean the PED (with larger mesh size and without clams and sediment inside) than to clean the culture bags. We recommend periodic inversion of bags to smother biofouling organisms.

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## RELATIONSHIP BETWEEN THE BURROWING WORM *POLYDORA* SP. AND THE BLACK CLAM *CHIONE FLUCTIFRAGA* SHOWERBY

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**ABSTRACT** The black clam *Chione fluctifraga* is collected for human consumption in both coasts of the peninsula of Baja California, México. An epibionts survey on the black clam from Bahía Falsa, B.C. and Bahía de Guásimas, Sonora revealed an association between the clam and the burrowing worm *Polydora* sp. Approximately 94% of the worms were located around the siphon area in both valves of the clams taken from Bahía Falsa and 54.5% in clams from Bahía de Guásimas. The number of worms per host varied from 1 to 48 in Bahía Falsa and 2 to 15 in Bahía de Guásimas. There was a trend of increased intensity of worm infestation with increased clam size. After a period of 10 months under aquarium conditions, mean percentage of occupation of the siphon area by the worm varied from 94.2% at the beginning of the observation period to 88.3% at the end in clams from Bahía Falsa, and from 54.5% at the beginning of the observation period to 43.4% at the end in clams from Bahía de Guásimas. There was an increase in the mean number of worms on the clams after the observation period, from 9 to 15.7 worms in organisms from Bahía Falsa and from 5.2 to 9.5 worms in clams from Bahía de Guásimas. Worms may survive on the shell after the host is dead. Temperature during observation period varied from 20 to 25.5°C. The U-shape channels of the worm result in a very porous and brittle host shell. In heavily infested clams the shell is broken and this impinges on the clams ability to close its valves. This is the first record of burrowing worms associated with the siphon aperture area of the shell of *C. fluctifraga*.

**KEY WORDS:** *Polydora*, *Chione fluctifraga*, burrowing worm, infestation

### INTRODUCTION

The named "polydorid" complex of the family Spionidae comprises a number of highly diverse but closely related species, all characterized by a modified fourth or fifth setiger (Light 1978). Among them, the genera *Polydora* and *Boccardia* contain a large number of species able to bore into calcareous substrates including shells of such commercially important bivalves as mussels, oysters, cockles, and scallops (Read 1975, Sato-Okoshi et al. 1990, Blake 1996, Handley and Bergquist 1997). These species live in a tube inside the hole bored in the shell of the host with two exterior apertures. The anterior end of the worm emerges from the tube and feeds from particles taken from the sediment surface or from the overlying water column with the aid of its palps (Daro and Polk 1973, Blake 1996). Its boring activity may reach the inner surface of the mollusk's shell and induce the host to secrete calcite and conchiolin layers, forming a blister to isolate the worm (Kent 1979, Lauckner 1983). As a consequence, these worm species are also named "mudworms" or "blisterworms" (Lauckner 1983). A deviation of host energy for growth and reproduction to build a blister has been suggested by some authors (Williams 1968, Kent 1979). In edible oysters, the blisters affect the half-shell market, because the blisters can be punctured and release anaerobic metabolites, including hydrogen sulphide (Handley 1995). A blister-

worm can also render oyster shells brittle and easily broken during shucking, packaging, and transport (Korringa 1951).

The black clam, *Chione fluctifraga* is a highly regarded food and supports an extensive sport and commercial fishery in southern California (Haderlie and Abbott 1980). This species is also gathered for human consumption on the Pacific coast of Baja California and the Gulf of California, México (Martínez-Córdova 1988, Martínez-Córdova 1996). A survey of clams from Bahía Falsa on the Pacific coast of Baja California, and Bahía de Guásimas, Sonora, México revealed the presence of polychaetes on shells. The aims of this study were to determine the identity of the polychaete and to document some aspects of the relationship between this worm and the black clam.

### MATERIALS AND METHODS

In April 1997, a sample of 48 live *Chione fluctifraga* from Bahía Falsa, Baja California was collected and subsequently, in June, a sample of 71 dead clams (empty paired shells) were obtained. Finally, in July 1997, a sample of 125 live black clams from Bahía de Guásimas, Sonora was also collected (Fig. 1). Bahía Falsa has a muddy bottom, and Bahía de Guásimas has a sandy and muddy bottom. After washing the clams in running seawater, each live clam was measured (length from the umbo to the posterior

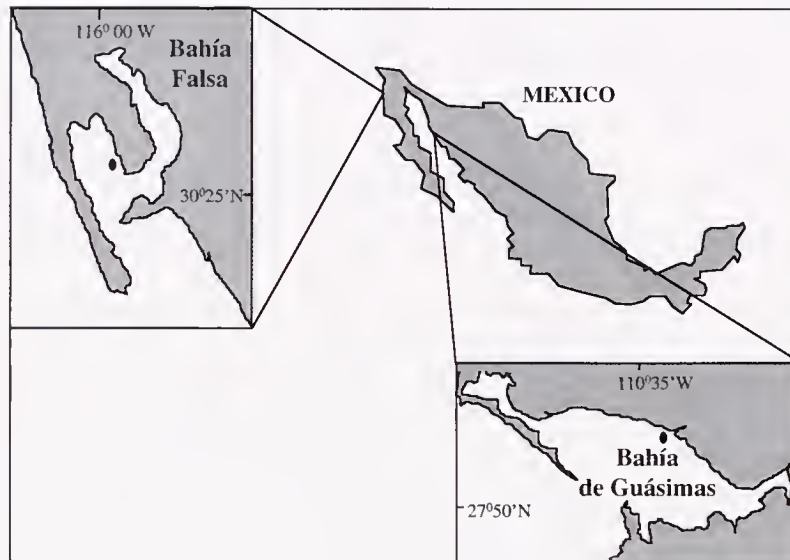


Figure 1. Map showing Bahía Falsa in Baja California and Bahía de Guásimas, Sonora, México. Black dots indicate sampling areas.

margin of the shell). The surface of the shell was delimited for examination in three zones (approximately 30% of the total surface area each one): zone 1 (Z1), around the siphon area; zone 2 (Z2) in the middle area, and zone 3 (Z3) opposite to the siphon area (Fig. 2). Clams were placed individually in Petri dishes filled with seawater, and the number of worms emerging from the shell were counted by zone under the dissecting microscope. Two holes from the same "U"-shaped channel were considered as one worm. Subsequently, clams were opened with a knife, and the meat was discarded. Then, the inner sides of the right and left valves were checked for worms by zone, and *Polydora* infestations visible in the inner shell were enumerated by zone. The total infestation intensity was determined by comparing the number of worms observed on both sides of the shell. Dead clams were cleaned under running tap water, and worm blisters and holes related to burrowing worms were counted by zone. Prevalence was considered as the percentage of infested clams in the sample. To determine the

degree of damage of the burrowing worm on the shell of the clam, X-ray radiographs were taken from clams with different infestation intensities. The number of worms was related to the size of the clams.

Fifteen worms were extracted from the shell of six clams collected in Bahía Falsa, and seven were extracted from six clams from Bahía de Guásimas by crushing them with nippers around the edge of the shell, where the worms were located. The worms were removed from the shell fragments with dissection tweezers and fixed in 70% ethanol for identification.

To assess worm behavior, 33 infested live clams from Bahía Falsa and nine infested live clams from Bahía de Guásimas were labeled and placed separately in aquaria without sand for 10 months. The water was changed every 3 or 4 days, and the temperature was recorded with a manual thermometer. The clams were fed once daily with *Isochrysis galvana* and *Chaetoceros* sp. The water was checked for release of burrowing worm larvae and the

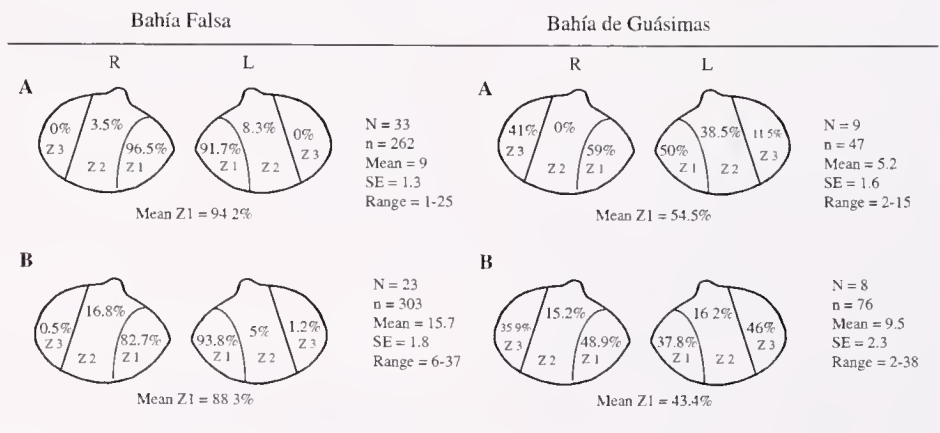


Figure 2. Zones delimited on the valves of *Chione fluctifraga* to determine distribution of *Polydora* sp. Percentage of *Polydora* sp. infesting different zones (Z1, Z2, Z3) of the right (R) and left (L) valves of the black clam from Bahía Falsa, B.C. and Bahía de Guásimas, Sonora, at the beginning of the observation period (A) and the end (B). N = number of clams studied; n = number of *Polydora* sp.; Mean Z1 = Mean of the percentages of occupation of worms in both valves; Mean = mean number of worms in both valves; SE = standard error of the mean number of worms in both valves; Range = minimum and maximum number of *Polydora* sp. found in both valves.



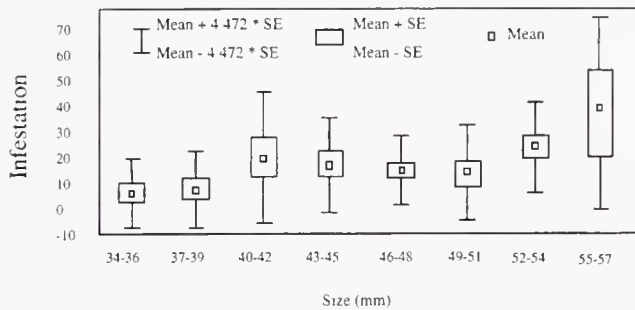


Figure 3. Relationship between the size of the clams and the number of worms infesting their shells.

number of living worms, and worm holes per zone in the shell of the clams were counted and compared to the initial numbers at the end of the period.

#### Statistics

Mean shell length and infestation between live and dead clams were compared using *t* and the Kruskal–Wallis tests. A nested effect model was carried out to determine infestation differences between: (1) clams from Bahía Falsa and Bahía de Guásimas; and (2) valve side; and (3) among valve zone, assuming that the number of worms per zone was nested to the corresponding valve, which was also nested to the corresponding bay. Finally, another nested effect model was applied to the study in aquaria conditions. The initial number of worms was incorporated as a covariate to analyze whether the number of worms changed at the end of the study.

### RESULTS

The mean size of live clams from Bahía Falsa was 43.3 mm (SE 0.48) and of dead clams was 46.4 mm (SE 0.56), the difference was significant ( $t = -39$ ,  $p < .01$ ). The mean size of clams from Bahía de Guásimas was 32.9 mm (SE 0.2).

Holes in the shells were occupied by a spionid polichaete from the genus *Polydora*, and the mean size of worms was 37.7 mm (SE 5.68). Their morphological characteristics agree with the description of *Polydora limicola* (Anenekova): prostomium weakly incised along anterior margin, caruncle extending to setiger 3, 4 eyes present; palps and dorsum of anterior setigers with black pigmented bands or without bands; major spines of setiger 5 with a small, triangular lateral tooth; posterior notopodial spines absent and pygidium disclike with dorsal notch. However, its borrowing behavior suggests the species *P. ciliata*. (Johnson) (See Blake 1996; 173). The worm produced U-shaped channels, which were filled with compacted mud. The burrows were extended by "chimneys" composed of detritus (algae remnant), which protruded from the surface of the clams' valves. Worms were very active, their palps were continuously extended from the burrows.

*Polydora* prevalence in live and dead clams from Bahía Falsa was 48.6 and 66%, respectively. The number of worms per host ranged from 1 to 25 in live clams and from 1 to 48 in dead clams, and infestation differences were not significant, *t*-test ( $t = 1.345$ ,  $p = .180$ ), Kruskal–Wallis test ( $\chi^2 = 0.110$ ,  $p = .740$ ). In general, there was a trend of more *Polydora* sp. in larger clams relative to small clams (Fig. 3), however, this trend was not significant, *t*-test ( $F = 1.750$ ,  $p = .112$ ), Kruskal–Wallis test ( $\chi^2 = 10.600$ ,  $p = .157$ ).

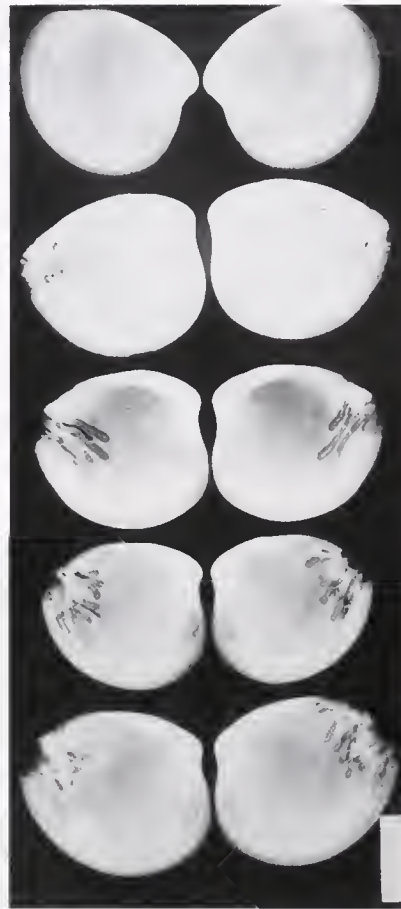
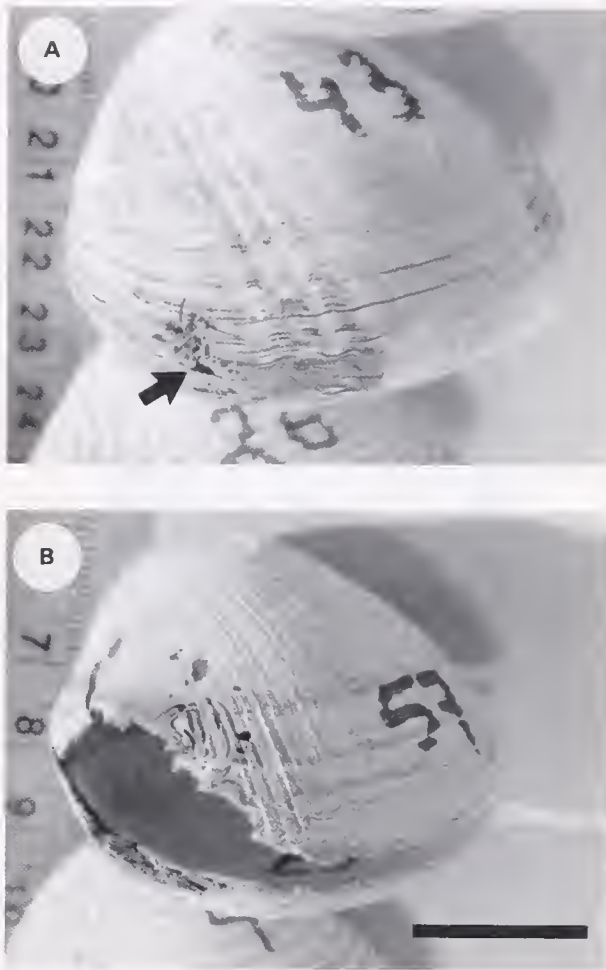


Figure 4. X-ray photograph of several black clams showing different degrees of damage in the shell; note the channels are touching each other in heavily infested clams and the borders of the shells are destroyed.

Worm prevalence in clams from Bahía de Guásimas was 15%, and the number of worms per host ranged from 2 to 15. Between 95 and 97% of the observed worms were placed in the Z1 of live and dead clams from Bahía Falsa, respectively. The corresponding intensity of infestations were 5 and 3% in Z2, there were no worms in Z3. The distribution of *Polydora* sp. in clams from Bahía de Guásimas was 54.5% in the Z1, 18.2% in Z2, and 27.3% in Z3. The right valve was slightly more infested than the left valve. The results of the nested effect model confirmed that the infestation in clams from Bahía Falsa was greater than in clams from Bahía de Guásimas ( $F = 229.370$ ,  $p < .0001$ ); the model also showed that there was a greater infestation in the right than in the left valve ( $F = 6.390$ ,  $p = .0017$ ); finally, it was confirmed that the infestation per valve zone was greater in Z1 than Z2 and Z3 ( $F = 72.660$ ,  $p < .0001$ ).

The damage on the shell depends upon the number of worms and size of the channels. Figure 4 shows different degrees of infestation and associated damage. The channels often extended toward the middle of the valve (Z2) were in close proximity with one another, resulting in a very brittle shell. The shell of heavily infested clams was often broken in the siphon area, hindering valve closure (Fig. 5).

In aquaria, where clams could not burrow into substrate, *Polydora* sp. showed a slight tendency to spread on all the surface of



**Figure 5.** Shell of *Chione fluctifraga* infested by *Polydora* sp. around the siphon area; note the holes in the area (A), heavy infestation results in a very brittle shell that is easily broken (B).

both valves of the host. Mean percentage of occupation of Z1 varied from 94.2% at the beginning of the observation period to 88.3% at the end of the observation period in clams from Bahía Falsa, and from 54.5% at the beginning of the observation period to 43.4% at the end of the observation period in clams from Bahía de Guásimas. The distribution of worms was similar in both valves (Fig. 2). There was an increase in the mean number of worms on the clams after the observation period from 9 (1–25) to 15.7 (6–37) worms in organisms from Bahía Falsa, and from 5.2 (2–15) to 9.5 (2–38) worms in clams from Bahía de Guásimas (Fig. 2). The results of the nested effect model showed that infestation in clams from Bahía Falsa and Bahía de Guásimas was slightly different ( $F = 3.070$ ,  $p = .081$ ); the same model revealed that infestation differences between both valves were not significant ( $F = 0.731$ ,  $p = .482$ ). However, infestation differences among valve zone showed that it was greater in Z1 than Z2 and Z3 ( $F = 9.250$ ,  $p < .0001$ ); finally, it was corroborated that there was an increase in the number of worms at the end of the study ( $F = 138.53$ ,  $p < .0001$ ). Approximately 30% of the clams from Bahía Falsa and 11% of the hosts from Bahía de Guásimas died during this observation period. Worm larval stages were observed during water exchange. The number of living worms at the end of the study from clams belonging to Bahía Falsa was 58 and in clams from Bahía de Guási-

mas was 35. Temperature ranged from 24 to 25.5°C in summer and from 19 to 21°C in winter.

## DISCUSSION

The polydorid species *Polydora limicola* and *P. ciliata* have been found in the northwest Pacific (Radashevsky 1993). *Polydora limicola* has been found in southern California (Hartman 1961, Blake 1996). However, the authors recognize that there are difficulties in their systematics. In accordance with Blake (1996), *Polydora limicola* is virtually identical to that of the well-known and widely distributed shell and limestone borer, *P. ciliata*. Manchenko and Radashevsky (1994) evaluated genetic differences between *P. limicola* and the superficially indistinguishable shell borer (*P. cf. ciliata*) in the Sea of Japan and found that, although there were no morphological differences, there were clear differences in 10 of 27 gene loci surveyed. These genetic differences support the separation of *P. ciliata* and *P. limicola*, which formerly had been based strictly on habitat (Blake 1996). In this sense, the species that we found in this study could be *P. ciliata*; however, detailed molecular genetic studies are needed to clarify its identity.

This is the first record of *Polydora* sp. associated with the siphon area of the black clam *Chione fluctifraga*; however, a similar observation has been recorded in *C. stutchburyi* infested by the polychaete *Boccardia acus* in Wellington Harbour, New Zealand. This worm is a common and conspicuous epibiont of the cockle *C. stutchburyi*. The U-shaped burrow usually follows the curve of the shell growth lines. The boring is not lined with sand apart from a sand-grain partition at the U-bend and a short external sand-grain chimney. The external chimney extends out around the siphons of the cockle, which lies buried just beneath the sediment surface (Read 1975). The polychaete *Boccardia chilensis* has also been recorded boring in *Chione stutchburyi* shells in association with *Broccardia syrtis* [Read (1975)]. The high percentages of worms sited around the siphon area may suggest a specialized relationship between worm and host. The specialized relationship between siponids and their hosts has been described in *Polydora commensalis*; it lives in a shallow burrow excavated along the columnella of the gastropod shell occupied by a hermit crab. This specialized species has short palps with an unusually narrow food groove that seems to be adapted to capturing food particles stirred up or suspended by the activities of the hermit crab (Blake 1996). Our results suggest that the relationship between the worm and the clam seems to be less specialized, because the worm may be sited out of the siphon area if the surface of the clam is available, as in aquaria conditions. Moreover, *Polydora* sp. remain alive on the shell after host death. The trend of more worms around the siphon area between clams from Bahía Falsa and those from Bahía de Guásimas suggest that, in the latter bay, clams are more exposed to colonization by *Polydora* sp. than in the former. Worm prevalence could be related to the type of substrate and with particular environmental factors of the two embayments we examined. Its placement, exclusively around the siphon aperture, allows the worm to feed on the particles inhaled or expelled by the clam, resulting in an advantageous position relative to other surface areas of the shell, if available. We observed great motility of worm palps while the clam protruded its siphons. The preference of worms for the right valve recorded in the field study remains unknown.

The prevalence and number of worms per host in studied clams could be related to age and size of the clams. Possibilities of infestation by *Polydora* sp. in older clams are higher than in younger clams, because the former have had more encounters with

the worms. In addition, larger surfaces provide more area for burrowing worm colonization. This observation could explain differences in prevalence and number of worms per host between larger and older clams from Bahía Falsa relative to smaller and younger clams from Bahía de Guásimas. The different environmental conditions of both bays may also play an important role in prevalence and abundance of the worm.

In aquaria conditions, the increase in number of worms through time and the presence of larval stages indicated reproduction and settling of the worm species. Temperature is one of the primary factors for determining the abundance of *Polydora* sp. (Lauckner 1983); in other words, generation time, reproduction and, hence, transmission. In this study, temperature was maintained near the values recorded in Bahía Falsa (see Cáceres-Martínez et al. 1998) and Bahía de Guásimas (Arreola 1998), this supported the reproduction, setting, and the increase in the number of worms recorded in this study. However, specific studies on temperature in relation to reproduction, growth, and transmission are needed. The mean number of worms per host (initial and final) was slightly higher in

larger and older clams from Bahía Falsa, than in those from Bahía de Guásimas. This observation also supports the observed relationship of surface area and intensity of worm infestation (Fig. 2). Mortality of both host and worms was detected at the end of the observation period. This could be related to deterioration of aquarium conditions (frequency of water renovation and nutrition). However, specific studies on clam mortality in relation to the presence of this worm are needed. Heavy infestation may result in severe damage to the clam shell, a brittle shell border may increase the potential for mortality because of enhanced predation as a result of holes in the valves, and problems handling the clam for packing.

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## ADHESION OF *VIBRIO TAPETIS* TO CLAM CELLS

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**ABSTRACT** The adhesive properties of *Vibrio tapetis*, the causative agent of the brown ring disease affecting cultured clams, were determined considering both the contribution of bacterial surface hydrophobicity and the attachment capability to different animal cells. Hydrophobicity of *V. tapetis* strains was evaluated by means of three different methods, most of the strains being highly hydrophobic for any of the methods used. *V. tapetis* showed higher adhesion capability toward the clam cells used (hemocytes and mantle cells), as compared to several fish cell lines. No significant relationship was obtained between hydrophobicity and cell adhesion, which suggests the existence of adhesion-specific mechanisms. In addition, different bacterial structures were investigated as potential adhesins of *V. tapetis*, including hemagglutinins, pili, flagella, and outer membrane proteins.

**KEY WORDS:** *Vibrio tapetis*, brown ring disease, adhesive capabilities, hydrophobic interaction, clam cells

### INTRODUCTION

*Vibrio tapetis* is the causative agent of brown ring disease (BRD), an epizootic disease that affects cultured clams (*Tapes philippinarum* and *T. decussatus*). Although experimental reproduction of BRD symptoms in healthy clams has been achieved by means of *V. tapetis* inoculation (Paillard and Maes 1990, Castro 1994, Novoa et al. 1998), the precise mechanisms involved in the *in vivo* infection have not yet been well established.

Bacterial attachment and ulterior colonization of the clam periostracal lamina seem to be the first steps in the pathogenesis of *V. tapetis* (Paillard and Maes 1995a, Allam et al. 1996). The colonization and disruption of the periostracal lamina provoke the bacterial accumulation on the inner surface of the clam shell, thereby producing the conchiolin deposit (Paillard and Maes 1995b), which constitutes the main gross symptom of this disease.

In BRD, as in other fish and shellfish diseases, bacterial adhesion to appropriate host surfaces is a key factor for infection establishment (Daly and Stevenson 1987, Santos et al. 1991). However, little is known about the factors contributing to *V. tapetis* adhesion to host surfaces. Several potential adherence factors have been described for *Vibrio* species, including surface proteins, hemagglutinins, and several types of pili (Jonson et al. 1991, Sperandio et al. 1995). These bacterial surface structures, named adhesins, interact with a broad variety of molecular host-cell receptors (Iijima et al. 1981, Christensen et al. 1985, Nakasone and Iwanaga 1990). On the other hand, it has been reported that hydrophobic interactions in addition to hemagglutinating capabilities could be responsible for bacterial adhesion to animate and inanimate surfaces (Bruno 1988, Clark et al. 1989, Savage 1992, Vazquez-Juarez et al. 1994). However, several *Vibrio* species showed an ability to adhere to host cells and cell lines, regardless of their degree of hydrophobicity (Santos et al. 1991). The aim of this work is to study the adhesion properties of *V. tapetis*, considering both the contribution of bacterial surface hydrophobicity and the specific attachment to different cells.

### MATERIAL AND METHODS

#### Microorganisms and Culture Conditions

Twenty-seven strains of *V. tapetis* were used for the hydrophobicity studies. Bacterial strains were grown in tryptone soya broth

or agar (Oxoid Ltd., Basingstoke, Hampshire, UK) supplemented with 1.5% NaCl (TSBS and TSAS, respectively), and incubated at 22°C for 18 h. Eight *V. tapetis* strains, representative of each *V. tapetis* group established previously by Borrego et al. (1996b) and Castro et al. (1997) were used for the adhesion experiments to cells. Bacteria were grown in TSBS for 18 h at 22°C, resuspended in sterile buffered saline (BS), and washed twice by centrifugation (at 24,000 × g for 5 min at 4°C). Bacterial pellets were resuspended in the same BS and adjusted at a bacterial concentration of 10<sup>8</sup> bacteria/mL.

#### Hydrophobicity Assays

To test the hydrophobic capabilities of *V. tapetis* strains, three different assays were performed: microbial adhesion to hexadecane (MATH); salt aggregation test (SAT); and nitrocellulose adhesion test (NCF).

MATH was performed as described by Rosenberg et al. (1980). Bacteria were centrifuged at 4,000 × g for 10 min at 4°C, washed, and resuspended in phosphate urea magnesium sulphate (PUM) buffer (22.2 g/L K<sub>2</sub>HPO<sub>4</sub> · 3H<sub>2</sub>O, 7.26 g/L KH<sub>2</sub>PO<sub>4</sub>, 1.8 g/L urea, 0.02 g/L MgSO<sub>4</sub> · 7H<sub>2</sub>O, pH 7.1), or phosphate buffered saline (PBS) (0.02 M, pH 7.2) to an absorbance at 400 nm of 0.9–1.1. Bacterial suspension aliquots (2 mL) were then transferred to clean round-bottom test tubes, and 0.3 mL n-hexadecane (Sigma Chemical Co., St. Louis, MO, USA) were added and incubated for 10 min. After the mix was homogenized for 2 min, the hydrocarbon phase was allowed to rinse completely, and the aqueous phase was removed to determine the absorbance at 400 nm. The percentage of adhesion to hydrocarbons was calculated using the following expression: Adhesion (%) = [A<sub>400</sub> (initial bacterial suspension) – A<sub>400</sub> (aqueous phase)]/[A<sub>400</sub> (initial bacterial suspension)] × 100.

The ability of the bacteria to bind to nitrocellulose filters (NCF) was determined according to the technique described by Lachica and Zink (1984). Bacterial cultures were centrifuged at 4,000 × g (10 min at 4°C), washed as above, and resuspended in saline solution (0.85% NaCl, pH 7.2) at an absorbance of 1 at 600 nm. Suspensions were filtered through a 13-mm NCF (type CS, 8.0-μm pore size) (Millipore Corp., Bedford, MA, USA). Optical density of the filtrates was measured at 600 nm, and the percentage of adhesion was expressed as: Adhesion (%) = [A<sub>600</sub> (initial bacte-

rial suspension) -  $A_{600}$  (filtrate)]/[ $A_{600}$  (initial bacterial suspension)]  $\times$  100.

The SAT, described by Lindhal et al. (1981), is based on bacterial precipitation in presence of salts. Bacterial cultures were centrifuged at  $4,000 \times g$ , washed, and resuspended in PBS (0.002 M, pH 8.6) to achieve a concentration of  $5 \times 10^9$  bacteria/mL. Then, 30- $\mu$ L aliquots of bacterial suspensions were mixed with equal volumes of decreasing molarities of buffered ammonium sulfate solutions ranging between 0.05 and 4 M. Hydrophobicity was expressed as the lowest molarity of ammonium sulfate that produced visual clumping. Kendall rank coefficients were calculated to determine the correlation between the different hydrophobicity tests assayed.

#### Hemocytes and Mantle Cells Collection

Hemolymph was taken from the posterior adductor muscle of two clam species, *Tapes decussatus* and *T. philippinarum*, using a 20-gauge needle attached to a 3-mL syringe, through a hole performed in the shell margin of the clams. Then, the collected hemolymph was diluted 1:3 in a modified anti-aggregant Alsever solution (MAS) (20.8 g/L glucose, 8.0 g/L sodium citrate, 3.36 g/L EDTA, 22.5 g/L NaCl, and 100  $\mu$ L/L distilled water). Hemolymph of 5 adult specimens of each clam species was pooled and the number of hemocytes was estimated using a Coulter-counter. Before the adhesion assays, hemocyte suspension in MAS was centrifuged at  $400 \times g$  for 10 min, supernatant removed, and hemocytes were resuspended in BS (0.58 m NaCl, 13 mm KCl, 13 mm  $\text{CaCl}_2$ , 26 mm  $\text{MgCl}_2$ , 0.54 mm  $\text{Na}_2\text{PO}_4$ , 50 mm Tris-HCl, pH 7.4). Then, the cells were fixed with 3.7% formaldehyde for 20 min at 4°C. Formaldehyde was removed by centrifugation ( $400 \times g$  for 10 min), and the pellet resuspended in BS at a concentration of  $10^6$  cells/mL.

Mantle cells were collected from healthy specimens of both clam species. Briefly, the clams were opened, and the mantle was extracted in aseptic conditions and washed for 15 min in BS, for 20 min in an antibiotic solution (Sigma, 10,000 IU/mL penicillin, 10 mg/mL streptomycin and 25  $\mu$ g/mL amphotericin) 10-fold diluted in PBS, and finally washed for 15 min in BS supplemented with 2.5% trypsin. Mantle tissue was disrupted using Pasteur pipettes, centrifuged at  $300 \times g$  for 5 min at 4°C, and the pellet was resuspended in saline solution (0.55 m NaCl in distilled water). Mantle cells were isolated using a continuous gradient of Percoll (Amersham Pharmacia Biotech GmbH, Barcelona, Spain) previously prepared by centrifugation (at  $25,000 \times g$  for 20 min at 4°C) of a 60% percoll in saline solution. Cells were separated by centrifugation at  $10,000 \times g$  for 10 min at 4°C. The band in the percoll gradient that contained the cells was collected, washed twice in BS (at  $300 \times g$  for 5 min at 4°C), and fixed with 3.7% formaldehyde for 20 min at 4°C. Then, the formaldehyde was removed by centrifugation ( $400 \times g$ , for 10 min), and the pellet was resuspended in BS at a concentration of  $10^6$  cells/mL.

#### Cell Adhesion Assays

The adhesion of *V. tapetis* to hemocytes or mantle cells of both clam species was evaluated by two different methods: the adhesion method described by Kumazawa et al. (1991), and by an ELISA test developed in the present study. Clam cells and *V. tapetis* were incubated at a concentration of  $1 \times 10^6$  cells/mL and  $1 \times 10^8$  bacteria/mL, respectively, in BS at room temperature (about 20°C) for 2 h with gentle agitation. After the incubation, the nonadhered

bacteria were removed by three cycles of centrifugation (at  $300 \times g$  for 5 min, 4°C) in BS, and the final pellet was resuspended in 500  $\mu$ L of BS and fixed with 0.7% formaldehyde overnight. Afterwards, volumes of 100  $\mu$ L were deposited in microplate wells to perform the ELISA test, and volumes of 200  $\mu$ L were disposed in slides, stained with Giemsa and observed under light microscopy.

In the indirect ELISA test, an anti-*V. tapetis* serum raised in rabbit (Castro et al. 1995) was used as the first antibody, and antirabbit IgG labeled with peroxidase (Sigma) as the second antibody. Mixtures of bacteria and clam cells without the first antibody, and clam cells with the first and second antibodies, were used as controls.

#### Adhesion to Fish Cell Lines

Three fish cell lines were used for adhesion assays, Chinook salmon embryo (CHSE), epithelioma papulosum of carp (EPC) and SAF-1 derived from fibroblast of gilt-head seabream fins. Cells were maintained in Eagle's minimal essential medium (MEM) (Gibco Life Technologies, Paisley, UK) or, in the case of SAF-1 cell line, in L-15 Leibovitz medium (Gibco) supplemented with 2% glutamine, both containing 10% fetal calf serum and antibiotics (1% penicillin/streptomycin). Semiconfluent monolayers were grown on 24-multiwell plastic dishes with glycerol-treated coverslips (12-mm diameter). Cell monolayers were fixed with 3.7% formaldehyde for 20 min at 4°C, and washed thoroughly with PBS.

To perform the adhesion assays, bacterial suspensions containing  $10^8$  bacteria/mL were placed in the multiwell dishes containing the cell-coated coverslips and incubated at 20°C with gentle shaking for 2 h. After being washed thoroughly with PBS, coverslips were air dried and fixed with formaldehyde for 20 min. Then, coverslips were stained with crystal violet, mounted onto microscope slides, and examined under light microscopy.

The adherence to fish cell lines was also evaluated using the ELISA test described above. Fish cell monolayers were trypsinized and resuspended in fresh medium without antibiotics. Then, the cells were fixed and washed as described, and resuspended in PBS at a concentration of  $10^6$  cells/mL. Adhesion assays and the ELISA test were conducted as mentioned for clam cells.

#### Hemagglutination Tests

Hemagglutination was determined using rat, horse, rabbit, and human erythrocytes according to the technique described by Larsen and Møllergaard (1984). Equal volumes (100  $\mu$ L) of bacterial suspension ( $10^9$  bacteria/mL) and erythrocyte suspension (3%, v/v) in PBS (0.01 m, pH 6.8) were mixed on a 96-well plate, and incubated at room temperature for 1 h. As negative controls, erythrocyte suspensions in PBS and bacterial suspensions in PBS were used. The test was considered negative if visible agglutination did not occur within 10 min.

Inhibition of hemagglutination was performed by mixing the bacterial suspensions with 10, 25, 50, 75, and 100 mM solutions in PBS of D-mannose, D-fucose, L-fucose, D-glucose, D-galactose, D-fructose, D-lactose, and raffinose (Sigma). A negative control of erythrocytes plus sugar in PBS was used.

#### Transmission Electron Microscopy (TEM)

The arrangement of flagella and fimbriae was examined under TEM in 24-h *V. tapetis* grown in TSAS. Briefly, the samples were fixed with 2.5% glutaraldehyde in 0.01 m cacodylate buffer (pH

7.2) for 2 h at 4°C. Then, they were stained with 1% uranyl acetate (pH 4.5) for 45 s on copper grids (400 mesh) covered with formvar, dried, and examined under TEM.

#### Outer Membrane Protein Analyses

Outer membrane protein (OMP) analyses were carried out by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), following the method described previously by Castro et al. (1996). Briefly, bacterial cells were disrupted by sonication (in an ice bath with 4 pulses of 30 s at 50 W), and the cellular envelopes were sedimented by centrifugation (100,000 × *g* for 1 h at 4°C). Cytoplasmic membranes were selectively solubilized with sodium lauryl sarcosinate (Sarkosyl, Sigma), and outer membranes (OM) were sedimented by centrifugation as above.

OM samples were electrophoresed in discontinuous polyacrylamide-SDS gels (4.5–12.5%), using the Laemmli's technique (1970). Proteins were detected in the gels by staining with Coomassie brilliant blue (Sigma).

## RESULTS

#### Hydrophobicity

Different results have been obtained depending on the method used to estimate cell surface hydrophobicity of *V. tapetis* strains (Table 1). Most of the isolates (77.8%) aggregated in the presence of less than 1 M ammonium sulfate. Adhesion to nitrocellulose filters yielded the highest values of hydrophobicity for most of the bacterial isolates, percentages of adhesion ranging between 94.9 and 99.9%. A variable degree of adhesion to n-hexadecane has been observed, percentages of adhesion ranging between 20.1 and 64.6% when PUM buffer was used an aqueous phase and between 21.4 and 63.8% when PBS was used.

The criteria of hydrophobicity proposed by Santos et al. (1990) and Lee and Yii (1996) were used to evaluate the hydrophobicity of the *V. tapetis* strains tested (Table 2). According to the criteria applied, 11.1 and 40.8% of the isolates were highly hydrophobic with MATH assay, using PUM and PBS buffers, respectively. However, 77.8% of the strains showed strong hydrophobicity with SAT and 100% with NCF assay (Table 2). Most of the isolates were included in the group of moderate hydrophobicity with MATH (88.9% for PUM buffer, and 59.2% for PBS buffer), and only 22.2% of the strains were included using the SAT test.

Kendall rank coefficients have shown the presence of significant correlation ( $p < .05$ ) between MATH assays in the presence of PUM and PBS buffers ( $p = .026$ ). These findings suggest that adhesion to n-hexadecane is not significantly influenced by the use of PUM or PBS buffers as aqueous phase. On the contrary, no significant correlation was obtained between the rest of tests assayed ( $p > .05$ ), with significance degree of  $p = .393$  for MATH (PUM) versus SAT,  $p = .884$  for MATH (PUM) versus NCF,  $p = .440$  for MATH (PBS) versus SAT,  $p = .491$  for MATH (PBS) versus NCF, and  $p = .348$  for SAT versus NCF.

#### Cell Adhesion Assays

*V. tapetis* adhesion to different cell systems, such as clam hemocytes, mantle cells of clams, and fish cell lines, has been evaluated by two different approaches, the microscopic determination of cellular adhesion percentages and by an ELISA (Tables 3 and 4). The results obtained varied depending both on the cellular system used and the *V. tapetis* strains tested. Thus, all the *V.*

TABLE 1.

Determination of cell surface hydrophobicity of *Vibrio tapetis* strains using different methods

Strains	MATH <sup>a</sup>			
	PUM	PBS	NCF <sup>b</sup>	SAT <sup>c</sup>
<i>V. tapetis</i> 2.1	47.5	61.0	99.1	0.50
<i>V. tapetis</i> 2.3	44.9	60.0	99.2	1.00
<i>V. tapetis</i> 8.1	47.3	58.9	99.9	0.46
<i>V. tapetis</i> 8.3	42.9	63.8	99.5	0.93
<i>V. tapetis</i> 8.4	32.2	55.7	99.5	0.70
<i>V. tapetis</i> 8.5	35.4	62.6	99.1	0.46
<i>V. tapetis</i> 8.6	22.0	22.3	99.5	0.80
<i>V. tapetis</i> 8.7	29.6	37.6	99.4	0.70
<i>V. tapetis</i> 8.17	24.8	35.4	99.9	1.20
<i>V. tapetis</i> 8.19	51.3	48.5	99.4	0.80
<i>V. tapetis</i> 9.3	54.0	53.3	99.4	0.70
<i>V. tapetis</i> 9.4	30.4	44.3	99.9	0.93
<i>V. tapetis</i> 9.5	46.2	53.7	99.7	0.66
<i>V. tapetis</i> 9.7	46.0	58.0	99.4	0.93
<i>V. tapetis</i> 11.1	64.7	50.0	99.1	1.00
<i>V. tapetis</i> 11.2	26.0	21.4	99.9	0.80
<i>V. tapetis</i> 11.4	29.1	56.3	97.9	0.93
<i>V. tapetis</i> 1S-1	20.1	37.4	94.9	0.86
<i>V. tapetis</i> 1S-5	46.9	33.5	99.4	0.80
<i>V. tapetis</i> 1S-7	9.7	44.8	98.1	0.83
<i>V. tapetis</i> 1S-8	27.0	35.6	99.8	0.80
<i>V. tapetis</i> 1S-9	46.2	50.7	99.9	0.80
<i>V. tapetis</i> CECT 4600 <sup>†</sup>	20.7	46.5	99.6	1.00
<i>V. tapetis</i> 1703	23.3	31.4	98.9	0.80
<i>V. tapetis</i> 6301	46.0	42.0	99.5	0.66
<i>V. tapetis</i> 0202	40.9	44.5	99.1	1.00
<i>V. tapetis</i> 0705	26.2	44.4	99.0	1.00

<sup>a</sup> Percentage of adherence to n-hexadecane.

<sup>b</sup> Percentage of adherence to nitrocellulose filters.

<sup>c</sup> Lowest molarity of ammonium sulphate producing visible aggregation. CECT: Spanish Type Culture Collection.

<sup>†</sup> Type strain.

*tapetis* strains did not adhere to the fish cell lines CHSE and EPC, but the adhesion percentage to SAF-1 cells ranged from 2 to 96% (Table 3). These values contrast with the values obtained for clam cells (hemocytes and mantle cells), varying between 68 and 100% (Table 3). Only two *V. tapetis* strains (8.6 and 0202) showed similar adhesion rates, regardless of the type of cells used. No significant differences ( $p > .05$ ) were obtained in the adhesion capability of *V. tapetis* strains depending on the origin of the clam cells used, except in the case of the strain 11.2 for hemocytes of *T. decussatus* and *T. philippinarum* (89% vs. 68%), and the strains 2.1 (76% vs. 100%) and CECT 4600<sup>†</sup> (77% vs. 97%) for mantle cells of both clam species.

Mean numbers of adhered bacteria per cell are also given in Table 3. SAF-1 cell line proved to be a poor system for *V. tapetis* adhesion, with mean values ranging between 1.5 and 6.8 adhered bacteria per cell. In contrast, mantle cells and clam hemocytes were better matrix systems for *V. tapetis* adhesion, ranging between 5.8 and higher than 25 adhered bacteria per hemocyte, and between 10.4 and 22.8 adhered bacteria per mantle cell. In this case, no significant differences were detected between the origin of the cells (source and species), although significant differences were obtained between the *V. tapetis* strains tested, strain 11.2 being the least adherent to hemocytes (Table 3).

TABLE 2.

Hydrophobicity degree of *Vibrio tapetis* strains using SAT, NCF, and MATH assays according to the criteria proposed by Santos et al. (1990) and Lee and Yui (1996)

Test	Values	Hydrophobicity degree	Percentage of <i>V. tapetis</i> Isolates
SAT	<1.0 M	Strong	77.8
	1.1–2.0 M	Moderate	22.2
	2.1–4.0 M	Weak	0
	>4.0 M	Negative	0
NCF	>75%	Strong	100
	50–75%	Moderate	0
	<50%	Negative	0
MATH (PBS) <sup>a</sup>	>50%	Strong	40.8
	20–50%	Moderate	59.2
	<20%	Negative	0
MATH (PUM) <sup>b</sup>	>50%	Strong	11.1
	20–50%	Moderate	88.9
	<20%	Negative	0

<sup>a</sup> PBS used as aqueous phase.

<sup>b</sup> PUM buffer used as aqueous phase.

Table 4 expresses the results of *V. tapetis* adhesion using an ELISA technique. In agreement with the results given in Table 3, *V. tapetis* strains possessed a low adhesion rate for SAF-1 cells, and even strain IS-7 was nonadherent. Adhesion to clam cells varied depending on the strains considered; thus, significant differences were found between the adhesion to clam hemocytes of both clam species for *V. tapetis* CECT 4600<sup>T</sup>.

No correlation was obtained between the hydrophobicity of the strains and their adhesion to several cells, except in the case of MATH (PUM) hydrophobicity and adhesion to hemocytes of *T. philippinarum* determined by ELISA (data not shown).

#### Hemagglutination and *V. tapetis* Appendages

None of the *V. tapetis* strains presented hemagglutination of horse, rabbit, and human erythrocytes; on the contrary, all of them agglutinated rat erythrocytes, and the hemagglutination was not inhibited by any of the sugars tested at the different concentrations assayed.

The presence of appendages on the *V. tapetis* surface was determined by TEM. In all the *V. tapetis* strains tested, the presence of a sheathed polar flagellum was recorded (Fig. 1), and sometimes several lateral flagella were also observed. Piliation of the strains or fimbria-like structures were not observed in the bacterial cultures tested. However, visualization of isolated and purified pili has not been performed yet.

#### Analysis of the Cellular Components of the Outer Membrane

The electrophoretic analyses of OMP showed that all *V. tapetis* strains tested, except the strain 0202, present the same band pattern, expressing proteins of molecular weight ranging between 78 and 15 kDa. The profile of these strains is dominated by a major outer membrane protein (MOMP) of an estimated molecular weight (MW) of 35 kDa. In the case of the strain 0202 the MOMP was of 37 kDa, and presented a high MW protein of 94 kDa (Fig. 2).

## DISCUSSION

In BRD, an epizootic disease affecting cultured clam species, mainly *T. philippinarum*, *V. tapetis* is predominantly detected on the clam periostracal lamina (Allam et al. 1996). The capacity of this pathogen to adhere to periostracum is obviously an essential step for the bacterial colonization. However, the mechanisms by which bacterial cells adhere to this clam surface has not been elucidated completely. One hypothesis for the mechanisms by which *V. tapetis* adheres to clam tissues involves the concept that filamentous appendages characterized as pili (Paillard and Maes 1995a) bind the cells to periostracal lamina. However, the presence of these bacterial appendages were visualized only in some colonizing *V. tapetis* in diseased clams (Borrego et al. 1996a). On the other hand, Arp (1988) pointed out that before the bacterial adhesion, it is necessary for the bacteria to maintain their position along a mucosal surface by establishing small numbers of noncovalent bonds between the bacterial and mucosal surfaces. These bonds depend on several physicochemical mechanisms, the hydrophobic interactions being the most important (Rosenberg and Kjelleberg 1986). For these reasons, we suggest that the adhesion of *V. tapetis* to clam tissues may be governed by two different but complementary mechanisms: (1) physicochemical forces of adsorption; and (2) specific adhesion depending on adhesive bacterial structures

TABLE 3.

Adhesive capabilities of *Vibrio tapetis* strains to different cell systems

Strains	Adhesion Percentages					Mean of Adhered Bacteria per Cell				
	SAF-1	Hemocytes		Mantle Cells		SAF-1	Hemocytes		Mantle Cells	
		<i>T.d</i> <sup>a</sup>	<i>T.p</i> <sup>b</sup>	<i>T.d</i>	<i>T.p</i>		<i>T.d</i>	<i>T.p</i>	<i>T.d</i>	<i>T.p</i>
2.1	2	88	91	76	100	1.9	6.6	11.2	21.0	21.3
8.6	96	100	94	88	98	6.8	11.7	10.9	19.0	17.6
9.4	25	98	94	90	99	5.1	>25	12.1	15.1	22.8
11.1	18	88	100	96	96	1.6	10.4	15.2	21.7	10.5
11.2	46	89	68	90	91	2.2	6.6	8.1	21.1	13.7
IS-7	58	89	99	94	98	1.5	>25	16.1	17.9	16.5
CECT 4600 <sup>T</sup>	59	100	95	77	97	3.7	11.3	5.8	10.4	17.4
0202	96	100	100	90	97	6.6	>25	15.2	14.6	20.1

<sup>a</sup> *Tapes decussatus*.

<sup>b</sup> *Tapes philippinarum*.



TABLE 4.

Adhesive capabilities of *Vibrio tapetis* strains to different cell systems using an ELISA technique

Strains	SAF-1	Hemocytes		Mantle Cells	
		<i>T. decussatus</i>	<i>T. philippinarum</i>	<i>T. decussatus</i>	<i>T. philippinarum</i>
2.1	0.21 <sup>a</sup>	0.23	0.24	0.28	0.34
8.6	0.19	0.22	0.25	0.28	0.33
9.4	0.18	0.23	0.24	0.28	0.31
11.1	0.17	0.24	0.24	0.24	0.30
11.2	0.15	0.20	0.26	0.26	0.30
IS-7	0.09	0.20	0.25	0.29	0.29
CECT 4600 <sup>T</sup>	0.22	0.17	0.38	0.24	0.30
0202	NT <sup>b</sup>	NT	NT	NT	NT

<sup>a</sup> Absorbance units at 450 nm.<sup>b</sup> NT: Not tested because of the lack of specificity of the antiserum against *V. tapetis* to strain 0202.

that interlock in a stereospecific manner with complementary structures on the opposing surface.

Cellular hydrophobicity is known to be associated with the capacity of microbial cells of many taxonomic groups to adhere to surfaces of numerous types, including those of animal tissues (Doyle and Rosenberg 1990). The results obtained in the present study demonstrate that even strains of *V. tapetis* closely related at biochemical, serological, and molecular levels (Borrego et al. 1996b, Castro et al. 1996, Castro et al. 1997) may vary significantly in surface hydrophobicity as measured by any of the three assays used (Table 1). As with other bacteria, consistency of results among the three hydrophobicity assays was only observed in strains with very hydrophilic surfaces (Mozes and Rouxhet 1987, Sorogon et al. 1991). Differences in the distribution of hydrophobic components on the bacterial surface may account for these disparities. Although the SAT may provide a measure of overall surface hydrophobicity, the MATH and NCF assays may indicate the presence of hydrophobic domains on an otherwise hydrophilic cell surface (van der Mei et al. 1987). The lower values of hydrophobicity obtained for *V. tapetis* strains using the MATH assay as compared to SAT and NCF techniques has been reported previously (Sorogon et al. 1991). These authors pointed out the possi-

bility that cell surfaces of the bacteria tested were modified by the hydrophobicity assay procedures. Thus, hexadecane used in the MATH assay may extract constituents from the bacterial envelope (Dillon et al. 1986, Rosenberg and Kjelleberg 1986).

Methods for measuring bacterial hydrophobicity differ somewhat in the precise properties they measure, and different types of interactions are considered when different methods to estimate hydrophobicity are used. Thus, Dickson and Koochmaria (1989) observed that the relative hydrophobicity estimated by hydrophobic interaction chromatography, MATH and contact angle measurements for different bacterial species was dependent on the specific method tested. The results obtained in the present study for *V. tapetis* strains isolated from diseased clams show that the experimental conditions imposed by the different methods used influence the observed hydrophobicity interactions to some degree.

Specific hydrophobicity assays may be useful predictors of adhesion for closely related strains of certain bacterial species (Martin et al. 1997). In the case of *V. tapetis* strains isolated from diseased clams, the results obtained in the hydrophobicity tests indicate that adhesion of these bacteria cannot be explained by hydrophobic interactions alone. Rather, adhesion is likely to be mediated by an interplay of hydrophobic and hydrophilic surface components (Dickson and Siragusa 1994), or specific interactions

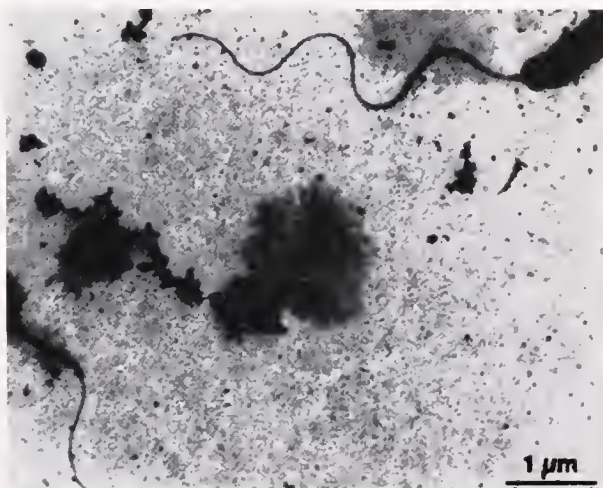


Figure 1. Electron micrograph of negative stained *Vibrio tapetis* CECT 4600 cells, showing the presence of a single sheathed polar flagellum.

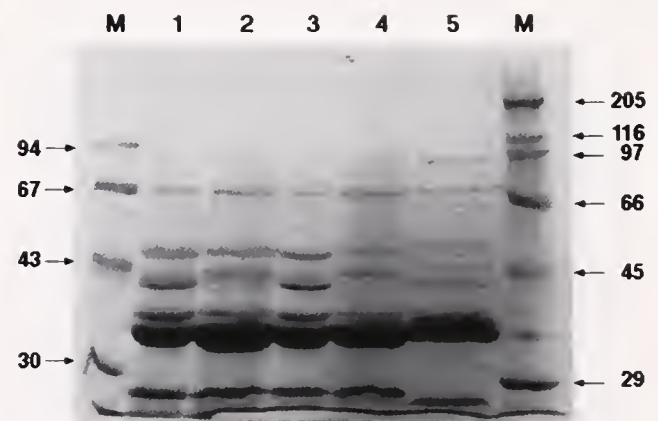


Figure 2. Electrophoresis in SDS-polyacrylamide gels of the outer membrane proteins from several *Vibrio tapetis* strains; lanes 1 to 5, strains CECT 4600<sup>T</sup>, 2.1, 11.2, IS-7, and 0202, respectively. In lanes M, the molecular weight (in kDa) of standard proteins is indicated.

between bacterial adhesins and cellular receptors (Christensen et al. 1985).

For many pathogenic bacterial strains, mucosal attachment is mediated by specific bacterial surface appendages. Pili are considered as the most relevant adhesins and colonization factors of host tissue surface. Many types of bacterial pili have been recognized in *Vibrio* species, such as toxin-coregulated pili (TCP), mannose-sensitive hemagglutinin (MSHA) pili, core-encoded pili (Cep), accessory colonization factor (Acf), NAGV 14, Na2, and Ha7 pili (Yamashiro and Iwanaga 1996). In the present study, we have not detected the presence of these appendages on *V. tapetis* surface, under TEM examination (Fig. 1). However, all *V. tapetis* cells examined showed the presence of a sheath flagellar structure, which may be important for attachment to host tissues (Attridge and Rowley 1983). In contrast, Paillard and Maes (1995a) reported that fimbria-like appendages were present in the *V. tapetis* colonizing the periostracal lamina of diseased clams. These contradictory results lead us to suggest the role of an unknown factor that promotes the fimbria synthesis in *V. tapetis* infection. Iijima et al. (1981) suggested that *V. parahaemolyticus* synthesize a cytotoxic factor that degenerates epithelial cells of the host and promotes its adherence. On the other hand, nutrient-limiting conditions may enhance the bacterial adhesion (Dai et al. 1992) or induce the formation of adherent structures (McCarter and Silverman 1989, Nakasone and Iwanaga 1990). The importance of the flagellum as a potential virulence factor has been demonstrated for several bacterial species. This structure has been involved in pathogenicity as either a motility organelle or an organelle that carries an adhesive component, both roles providing an advantage to the bacterium for its invasive capabilities (Norqvist and Wolf-Watz 1993, Milton et al. 1996).

The agglutination of bacteria to erythrocytes has been proposed as an efficient *in vitro* system to demonstrate the bacterial adhesive activity (Santos et al. 1990) and as a system to characterize the type of adhesins (Evans et al. 1980, Zunino et al. 1994). None of the *V. tapetis* strains tested showed hemagglutination of horse, rabbit, and human erythrocytes, but all of them were positive for rat erythrocytes. This apparent contradiction in the hemagglutination results obtained may be explained by the fact that the hemagglutination depends on the presence of specific receptors on the erythrocyte surface, and such receptors contain oligosaccharides that varied depending on the animal species (Jones and Freter 1976).

Previously, adhesion to clam cell systems by *V. tapetis* has not been demonstrated. Therefore, this study constitutes the first report of adhesion to hemocytes and mantle cells of two clam species. All the *V. tapetis* strains tested showed a higher degree of adhesion to clam cells, both hemocytes and mantle cells, than to fish cells (Table 3). These findings suggest the existence of a host or tissue specificity. According to Christensen et al. (1984), the adhesion of a particular bacterial species may vary considerably depending on the host species, physiology, phenotype, and tissue.

Miller and Mekalanos (1988) described the role of two outer membrane proteins, OmpU and OmpT, as colonization factors of *V. cholerae*. Later, Sperandio et al. (1995) verified that OmpU acts as an adherence factor involved in the colonization of epithelial cells by *V. cholerae*. The amino-terminal amino acid sequence of OmpU was similar to the sequences of *Haemophilus influenzae* HMW1 and HMW2 adhesins, and shared also similarities with the *Bordetella pertussis* FHA. As it can be seen in Fig. 2, all the *V. tapetis* strains tested presented the same OMP profile, except for 0202 strain. The MW of OmpU (32–38 kDa) is similar to the major OMP (MOMP) detected in *V. tapetis* (35–37 kDa), which induces to speculate about a similar function of this protein in *V. tapetis* strains. To verify this hypothesis, further studies of adhesion to cells using isolated MOMP and inhibition with anti-MOMP anti-serum are necessary.

In short, the adhesion mechanisms of *V. tapetis* are complex and depend on different processes that act in several steps. In a hypothetical model, *V. tapetis* is directed to specific substrate of the clam tissue by means of their motility organelles, which also help the bacterium adhere to clam cells. Then, the hydrophobic forces maintain the bacterial position along a mucosal surface by establishing small numbers of non-covalent bonds, and, finally, bacterial adhesins or surface proteins of *V. tapetis* interlock specific attachment with complementary structures on the opposing surface.

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## ASSESSING MANIPULATIONS OF LARVAL DENSITY AND CULLING IN HATCHERY PRODUCTION OF THE HARD CLAM, *MERCENARIA MERCENARIA*

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**ABSTRACT** Studies of the hard clam, *Mercenaria mercenaria*, indicate that the hatchery practice of larval culling may be counterproductive because of an inverse relationship in growth between larvae and postsettlement juveniles. The effects of larval culture manipulations were explored with two parallel studies: one set up in a commercial hatchery and the other in the laboratory. Both laboratory and hatchery studies started with the same cohort of larvae produced from spawning hatchery broodstock. Except for culling, these larvae were raised using standard practices in the hatchery for the first 10 days. On the tenth day of development, the cohort was sieved through 105- $\mu$ m mesh and separated into two treatments, large larvae and small larvae. In the hatchery, these samples were followed through settlement, and growth was monitored for 174 days postfertilization. In the laboratory, the two samples of larvae were raised under high-density (20 larvae/mL) or low-density (4 larvae/mL) conditions and thus assigned to one of four larval treatments: large/high-density, large/low-density, small/high-density, and small/low-density. These treatments were replicated (10 times each) to yield a 2  $\times$  2 factorial, randomized block, repeated-measures analysis of variance (ANOVA) experiment. Growth in the laboratory was monitored for 276 days. Both the hatchery and laboratory studies indicate that larval culling does not increase productivity and may be counterproductive. Furthermore, larval density has an effect on subsequent juvenile growth. Larvae raised at low density produced larger clams in both the small and large larval treatments.

**KEY WORDS:** *Mercenaria mercenaria*, hard clam, larval culling, aquaculture, growth

### INTRODUCTION

... it is reasonable to question the merits of the larval culling practices carried out worldwide in bivalve hatcheries ... a substantial proportion of the larvae routinely discarded during hatchery cullings would turn out to be individuals which would have grown to a market size more rapidly than many of those retained by the culling practice. (Heffernan et al. 1991, p. 201)

Larval culling is the elimination of smaller, slow growing individuals from larval cultures during the drain-down process in the aquaculture production of many species of bivalves. Culling is a common practice used in hatcheries producing the hard clam, *Mercenaria mercenaria* (Linnaeus 1758), and this process eliminates individuals that are diseased and not developing properly within a day or two of setting up a larval culture (Castagna and Kraeuter 1981, Rice 1992). However, continued culling of larval cultures is also used to select for the fastest growing individuals in a cohort to increase hatchery productivity by reducing the time to market size. Use of larval culling for the latter goal was called into question by Heffernan et al. (1991) in their rigorous study of selection for increased growth rate in *M. mercenaria*. They found an inverse relationship between postsettlement and larval growth rates; fast-growing clams produced slow-growing larvae.

Studies of other mollusks also suggest that there is either a negative relationship, or no relationship, between larval growth rates and postsettlement juvenile growth rates. For example, there is a negative relationship between larval and juvenile growth in the oyster *Ostrea edulis* (Newkirk and Haley 1982, Newkirk and Haley 1983) and in the bay scallop *Argopecten irradians* (Heffernan et al. 1992). Furthermore, no relationship between larval and juvenile growth was found in the mussel *Mytilus edulis* (Strömngren and Nielsen 1989) and in two species of the marine gastropod *Crepidula* (Pechenik et al. 1996). These studies indicate that fur-

ther work on larval rearing practices is needed to improve over-all bivalve aquaculture productivity.

Other than the physical conditions of the culture water, pedigree of the broodstock, and levels of food, hatchery managers can control only two larval variables when maintaining their cultures—density and size of individuals (through culling). Here, we report a factorial experiment where both of these variables were manipulated to assess the consequences of larval culling on the hatchery production of *M. mercenaria*.

### MATERIALS AND METHODS

#### *Hatchery Phase (5 June to 26 November, 1996)*

We carried out the hatchery phase at Biosphere, a clam hatchery in Tuckerton, New Jersey, USA. We established a single cohort of *M. mercenaria* larvae by spawning Biosphere broodstock (31 females and six males) at the hatchery on June 5, 1996 (Table 1). These clams were a combination of wild and hatchery-produced broodstock. Although beginning with purely wild broodstock has advantages (some hatchery broodstock are the result of larval culling); we wanted to emulate current industry practices as much as possible and, therefore, used the same broodstock as the hatchery. On day 2 of development, we discarded the poorly developing trochophores, and for the next 8 days, followed standard larval rearing procedures (Castagna and Kraeuter 1981), except for larval culling. During this period we maintained the culture in a single 3,500 Liter tank (mean density = 7.1 larvae/mL) at 25°C, 22–26 ppt, and fed the larvae *Isochrysis galbana* (both T. ISO and C. ISO strains). On June 15, we separated the larvae into two samples based on size by sieving on 105- $\mu$ m mesh. The “large” (~60% of cohort) or faster growing larvae were retained on the 105- $\mu$ m mesh, and the “small” (~40% of cohort) or slower growing larvae were retained on 70- $\mu$ m mesh placed beneath the larger sieve. Immediately after separation, we transported approximately 2% of the larvae from Biosphere to the laboratory at Villanova and set up the factorial experiment (see below). We reared the remainder of the hatchery larvae in two separate 3,500-L tanks at a

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TABLE 1.

Timetable of events from June 5, 1996 to March 15, 1997 in the hatchery and laboratory phases of this study.

Date	Age (Days Postfertilization)	Hatchery	Laboratory
June 5	0	Spawn	—
June 7	2	Diseased larvae culled	—
June 15	10	Larval separation	Set up factorial experiment
June 21	16	—	Tetracycline treatment
June 22–25	17–20	Downwellers (131 $\mu\text{m}$ )	—
June 28	23	—	Downwellers (120 $\mu\text{m}$ )
June 29	24	—	Three replicates eliminated
July 2	27	Upwellers (200 $\mu\text{m}$ )	—
July 5	30	—	Tetracycline treatment
July 7	32	Upwellers (300 $\mu\text{m}$ )	—
July 17	42	—	Tetracycline treatment
July 25	50	—	Upwellers (200 $\mu\text{m}$ )
July 29	54	Upwellers (500 $\mu\text{m}$ )	—
Aug. 14	70	—	Upwellers (400 $\mu\text{m}$ )
Sept. 25	113	—	Tetracycline treatment
Oct. 1	118	Moved to raceways	—
Nov. 1	149	Moved to field cages	—
Nov. 26	174	End experiment	—
Jan. 25	234	—	Upwellers (1 mm)
March 15	276	—	End experiment

Movement to upwellers or downwellers also includes the mesh size of the containers. To reduce bacterial levels in the laboratory, four antibiotic treatments of tetracycline were applied. Despite these efforts, three replicates were eliminated because of mortality.

density of 4 larvae/mL until settlement. These larvae settled between June 22 and June 25 and were then placed in downwelling units and fed the larger green alga, *Tetraselmis* sp., in addition to T. ISO and C. ISO. On July 2, the postsettlement juveniles were moved to an upwelling nursery system (Manzi et al. 1986) for 91 days, where they fed on natural levels of phytoplankton supplied by a direct seawater line from Little Egg Harbor. We transferred the seed clams (October 1) to raceways for the next 31 days and then placed all clams in field cages (November 1) in a tidal creek fed by Little Egg Harbor adjacent to the hatchery. We recorded the final measurements of the hatchery-reared clams on November 26, 1996, 174 days after fertilization (Table 1).

#### Laboratory Phase (15 June 1996 to 15 March, 1997)

After the separation on June 15, we transported both large and small larval samples to Villanova to set up a randomized complete block, two-way factorial experiment. Larval size at 10 days postfertilization was one fixed factor with two levels: large and small. Larval density after 10 days postfertilization was the other fixed factor, which also had two levels: high density (20 larvae/mL) and low density (4 larvae/mL). We replicated each of the four treatments (small larvae at both high and low density and large larvae at both high and low density) 10 times in 40 separate experimental units (see below). For the duration of the laboratory experiment, the animals were maintained in a 1,200 L recirculating seawater system, which was set up in an environment chamber. The experimental units were arranged in a block design in a single fiberglass sea-table and shared a common seawater reservoir. After circulating through the sea-table, the seawater was filtered through activated carbon, crushed coral gravel (biological filtration), protein skimmers, two cartridge filters (20 and 0.5  $\mu\text{m}$ ), and a UV sterilizer. The system was sanitized at least once each week; all tubing and filters were replaced, and all sea-table surfaces were washed in a dilute solution of sodium hypochlorite (~4%) and thoroughly rinsed. We monitored seawater chemistry (temperature, salinity, pH, and ammonia, nitrate, and nitrite levels) daily and maintained the temperature between 25 to 26°C and the salinity at 28 ppt. These physical characteristics of the seawater did not change throughout the experiment.

We constructed the 40 experimental units from 15-cm segments of 10-cm diameter PVC pipe, each with a mesh bottom and cut-out tripod supports (Fig. 1). These units were scaled-down versions the downwellers and upwellers used in hatcheries. The water level in the sea-table was adjusted so that each unit held approximately 800 mL. The same experimental units were used as larval corrals and as postsettlement juvenile downwellers and upwellers; the only difference among the three types of containers was the mesh size on the bottom and the direction and degree of water flow (Fig. 1, Table 1).

We fed larvae both T. ISO and C. ISO twice a day at concentrations of  $7 \times 10^4$  cells/mL (Rhodes et al. 1984, Riisgård 1988). Postsettlement juveniles were fed four species of phytoplankton twice a day: T. ISO, C. ISO, *Tetraselmis* sp., and a diatom, *Chaetoceros* sp. Feeding concentrations of phytoplankton were based on the total volume of the sea-table. During these feeding bouts (2 to 6 hours) the filter pump was turned off; however, the recirculating pump in the sea-table remained on and food was equally available among all experimental units because of the flow-through system of the downwellers and upwellers (Fig. 1). Initially, postsettlement phytoplankton feeding concentrations were set at  $7 \times 10^4$  cells/mL but were increased to  $10 \times 10^4$  cells/mL on day 76 postfertilization (see Deming 1998 for data on feeding times and concentrations for the 9-month laboratory experiment).

We suspected the initial high mortality observed in the laboratory was associated with bacterial levels; therefore, all individuals were treated with the antibiotic, oxytetracycline, four times between days 16 and 113 postfertilization (Table 1). An antibiotic treatment consisted of placing all individuals from an experimental unit in a bath of 0.02 g of oxytetracycline per L of seawater for 1 to 2 hours (Zodl, pers. comm., Castagna and Kraeuter 1981).

#### Quantitative Estimates and Statistical Analyses

We used shell length (SL) to quantify growth for both larvae and postsettlement juveniles in the laboratory and hatchery. All SL

TABLE 2.

Repeated-measures ANOVA of shell length from the laboratory factorial experiment with block treated as a random effect.

Source	df	SS	MS	F	p
Between-subjects analysis					
Larval size	1	3.651	3.651	5.02	.10
Larval density	1	5.172	5.172	5.90	.08
Block	9	8.184	0.909		
Larval size × larval density	1	0.706	0.706	1.10	.34
Larval size × block	9	6.547	0.727		
Larval density × block	9	7.889	0.877		
Postsettlement density	1	0.577	0.577	0.90	.39
Residual	5	3.220	0.644		
Within-subjects analysis					
Age	30	445.267	17.811	128.14	<.0001
Age × larval size	30	4.222	0.169	1.48	.19
Age × larval density	30	3.390	0.136	0.80	.44
Age × block	267	31.200	0.139	1.05	.40
Age × larval size × Larval density	30	4.117	0.165	1.24	.22
Age × larval size × block	267	25.577	0.114	0.86	.84
Age × larval density × Block	267	37.975	0.169	1.27	.07
Age × postsettlement density	30	3.334	0.133	1.01	.47
Error (age)	149	16.573	0.133		

The Huynh-Feldt  $\epsilon$  was used to correct the probability levels. Residual = Block × larval size × larval density interaction. No significant differences were found at the  $\alpha = 0.05$  level for larval size and larval density; however, for both variables  $p \leq .1$  (see text).

measurements were obtained using compound or dissecting microscopes fitted with ocular micrometers (calibrated before each sampling date). Measurements from the initial larval separation (day 10 postfertilization;  $n = 200$  for both large and small samples) were facilitated by fixing the larvae in 2% glutaraldehyde; all other measurements were conducted with replacement. In the hatchery, we measured larvae on days 17, 19, and 21 postfertilization ( $n: 200\text{--}400$ ) and juveniles on nine separate dates ( $n: 200\text{--}1,600$ ) up until the last measurement on November 26, 1996. In the laboratory, we measured 25 individuals from each replicate

on twenty-six dates until the last measurement on March 15, 1997. For the final measurement in the laboratory, we assigned each juvenile a number and selected the samples for this date using a random numbers table. We used a  $t$ -test to compare the large and small larvae from the initial separation. To compare treatments in the laboratory experiment, we used a repeated-measures randomized block analysis of variance (ANOVA) (the mean SL for a replicate represented the repeated measures). Average postsettlement density in each replicate was calculated from counts on nine dates (days 36, 56, 77, 91, 98, 105, 112, 190, and 273 postfertil-

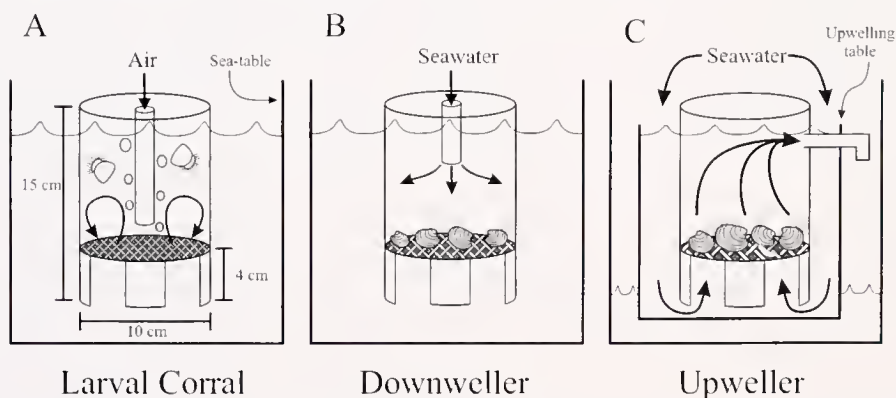


Figure 1. Experimental units. Each replicate was a 15-cm segment of PVC pipe with three 4-cm sections cut out of the bottom to form a tripod support. As the cohort developed, the size of the mesh on the bottom was increased (Table 1) to enhance flow within the approximately 800 mL volume held by each unit. The units are drawn to scale; however, the sea table and upwelling table are not—all 40 experimental units were in the sea table and then moved to the upwelling table (which was held in the sea table). The thick arrows represent air and water flow. Flow rates were monitored regularly and adjusted to no less than 1 L/min in the downwellers and upwellers. A. Larval Corral. Filtered air entered the bottom of the experimental units through a 2-mm plastic tube positioned approximately 2 cm from the bottom and was regulated to 1 bubble/s. Seawater circulated within the unit and between the unit and the sea table through the bottom mesh (105  $\mu\text{m}$ ). B. Downweller. Seawater entered through a 1-cm tygon tube positioned directly above each experimental unit. C. Upweller. All 40 experimental units were placed in the same blocked arrangement, in an upwelling table nested within the sea table. Each unit was plumbed near the top with a sealed tygon connector attached to the upwelling table. Seawater flowed into the upwelling table, up through the bottom of each unit, and out to the sea table via the tygon connector.

ization) and used as a covariate in the repeated-measures ANOVA to account for the effects of juvenile density on growth. In addition, an ANOVA was performed on the means of SL from each replicate on the last set of measurements (day 276 postfertilization) with average postsettlement density as the covariate. Differences in survivorship were tested using a randomized block ANOVA on the number of individuals in each replicate on the last sampling date (start density / final density). All analyses were performed using SAS (1989).

## RESULTS

There was a significant difference ( $p < .0001$ ,  $t = 18.06$ ) in SL between the large and small larval treatments on day 10 postfertilization (Fig. 2A). On this date, approximately 5% of the culture was in the pediveliger stage of development, and all pediveligers were part of the large larval treatment. Settlement occurred earlier in the hatchery-reared clams (days 17–20 postfertilization) than in the laboratory experiment (days 23–25 postfertilization, Table 1). Although we tracked the growth of clams in the hatchery phase of the experiment (Fig. 3), we did not compare the two groups statistically, because this part of the experiment was pseudoreplicated (Hurlbert 1984). At the end of the hatchery study (174 days postfertilization), there did not seem to be a difference in SL between

the two larval size groups; the mean size of clams from the large larval treatment was 4.21 mm, and the mean size of clams from the small larval treatment was 4.31 mm (Fig. 2B).

The repeated-measures ANOVA revealed that there were no significant differences in SL among the four treatments attributable to larval size or larval density ( $0.10 \geq p > .05$ , Table 2) over the course of the 276-day experiment. However, the ANOVA from the last sample revealed that although larval size was not significant ( $p = .22$ ,  $F = 1.69$ ) larval density was significant ( $p = .02$ ,  $F = 7.65$ ). In both the large and small larval treatments, larvae raised at lower densities produced bigger juveniles at the end of the experiment on day 276 postfertilization (Fig. 2C). This difference in growth cannot be attributed to differences in postsettlement density ( $p = .39$ , Table 2). For most of the experiment, postsettlement juvenile density was highest in the large/low-density larval treatment (Fig. 4), and this treatment produced the biggest juveniles (Fig. 2C).

Figure 4 shows the average number of individuals per replicate in the four treatments over the course of the experiment. Mortality levels were highest among all treatments between days 21 and 42 postfertilization. For most of the experiment (days 42–270), survivorship remained constant among all four treatments. Mortality was highest in the high larval density treatments and lowest in the large/low-density larval treatment ( $p < .0001$ ,  $F = 4.12$ , Fig. 4).

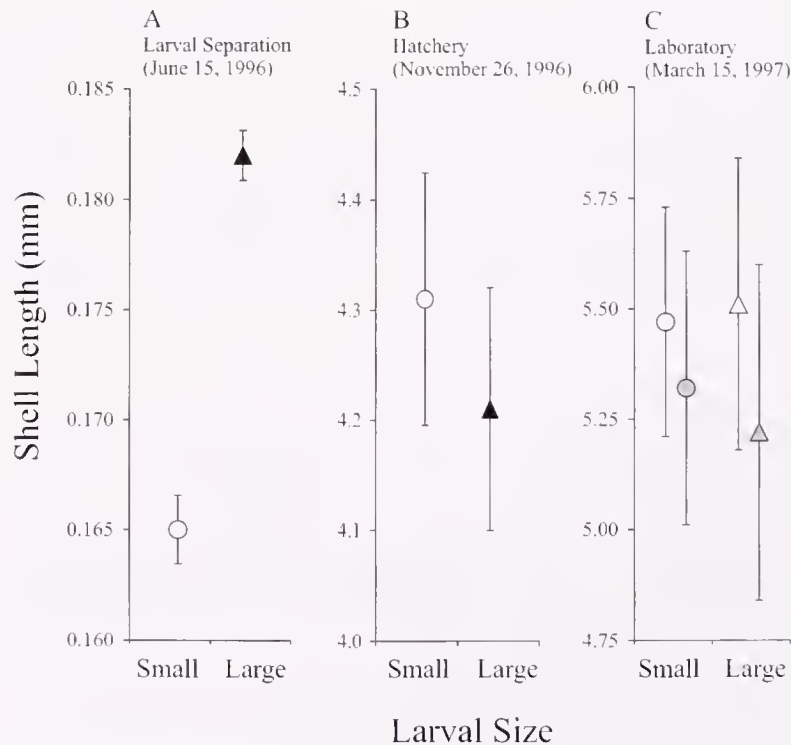


Figure 2. Means ( $\pm 2$  standard errors) of shell lengths at the beginning and end of the hatchery and laboratory phases of the study. The circles represent the small larval treatment; larvae that passed through the 105- $\mu$ m mesh and were caught on the 70- $\mu$ m mesh on June 15, 1996. The triangles represent the large larval treatment; larvae caught on the 105  $\mu$ m mesh. A. Initial separation. These samples ( $n = 200$  each for large and small) are significantly different ( $p < .0001$ ,  $t$ -test). B. Final measurements of hatchery-reared samples on day 174 postfertilization ( $n = 200$  each for large and small). No statistical analysis was performed on the hatchery-reared samples, because the design of this experiment was pseudoreplicated (Hurlbert 1984, see text). C. Final measurements of laboratory samples. These values are means of replicates ( $n = 10$  for small larvae at low density, and  $n = 9$  for the other three treatments, see text). There were 25 clams measured from each replicate. The open symbols (Lo) represent larvae raised at low density (4/mL) and the shaded symbols (Hi) represent larvae raised at high density (20/mL). Within larval size treatment, the low-density larval treatments produced larger postsettlement juveniles. There was no difference in SL associated with larval size treatment ( $p < .05$ , see text) on this date.



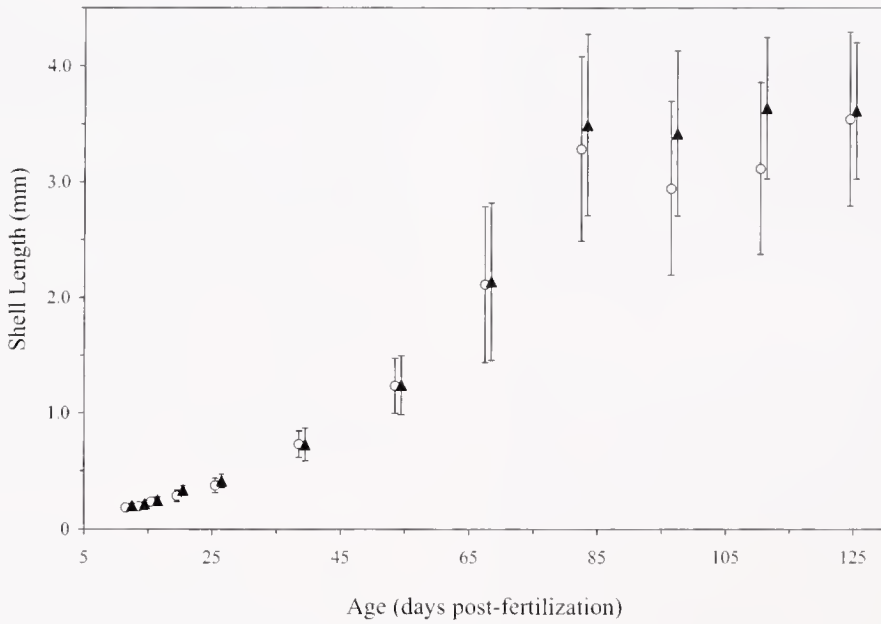


Figure 3. Means and standard deviations ( $\pm 1$ ) of shell lengths from the two experimental groups raised in the hatchery between the initial separation and the final measurements at the end of the experiment. The circles represent the small larval treatment and the triangles represent the large larval treatment (n: 200–1,600).

DISCUSSION

Our results are consistent with previous work showing either no correlation or a negative correlation between larval and juvenile growth rates in mollusks (e.g., Newkirk and Haley 1982, Newkirk and Haley 1983, Strömngren and Nielsen 1989, Pechenik et al. 1996). Hilbish et al. (1993) found no evidence for “positive genetic covariation between larval and juvenile growth” in *M. mercenaria*

and recommended against larval culling as a method for enhancing juvenile growth and improving hatchery productivity (Hilbish et al. 1993, p. 102). In contrast, Heffernan et al. (1991) found a strong negative relationship between larval and juvenile growth, but they also questioned “the merits of hatchery culling practices for smaller larvae” (Heffernan et al. 1991, p. 199). We did not address the issue of heritability of growth characteristics in this study. Instead, we focused on the practical issues and consequences of

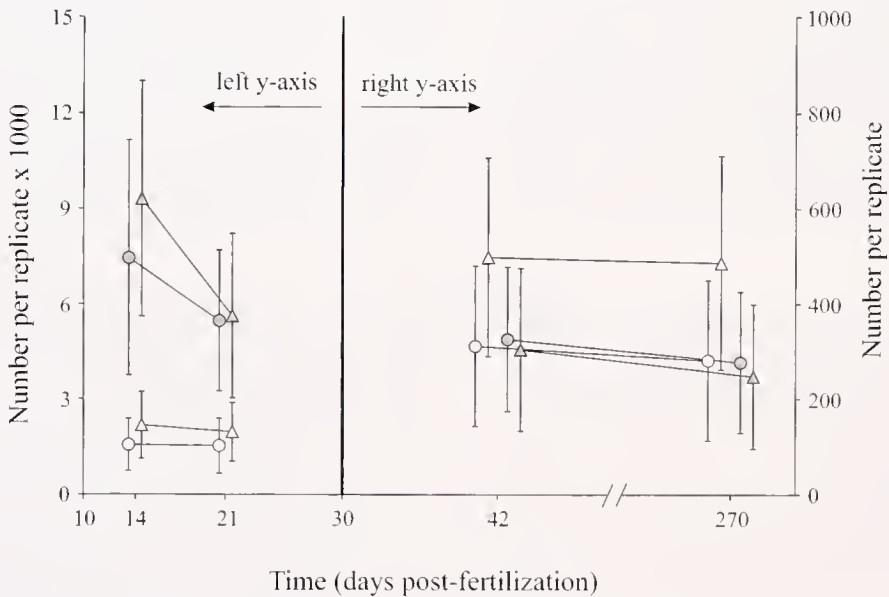


Figure 4. Means and standard deviations ( $\pm 1$ ) of number of individuals per replicate among the four treatments over the course of the experiment. The circles represent the small larval treatments and the triangles represent the large larval treatments. The shaded symbols represent the high-density larval treatments, and the open symbols represent the low-density larval treatments. The thick vertical line separates data plotted on the left y-axis from data plotted on the right y-axis. The left y-axis is more than an order of magnitude greater than the right y-axis.

manipulating larval cultures in a hatchery. Our results suggest that larval densities have subsequent effects on postsettlement growth rates. Attempts to increase productivity by "pushing" larval culture density higher may backfire, because larvae raised at higher densities seem to produce slower growing juveniles (Fig. 2C). In addition, there was no difference between the final sizes of the large/low-density and small/low-density larval treatments (mean final SL = 5.51 and 5.47 mm, respectively, Fig. 2C). Growth rates in the small larval treatments must have been higher for there to be no difference in final size, because SL of the small larval treatment was significantly smaller at the start of the experiment on day 10 postfertilization (Fig. 2A).

One potential criticism of the laboratory experiment is that use of 10 × 15 cm experimental units unrealistically emulates hatchery conditions because of the difference in scale between these units and hatchery upwelling and downwelling containers. The purpose of the hatchery phase of this study was to provide comparative data to the laboratory experiment. However, we realized at the outset that the design of the hatchery phase of the study was pseudoreplicated (Hurlbert 1984), because it was not possible to duplicate larval tanks beyond the two used for raising the large and small larval treatments (therefore, no statistical analyses were performed on these data). Despite these constraints, the results of the hatchery study are consistent with, and support the conclusions derived from the laboratory experiment (compare Figs. 2B and 2C).

We overestimated the number of animals the recirculating seawater system in the laboratory could accommodate and observed increased levels of bacteria and protozoans soon after the experiment began. This combination of factors probably contributed to the high levels of mortality (Fig. 4), which forced us to eliminate three replicates (Table 1, these replicates were from different treatments and different blocks). In addition to the oxytetracycline treatments (Table 1), the UV lights were increased from 8 watt to 25 watt to reduce mortality. After 1 month, mortality dropped off, and the density of clams per replicate stabilized and remained constant for the duration of the 9-month experiment (Fig. 4).

It is well documented that density of clams can adversely affect growth rates by limiting the amount of food available to individuals (e.g., Hadley and Manzi 1984, Manzi et al. 1986, Peterson and Beal 1989). The large/low-density larvae displayed the highest survivorship among the four treatments in the laboratory (Fig. 3), and because of this higher survivorship, juvenile clams from this treatment were reared at the highest postsettlement densities. How-

ever, despite nearly double the postsettlement density of this treatment, these clams were larger than the large/high-density larval clams (Fig. 2C). These observations indicate that "crowding" and competition for food was not a factor in the postsettlement period or, at least, that food was equally limiting for individuals among treatments.

In conclusion, our results indicate that the optimal procedures for maintaining larval cultures include eliminating larval culling after the initial disposal of poorly developing trochophores. In addition, maintaining cultures at low densities seems to enhance subsequent postsettlement growth. Our study focused on hatchery procedures; however, bivalve aquaculture includes both hatchery and growout phases of production. A superior experimental design would have included tracking juvenile growth to market size for both the large and small larval groups. Logistic constraints prevented us from extending our study, but we do plan to address this question in the future.

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## COMPARATIVE GROWTH OF THE EASTERN OYSTER *CRASSOSTREA VIRGINICA* (GMELIN) REARED AT LOW AND HIGH SALINITIES IN NEW BRUNSWICK, CANADA

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**ABSTRACT** A comparative study was conducted in 1993 and 1994 to determine the effects of salinity and temperature on 2- and 4-year-old eastern oysters *Crassostrea virginica* (Gmelin) reared in suspension and on the bottom, in a low- and a high-salinity environment in New Brunswick, Canada. Growth rates, glycogen concentrations, and condition indices were recorded throughout the study. Oysters cultured near the surface in the high-salinity environment displayed superior growth (16 mm/year) as compared to all other rearing combinations. In contrast, oysters cultured on the bottom at low salinity exhibited poorest growth (7.9 mm). In all instances, growth of 2-year-old oysters was superior to that of 4-year-old oysters. Oysters cultured on the bottom at high salinity displayed comparable growth to those reared in suspension at low salinity (12.6 and 13.4 mm, respectively). Although condition indices were not always significantly different among oysters of different sizes and reared under different conditions, for the most part, results were generally consistent with patterns observed for growth and glycogen concentrations.

**KEY WORDS:** Eastern oyster, growth, glycogen concentration, *Crassostrea virginica*

### INTRODUCTION

The Gulf of St. Lawrence and its associated tributaries represent the northernmost geographic distribution of the eastern oyster *Crassostrea virginica* (Gmelin). Although optimal salinity for purposes of growth for this species ranges between 14 and 28‰, oysters can tolerate extremes varying from 0 to 42‰ (Shumway 1996). In Atlantic Canada, oyster culture is primarily practiced in estuaries and bays where salinities range between 20 and 30‰. Such levels are consistent with data suggesting that suitable growth is best achieved at salinities between 14 and 28‰, and that slower growth, poorer spat production, and excessive valve closure result when salinities drop below 14‰ (Shumway 1996). Temperature is another critical factor that affects growth of *C. virginica*. Although oysters can also tolerate considerable temperature variations ranging between -4 and 49°C, effective filtration and optimal food ingestion are best accomplished at temperatures between 13 and 32°C; although, ingestion can occur at temperatures as low as 6°C (Shumway 1996).

Although both temperature and salinity are important, the synergistic effects of these two variables, along with such factors as primary production and seston, can have a significant effect on oysters (Alderdice 1972, Vernberg and Vernberg 1972, Shumway 1996). Our study compares growth and glycogen accumulation for oysters of two age classes cultivated in suspension and on the bottom at low and high salinities.

### MATERIALS AND METHODS

The study was conducted between May and October of 1993 and 1994 at two sites in the Richibucto river drainage basin in eastern New Brunswick, Canada (Fig. 1). Site A (low salinity) was situated 20 km from the mouth of the Richibucto river, adjacent to the community of Big Cove, and site B (high salinity) was situated in Northwest Branch, a body of water that houses a major oyster

culture operation, Aquaculture Acadienne, which provided the cultured oysters used in this study.

Eight plastic-coated wire platforms (1.2 × 1.2 m, 5 cm mesh) surrounded by an 8 cm lip were constructed. Two standard Vexar bags (0.6 × 1.2 m, 1 cm mesh) were affixed by electrical plastic ties to each platform, with a smaller Vexar sack (0.3 × 0.3 m, 1-cm mesh) positioned between them (Fig. 2). At the beginning of each sampling year, 1,700 2- and 4-year-old oysters, respectively were distributed as follows: 200 2-year-olds in each of the large bags affixed to four, platforms and 200 4-year-olds in each of the large bags mounted on the four remaining platforms. These oysters were used to assess glycogen content and condition index. The smaller bags on each platform contained 25 oysters (each marked with waterproof labels cemented to the right valve) of the corresponding age classes and were routinely monitored for growth and survival. Two of the platforms were positioned at each site, with one platform used to maintain oysters in suspension and the other for bottom culture.

Oysters were divided into four groups. Groups I and II were 2- and 4-year-old oysters respectively, and were sampled in 1993. Groups III and IV, also made up of 2- and 4-year-old oysters, respectively were sampled in 1994. These oysters were assigned to each of the following stations: low-salinity surface (LSS) and low-salinity bottom (LSB) at site A and high-salinity surface (HSS) and high-salinity bottom (HSB) at site B.

Growth (shell length) of individually marked oysters in the smaller bags was recorded monthly. In addition, 10 oysters were removed every 2 weeks from each of the two large bags on an alternating basis. Five oysters were analyzed for glycogen content using a YSI® (model 27) glycogen analyzer as described by Carr and Neff (1984) and five others were used to determine condition index according to the following formula: dry meat weight (100°C, for 12 h)/dry shell weight × 1,000 (Lawrence and Scott 1982).

Our findings focus on three critical periods for the oyster: before spawning (15 June 1993, 26 June 1994), after spawning (26

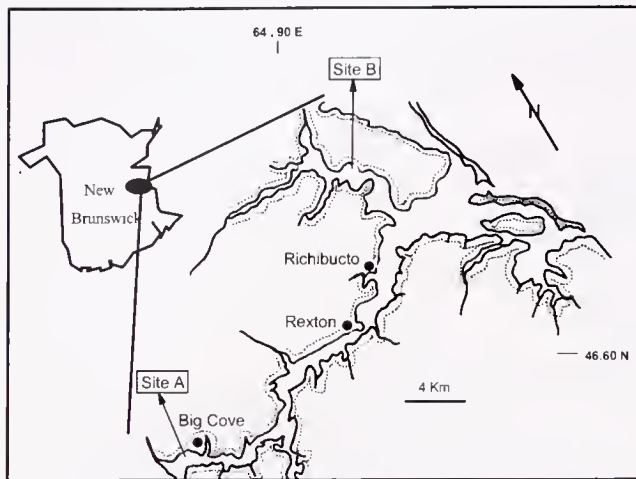


Figure 1. A portion of the Richibucto watershed displaying study sites A (low salinity) and B (high salinity).

June 1993, 12 July 1994) and before winter (27 September 1993 and 1994). Data based on oysters constituting group II "before spawning" were excluded because of vandalism. This group was immediately replaced with new oysters for subsequent analyses. Water temperature, salinity, dissolved oxygen, and pH were recorded for each sampling. During each sampling period, 3 L of water were collected using a horizontal sampling bottle and later analyzed for seston (Wetzel and Likens 1979, Widdows et al. 1984) and chlorophyll *a* (Stickland and Parsons 1972). Data were statistically treated using *t*-tests and 1- and 2-way ANOVA tests (Zar 1984).

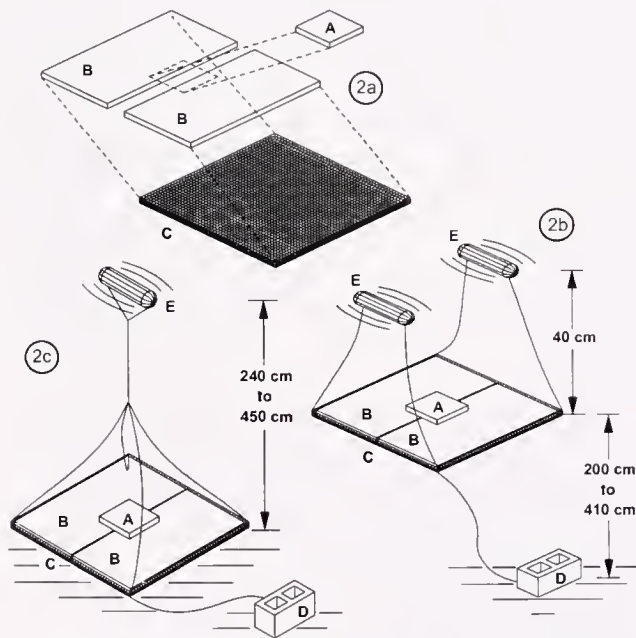


Figure 2. Rearing methods employed for 2- and 4-year-old oysters at each of two experimental sites in 1993 and 1994. 2a: enlarged view of a typical platform (four per site). 2b: surface platform arrangement (two per site). 2c: bottom platform arrangement (two per site). A: small vexar bag (30 × 30 cm) containing 25 marked 2- or 4-year-old oysters. B: large vexar bags (60 × 120 cm) each containing 200 2- or 4-year-old oysters. C: 5-cm wire mesh platform (120 × 120 cm). D: concrete block (5 kg). E: buoy.

## RESULTS

Water temperature and salinity data are shown in Table 1 and Figure 3. High variability in salinity is evident at each site except HSB. Such findings reflect the different weather patterns at any given time.

In general, primary productivity at our sites ranged from 0.2 to 2.5  $\mu\text{gC}/\text{m}^3$ . There were no significant differences between the stations, either in the availability of food as determined by the chlorophyll concentration in water ( $.29 < p < .89$ ), or by the amount of available carbon as measured by the ratio of particulate inorganic to organic material (PIM/POM:  $0.14 < p < .94$ ). A significant difference, however, between years was observed for the PIM/POM ratio (Galtsoff 1964, Bayne and Newell 1983, Wallace Reinsnes 1985).

Two-way analysis of variance (ANOVA) confirmed that growth (Fig. 4) for all groups (I, II, III, and IV), was significantly ( $p < .002$ ) influenced by salinity as well as by culture method (surface vs. bottom). Furthermore, one-way ANOVAs showed that there were significant differences ( $p < .05$ ) among stations (HSS, LSS, HSB, LSB) for each of the groups. Multiple comparison tests demonstrated that for each group, oysters from station HSS displayed superior growth as compared to all other stations. Moreover, growth of oysters sampled from stations HSB and LSS was comparable, but superior to oysters held at station LSB.

Results of glycogen analyses of oysters sampled before spawning indicate that neither site nor culture method played a significant role for group IV (Table 2). Glycogen concentrations for oysters from groups I and III, however, were affected by culture method and site, respectively (Table 2). Findings revealed that, after spawning, glycogen concentrations for groups I and II oysters were influenced by culture method and site, respectively. In contrast, oysters from groups III and IV were influenced by both variables (Table 2). Finally, the data showed that glycogen concentrations for oysters sampled just before winter for groups I, III, and IV were primarily site dependent (high vs. low salinity) and that group II was affected by both variables (Table 2). One-way ANOVA were undertaken on each of the groups (I, II, III, and IV) over the three sampling periods, demonstrating differences between stations in some instances (Fig. 5). Multiple comparison tests on oysters sampled before spawning for groups I, II, and IV indicated that glycogen concentrations were similar for all stations. Glycogen concentrations of oysters from station HSS and HSB, sampled from group III, were higher than those from stations LSS and LSB.

For oysters collected after spawning, multiple comparison tests revealed that for group I, glycogen concentrations recorded from stations HSS, HSB, and LSB were similar to each other, but superior to station LSS. For group II, glycogen concentrations of stations HSS and HSB are similar to each other and superior to those from station LSB. For group III, glycogen concentrations from station HSS are superior to those from stations HSB, LSS, and LSB, which are similar to each other. For group IV, glycogen concentrations for oysters from station HSS were similar to those of station HSB, which, in turn, were similar to those of stations LSS and LSB. No detectable differences in glycogen concentrations were noted for oysters sampled from the two latter stations.

Multiple comparison tests on oysters sampled before winter demonstrated that glycogen concentrations were higher for animals collected from high-salinity stations versus those from the low-salinity stations for groups I and IV. In group I, glycogen

TABLE 1.

Temperature, salinity, chlorophyll *a*, and seston data recorded at stations HSS (high-salinity surface), HSB (high-salinity bottom), LSS (low-salinity surface), and LSB (low-salinity bottom) during 1993 and 1994.

Station	Sample		Temperature (°C)		Salinity (‰)		Chlorophyll <i>a</i> (mgC/m <sup>3</sup> )		PIM/POM <sup>c</sup>	
	Number <sup>a</sup>	Date <sup>b</sup>	1993	1994	1993	1994	1993	1994	1993	1994
HSS	1	16 May	13.00	9.50	20.00	11.50	2.08	0.65	4.30	2.30
	2	30 May	17.00	12.50	6.00	13.00	2.50	1.12	5.70	9.20
	3	15 June	18.50	17.00	14.00	24.00	1.66	1.55	5.85	36.00
	4	26 June	20.00	20.00	15.50	22.00	1.34	1.76	4.40	15.20
	5	12 July	19.00	20.00	18.50	24.00	1.18	1.52	4.60	14.50
	6	25 July	20.50	24.00	19.50	25.50	0.97	1.49	4.90	—
	7	09 Aug	21.50	21.50	22.00	23.00	0.62	—	3.60	11.90
	8	22 Aug	15.00	20.00	21.00	22.00	0.70	—	6.70	14.40
	9	27 Sept	13.50	—	23.00	—	1.26	—	5.70	—
	10	11 Oct	6.00	12.00	21.00	24.00	—	—	—	—
HSB	1	16 May	11.00	6.50	23.00	20.00	—	1.01	—	15.50
	2	30 May	11.00	8.00	23.00	21.50	0.71	0.42	7.40	9.60
	3	15 June	12.50	14.00	24.00	23.00	0.84	1.48	15.30	12.10
	4	26 June	16.00	15.00	23.00	25.00	0.95	0.96	6.70	11.90
	5	12 July	17.50	18.50	19.50	25.50	1.36	0.82	4.90	11.20
	6	25 July	18.00	23.50	23.00	26.00	0.79	1.44	6.90	—
	7	09 Aug	21.00	20.50	23.00	24.50	0.42	—	5.00	13.90
	8	22 Aug	15.00	19.50	20.00	23.50	0.75	—	4.10	15.70
	9	27 Sept	13.00	—	24.00	—	1.36	—	4.10	—
	10	11 Oct	8.50	12.00	22.00	25.50	—	—	—	—
LSS	1	16 May	14.00	8.50	12.00	1.00	1.64	0.49	6.60	12.20
	2	30 May	18.00	12.50	2.00	8.00	1.89	0.74	10.90	11.70
	3	15 June	18.00	10.00	4.00	16.00	0.22	1.02	10.74	19.30
	4	26 June	21.50	19.50	8.50	15.00	1.15	1.10	4.50	12.90
	5	12 July	20.00	21.00	11.00	21.00	1.70	1.09	7.60	14.50
	6	25 July	22.00	22.50	13.50	21.50	0.98	1.36	6.20	—
	7	09 Aug	22.00	20.50	18.50	24.00	0.79	—	4.20	13.00
	8	22 Aug	17.00	19.00	16.00	22.50	—	—	5.70	16.40
	9	27 Sept	15.00	17.00	21.50	22.00	1.16	—	6.80	—
	10	11 Oct	9.00	13.00	18.00	22.00	—	—	—	—
LSB	1	16 May	14.00	7.50	12.00	1.00	—	0.80	—	16.00
	2	30 May	11.00	10.00	20.00	15.00	—	0.71	—	13.80
	3	15 June	17.00	7.00	5.00	25.00	1.87	0.99	12.60	20.30
	4	26 June	19.00	17.50	17.00	20.00	0.65	1.51	14.50	17.08
	5	12 July	20.00	20.00	11.50	22.50	0.67	0.98	5.30	23.65
	6	25 July	22.00	22.00	14.00	23.00	1.60	1.54	9.50	—
	7	09 Aug	22.00	20.50	17.50	24.00	1.19	—	7.50	11.20
	8	22 Aug	17.00	20.00	16.00	22.50	0.79	—	3.60	15.50
	9	27 Sept	14.00	19.00	22.00	22.50	0.58	—	4.50	—
	10	11 Oct	9.50	13.00	20.00	22.00	1.08	—	7.20	—

— Data missing attributable to apparatus breakdown.

<sup>a</sup> When doing statistical tests, data used for before spawning (BS), after spawning (AS), and before winter (BW) were those of sample times number 3, 4, and 9, respectively, for 1993 and 4, 5, and 9, respectively, for 1994.

<sup>b</sup> Sampling dates not exactly the same for each of the 2 following years, but fall into a range of 5–8 days.

<sup>c</sup> Particulate inorganic (PIM) and organic (POM) matter (mg/L).

concentrations of oysters from station HSS were significantly higher than those from station HSB. Glycogen concentrations of oysters constituting group III were lower for oysters sampled at station LSB as compared to those from the three other stations (HSS, HSB, and LSS). No significant differences were detected among the latter stations. In contrast, glycogen concentrations of oysters in group II sampled from stations HSS were higher than those from station HSB, which, in turn, were higher than those of station LSB (Fig. 5).

Glycogen concentrations for oysters sampled from all stations

for groups I and II (1993) displayed a characteristic decrease after the spawn. The recovery of glycogen reserves in 2-year-old oysters from the time after the spawn to the onset of winter (Fig. 5), was greater for bivalves sampled from stations HSS and HSB as compared to LSS and LSB. Nevertheless, some recovery was observed for oysters sampled from station LSS, but no observable recovery was noted from station LSB in group I.

As for glycogen analyses, comparisons of condition index were conducted on oysters sampled before and after spawning and just before the onset of winter (Fig. 6). Group IV oysters examined

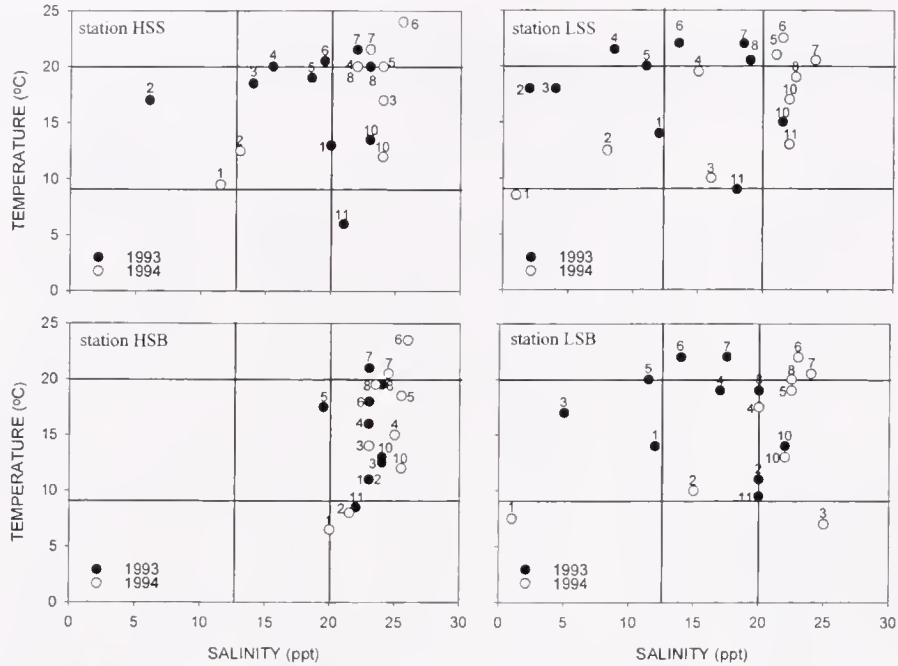


Figure 3. The relationship between temperature and salinity at each station for 1993 and 1994. The points and their corresponding numbers represent specific sampling times as outlined in Table 1. HSS: high-salinity surface; LSS: low-salinity surface; HSB: high-salinity bottom; LSB: low-salinity bottom. The vertical and horizontal lines represent salinities and temperatures, respectively, important for oyster growth and survival. The horizontal line at 20°C represents the temperature of the water required to induce spawn in the oyster, and the second line, at 8°C, represents the water temperature at which the oyster filtration is considerably reduced or stops completely. Salinities on the right of the vertical line at 20‰ are optimal for the eastern oyster and those at the left of the 13‰ line induce excessive valve closure and lower growth.

before spawning were only influenced by site (low vs. high salinity); whereas, group I and III were affected by both culture method (suspension vs. bottom) and site (Table 2). The data also indicate that group II oysters sampled after the spawn were not affected by site or culture method. In contrast, group IV oysters were influenced by culture method, and groups I and III by site and culture method. Analyses of oysters sampled before winter demonstrate that groups I and II were influenced by both culture method and site, with groups III and IV influenced only by site.

One-way ANOVA were undertaken on each of the groups (I, II, III, and IV) over the three sampling periods to compare stations (Fig. 6). Multiple comparison tests showed that for group IV oysters sampled before spawning, the condition indices of oysters at stations HSB and HSS were similar to each other, with station HSS similar to station LSS, which, was, in turn, comparable to data recorded for oysters from station LSB. In the case of Groups I and III oysters, however, the condition indices at station HSS were superior to HSB, LSS, and LSB. No significant differences were detected between oysters from the latter three stations. The condition indices sampled from all stations (HSS, HSB, and LSB) displayed no differences for group II.

For group II oysters collected after spawning, multiple comparison tests revealed that all stations were similar. There were no significant differences among stations HSS, HSB, and LSS for groups III and IV oysters. Oysters from station HSS, however, showed higher condition indices than those sampled from station LSB. Likewise, no significant differences were detected between stations HSS, HSB, and LSB for group I oysters. Moreover, there was no significant difference between oysters from stations HSS and LSS, although HSB and LSB were superior to HSS.

Results of multiple comparison tests on groups I and II oysters

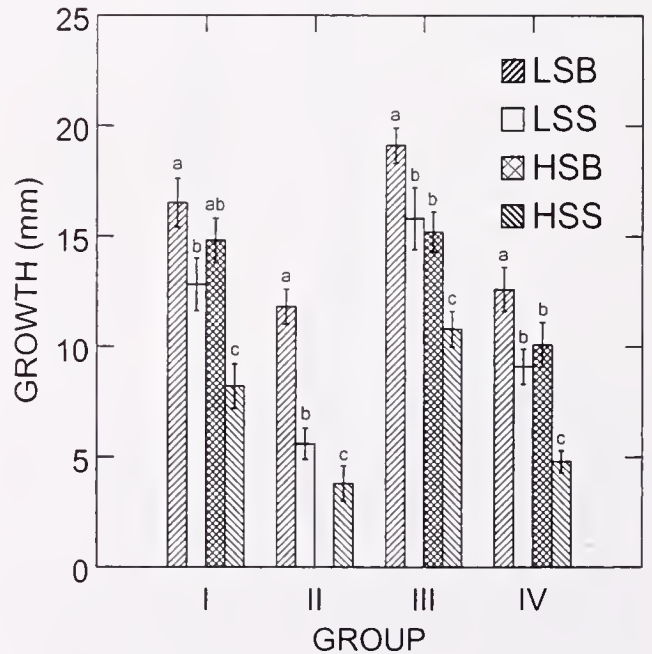


Figure 4. Total growth of oysters from stations HSS (high-salinity surface), HSB (high-salinity bottom), LSS (low-salinity surface), and LSB (low-salinity bottom) for groups I (2-year-old oysters in 1993), II (4-year-old oysters in 1993), III (2-year-old oysters in 1994), and IV (4-year-old oysters in 1994). Bars with similar letters are not significantly different from each other ( $p > .05$ ). Missing data for group II are attributable to oysters lost (see Materials and Methods).



TABLE 2.

Probabilities obtained with two-way ANOVA tests on data for condition indices and glycogen concentrations values of eastern oysters in relation to site and culture method, for each of four groups (I: 2-year-old oysters in 1993, II: 4-year-old oysters in 1993, III: 2-year-old oysters in 1994, and IV: 4-year-old oysters in 1994).

Factors	Probabilities											
	Before Spawning				After Spawning				Before Winter			
	Group				Group				Group			
	I	II	III	IV	I	II	III	IV	I	II	III	IV
Condition Indices												
Site	.040*	—	.006*	.006*	.019*	.571	.012*	.102	.002*	.004*	.000*	.000*
Culture method	.002*	—	.000*	.797	.003*	1.000	.017*	.043*	.017*	.017*	.801	1.000
Site* method	.100	—	.114	.512	.517	.909	.352	.602	.263	.077	.339	.844
Glycogen Concentrations												
Site	.188	—	.000*	.092	.737	.027*	.001*	.001*	.000*	.000*	.006*	.000*
Culture method	.001*	—	.197	.522	.015*	.187	.031*	.032*	.248	.000*	.110	.660
Site* method	.087	—	.125	.934	.002*	.203	.010*	.889	.624	.009*	.045*	.511

\* Significant difference ( $p < .05$ ).

— data not available.

collected before winter revealed that condition indices were higher for oysters sampled from stations HSS than for those from stations HSB, LSS, and LSB, all of which were similar to each other. The condition indices were higher for oysters sampled from high-salinity stations (HSS and HSB) than for the low-salinity stations (LSS and LSB) for groups III and IV oysters.

DISCUSSION

We focused on similarities and differences in growth and body condition between 2- and 4-year-old oysters cultured in suspension and on the bottom in a high- and a low-salinity environment during two consecutive growing seasons. Indices employed in our work.

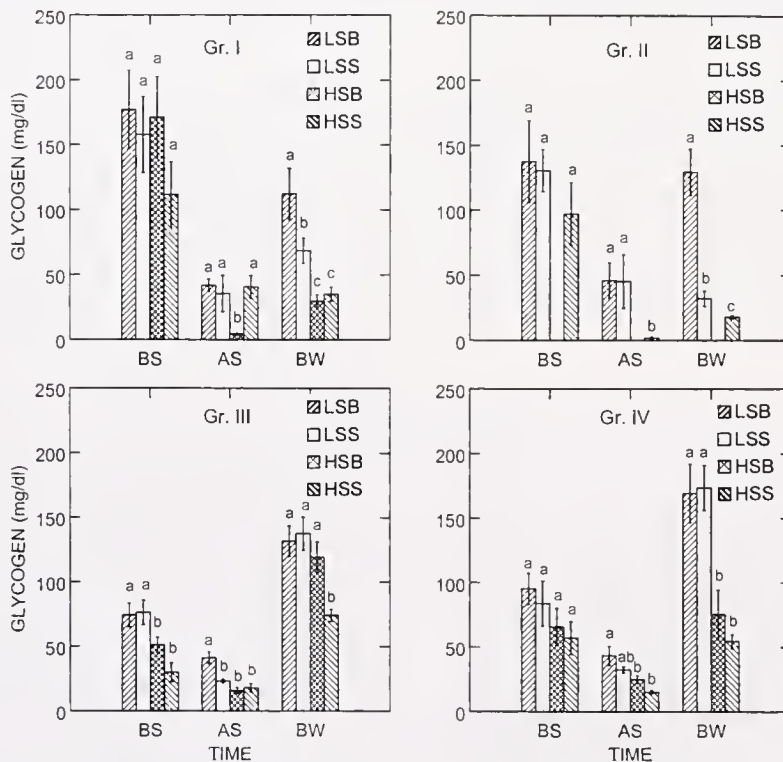
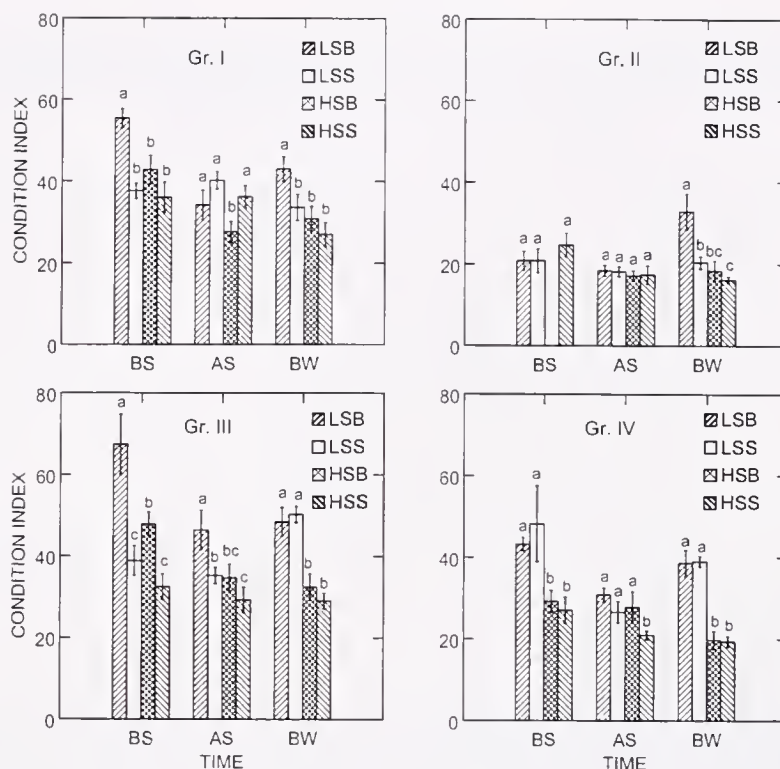


Figure 5. Glycogen concentrations in oysters sampled from stations HSS (high-salinity surface), HSB (high-salinity bottom), LSS (low-salinity surface), and LSB (low-salinity bottom) for groups I (2-year-old oysters in 1993), II (4-year-old oysters in 1993), III (2-year-old oysters in 1994), and IV (4-year-old oysters in 1994), before (BS) and after spawning (AS) and before winter (BW). Sampling dates for each group are shown in Table 1. Bars with similar letters are not significantly different from each other ( $p > .05$ ). Missing data for group II are attributable to oysters lost (see Materials and Methods).



**Figure 6.** Condition indices for oysters sampled from stations HSS (high-salinity surface), HSB (high-salinity bottom), LSS (low-salinity surface), and LSB (low-salinity bottom) for groups I (2-year-old oysters in 1993), II (4-year-old oysters in 1993), III (2-year-old oysters in 1994), and IV (4-year-old oysters in 1994), before (BS) and after spawning (AS) and before winter (BW). Sampling dates for each group are shown in Table 1. Bars with similar letters are not significantly different from each other ( $p > .05$ ). Missing data for group II are attributable to oysters lost (see Materials and Methods).

such as growth rate, glycogen concentration, and condition index have been used in the past to gauge the relative growth and health of oysters (Muniz et al. 1986, Brown and Hartwick 1988, Littlewood and Gordon 1988, Austin et al. 1993, Boghen et al. 1993).

Our work is consistent with those of others who demonstrated that oysters cultured in suspension display superior growth as compared to other growout methods. Although this proved to be the case at each site, when the sites were grouped together, we noted that oysters cultured on the bottom at high-salinity (HSB) displayed growth comparable to those reared near the surface at low-salinity (LSS).

Salinity and temperature, acting either independently or in synergy, are known to have greater effects on growth of the eastern oyster than do other factors (Butler 1949, Wells 1961, Alderdice 1972, Vernberg and Vernberg 1972). This, however, does not preclude the fact that such environmental variables as seston and primary production, may, either in combination with or separate from salinity and temperature, have an important effect on the physiology and growth of the eastern oyster (Bayne and Newell 1983, Brown and Hartwick 1988, Deksheniaks et al. 1993, Shumway 1996).

The significant difference that has been observed between years for the PIM/POM, may partially explain the differences between 1993 and 1994 for certain recorded values, such as growth, condition index, or glycogen content ratio (Galtsoff 1964, Bayne and Newell 1983, Wallace and Reinsnes 1985).

Although there were no significant differences in primary production among stations, this does not preclude the fact that the nutritional value of individual species of phytoplankton occurring at a given site or station at any particular time, may, in the long

run, represent a more critical factor in determining the suitability of a particular culture location (Haven 1960, Dunathan et al. 1969, Castell and Trider 1974).

Establishment of an halocline, persisting for up to 4 weeks, in the high-salinity water column during May and June for HSS and HSB (Fig. 3 and Table 1), is attributable to the effects of successive periods of melting snow and heavy rain (Environment Canada 1993, 1994). Although better growth is associated with higher salinity (Butler 1949, Chanley 1958, Galtsoff 1964) as observed at HSB, water temperature was lower (Fig. 3, points 1 to 5), and may, therefore, have had an opposite effect on oyster growth. The difference in temperature between bottom and surface at HSB and HSS persisted during the early growing period, which may help explain the reason for better growth at HSS over the growing season. Salinity fluctuations (5–15‰) were evident at LSB at the end of May and beginning of June (Fig. 3), and this may be attributable to heavy precipitation and tidal action.

From early August to mid-October, a phase representing optimal growing conditions for oysters (Shumway 1996), all four stations (HSS, HSB, LSS, and LSB) displayed comparable temperature and salinity profiles. The 1994 data demonstrated tendencies similar to those reported for 1993, although differences in weather patterns resulted in a slight shift in temperature and salinity variations. In all instances, 2-year-old oysters displayed superior growth to 4-year-olds, similar to findings reported by Carriker (1996).

Interpretations of findings related to glycogen concentrations and condition index (dry meat weight/dry shell weight), were considered for the three critical periods during the oysters' growing season: before spawning, after spawning, and before winter, as previously mentioned. For this study, animals were sampled at

specific dates on a biweekly basis. Therefore, it is possible that the data reported for glycogen and condition index do not necessarily represent the absolute maximum and minimum values. This may help to explain differences in recorded values between 1993 and 1994.

The less favorable environmental conditions (Fig. 3) for oysters at stations LSS and LSB for groups I and II after spawning may be responsible for their reduced capacity to rebuild glycogen reserves and their increased vulnerability at a critical time during their growing cycle. In general, however, oysters grown in suspension at HSS and LSS, during the same period, displayed higher glycogen concentrations than those cultivated on the bottoms at HSB and LSB, respectively. This is consistent with our growth data.

Results of the condition indices measured for oysters sampled from all stations for groups I to IV generally support the findings recorded for glycogen and growth (Fig. 6). One notable exception was that the condition index proved to be inconsistent with the glycogen data for oysters cultivated in the low-salinity environment. This index may prove to be an effective tool that could correlate positively with reported glycogen concentrations, depending upon environmental conditions (Ingle 1949, Walne 1970, Gabbott and Stephenson 1974). The absence of a detectable relationship between the condition index and glycogen concentration or even growth in certain instances may be attributable to several factors, ranging from low calcium levels in less saline waters to poor substrates and/or inferior quality and availability of food. These factors may, in turn, contribute to improper shell formation and may alter shell form and thickness, thus biasing the relevancy of condition index as based on conventional methods (Riley and Chester 1971, Wilbur and Saleuddin 1983). Moreover, a pronounced asynchronism in the growth rate of shell versus soft tissue may likewise influence the accuracy of interpretation of values for condition index, as has previously been demonstrated for mussels (Hilbish 1986, Rainer and Mann 1992).

Various authors (Lucas and Beninger 1985, Brown and Hartwick 1988, Rainer and Mann 1992) demonstrated that static indices based on the ratio of dry meat weight/shell cavity volume or dry meat weight/shell weight are less efficient than such dynamic indices as biochemical indicators. The value of such biochemical indicators as glycogen concentration, carbohydrate:nitrogen, and carbon:nitrogen have been discussed by Mann (1978).

Our results demonstrate that oysters grown in suspension under

high-salinity conditions display growth and development superior to those cultured in a low-salinity environment. Despite the superiority of oyster growth recorded at station HSS, the effectiveness of oyster culture in less saline waters as determined from our study should not be overlooked. Depending upon the specific environmental and hydrographic conditions characterizing a given site, bottom culture may be comparable to surface culture, if not more appropriate. This possibility is supported by Newell et al. (1998), who demonstrated that the contribution of detritus as a source of nutrient for mollusks may be more important than the phytoplankton available in the upper portions of the water column. Such an outcome may explain the reason for superior growth of bottom-cultured oysters versus oysters grown in suspension. A comparable situation may explain the similarity in growth of oysters from stations LSS and HSB.

The implications of our findings lend credence to the potential advantages of identifying new aquaculture sites, which may have been rejected for commercial oyster culture up to now. The importance of such studies is reinforced by the decreasing availability of traditional culture sites in Atlantic Canada because of excessive coastal development and enhanced organic pollution.

Finally, the appropriateness of certain sites may be more applicable for the culture of one age group versus another, as reported by Ortega and Sutherland 1992. Alternatively, less traditional sites may also prove to be useful as secondary or provisional storage areas for established operations, particularly at certain times of the year.

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## HIGH-RESOLUTION ANALYSIS OF KARYOTYPES PREPARED FROM DIFFERENT TISSUES OF THE EASTERN OYSTER *CRASSOSTREA VIRGINICA*

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**ABSTRACT** Establishment of chromosome identity is the first step of physical genome mapping. This step is hindered by a lack of banding techniques and size homogeneity in the chromosomes of the eastern oyster, *Crassostrea virginica*. In this study, chromosomes prepared from adult gill, ripe gonad tissues, and embryos were analyzed with a computer-based measurement system. Chromosomes from embryos were elongated with excellent morphology and identifiable secondary constrictions, although homologous pairs were difficult to establish because of asymmetric sizes found between homologues. Meiotic chromosomes at the stages of diakinesis (testis) and pachytene (ovary) offered distinct advantages for karyotyping. These chromosome bivalents possessed a haploid chromosome number ( $n = 10$ ) with transverse chromomere bands analyzable by microdensitometry. Chromosomes derived from gill tissue were highly condensed and few spreads were analyzable. Idiograms of each chromosome were developed in this study based on size, centromere position, and chromomere bands. These results indicate that mitotic and meiotic cells are each important for the study of chromosomes of the eastern oyster and that computer-assisted analysis will be useful for establishment of karyotypes and idiograms.

**KEY WORDS:** Chromomere bands, microdensitometry, karyotype, *Crassostrea virginica*

### INTRODUCTION

Identification of individual chromosomes is the prerequisite step for *in situ* detection and location of specific DNA sequences. The genome of most oyster species is composed of 10 metacentric and submetacentric chromosomes, comprising three groups based on size (Longwell and Stiles 1967). Techniques need to be developed for further identification of specific chromosomes within the oyster genome. Identification of individual mammalian chromosomes has been accomplished by techniques such as Giemsa (G)-banding or reverse (R)-banding; however, these techniques do not produce consistent banding patterns in lower vertebrates (Zhang and Tiersch 1998a) and invertebrates, including oysters.

It is well established that the quality of chromosome preparations is directly related to the source material. Embryos and somatic tissues (such as gill filament) are most commonly used for cytogenetic analysis in oysters. Karyotypes of the eastern oyster, *Crassostrea virginica*, based on somatic (Rodriguez-Romero et al. 1978) and meiotic chromosomes (Longwell et al. 1967), have been described. However, none of these studies examined structural markers on the chromosomes; therefore, chromosomes of similar size could not be distinguished in these studies. Although "G-like" chromosome bands were studied in this species (Rodriguez-Romero et al. 1979), routinely useful banding patterns and analysis techniques have not been established.

In fish, meiotic chromosomes (at early prometaphase I) without colchicine treatment, are typically extended with knot-like structural markers (chromomeres), which are useful for identifying individual chromosomes (Yu et al. 1994). Most spreads prepared from ripe gonad tissue are composed of chromosome bivalents, and no pairing is required to establish a karyotype. With the assistance of microdensitometric methods (Zhang and Tiersch 1998b), these chromomere bands could be identifiable as markers for chromosomes of oysters.

In the present study, we developed haploid karyotypes for the eastern oyster from meiotic chromosomes (in diakinesis and

pachytene stages). By computer-based measurements and statistical analysis, we compared these karyotypes with diploid karyotypes prepared from gill and embryonic cells, and documented naturally occurring chromomere structures useful for chromosome identification.

### MATERIALS AND METHODS

#### Materials

Eastern oysters were obtained from Grand Isle, Louisiana, and were maintained in an indoor recirculating system until use (Buchanan et al. 1998). Twenty ripe females and 15 ripe males were used to sample gonad and gill tissues in this study. Embryos were produced by artificial fertilization based on methods described in Paniagua-Chavez et al. (1998).

#### Chromosome Preparation

##### Gonad Tissue

About 0.2 g of testis or ovary tissue were removed and cut into ~2-mm<sup>2</sup> pieces, which were placed in 0.9% sodium citrate (prepared in deionized water) with continuous swirling for 5–7 h. The tissue fragments were fixed twice for 20 min with cold Carnoy's fixative I (methanol:chloroform:acetic acid; 6:3:1), and fixed three times for 20 min in cold Carnoy's fixative II (methanol:acetic acid; 3:1). The tissues were ground and passed through a 70- $\mu$  cell strainer. Cells were collected, pelleted, and resuspended in Carnoy's fixative II overnight. Chromosomes were prepared on microscope slides using standard air-drying methods.

##### Embryos

Embryonic cells collected 5 h after fertilization were used in this study. Techniques for preparation of chromosomes of oyster embryos have been described in Guo and Allen (1997). Embryos were concentrated on a 15- $\mu$  nylon filter after the colchicine treat-

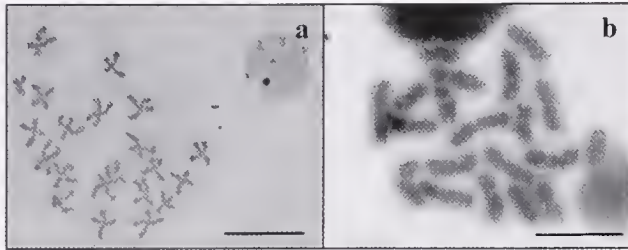


Figure 1. Preparation of mitotic (somatic) metaphase chromosomes from eastern oyster (*C. virginica*); (a) gill; and (b) male gonad tissue; bar = 10  $\mu$ m.

ments. Chromosomes were prepared using standard air-drying methods.

**Gill Filaments**

Gill tissues were incubated for 3 h in the medium JL-ODRP-4 developed for culture of oyster cells (J. LaPeyre pers. comm.). Colchicine was added to the medium at a final concentration of 0.01%. Chromosomes were prepared with the same method used for gonad tissues, except that hypotonic (sodium citrate) treatment was 30 min.

**Computer-Assisted Karyotyping**

Chromosome images were digitized with a 24-bit video capture board (Imaging Technology Inc., Bedford, MA) using a light microscope (Microphot-SA, Nikon Inc.) equipped with a high-resolution RGB color video camera (model A206A, Microimage Video Systems Co., Inc., Boyertown, PA). The Optimas® com-

puter software packages (Bioscan, Inc., Edmonds, WA), a Windows™ based application, was used to capture and process the chromosomal images. Total length, arm length, and banding patterns (chromomeres) of chromosomes were measured with the linear functions of Optimas (Zhang and Tiersch 1998b). Meiotic chromosomes ( $n = 10$ ) were arranged by order of descending size. For comparisons, mitotic chromosomes ( $2n = 20$ ) were ordered by size, and pairs were established based on relative length and ratio of short arm to entire length (i.e., centromeric index) (Levan et al. 1964). Idiograms were created using Microsoft Powerpoint™ for each chromosome based on size, centromere position, and banding patterns.

Repeated measurements ( $n = 10$ ) were taken from representative spreads, and statistical analysis (see below) was performed to estimate technical variation using data derived from individual spreads, and for biological variation using data derived from different spreads of a particular tissue type or from different tissue types.

**Statistical Analysis**

One-way analysis of variance (ANOVA) was performed to compare: (1) 10 repeated measurements of total length of chromosomes within a representative spread, used to examine the accuracy of the image analysis system; (2) relative length of individual chromosomes derived from different spreads from the same tissue source (embryo); and (3) relative length of individual chromosomes derived from four different tissue sources (gill, embryo, testis, and ovary). The relative length data were transformed to arcsine square root values before analysis. Data were collected

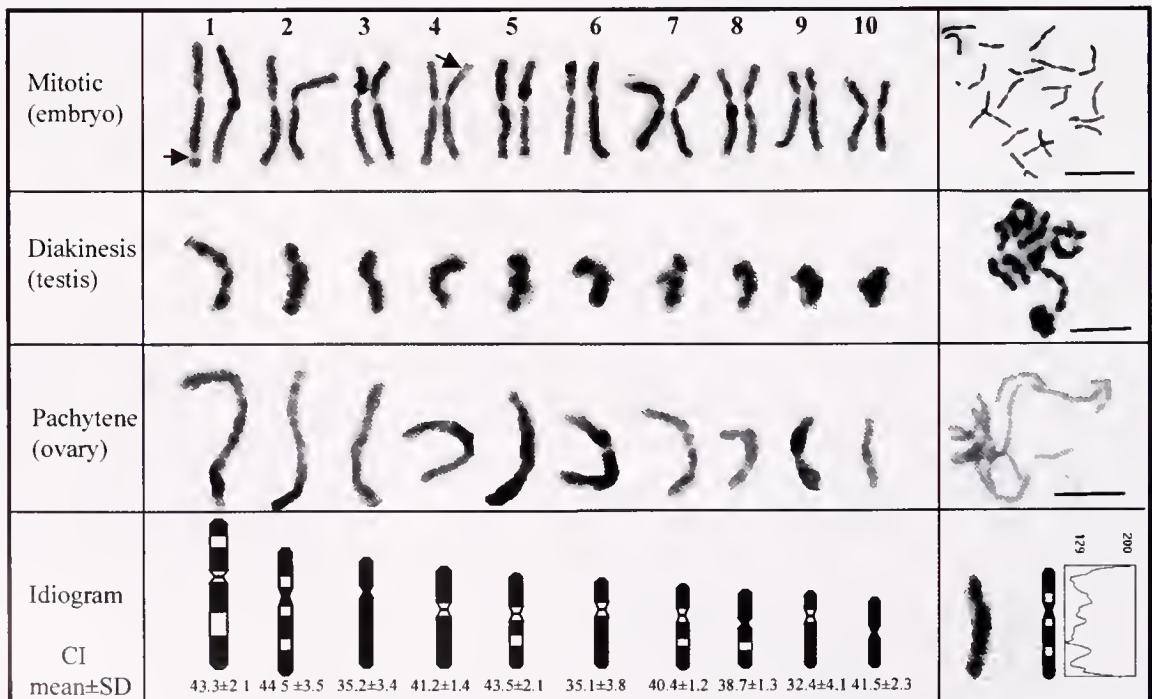


Figure 2. Karyotypes developed from different tissues of the eastern oyster; arrowheads point to secondary constrictions; insets to the right for mitotic, diakinesis, and pachytene chromosomes were the original spreads used for karyotyping; to the right of the idiogram is a demonstration of the method of microdensitometry; CI: centromeric index; bar = 10  $\mu$ m.

TABLE 1.

Repeated measurements (expressed in arbitrary computer units) of total length of chromosomes (in descending order of size) within a representative spread derived from eastern oyster embryos; one-way ANOVA was used to examine the variation among these measurements ( $p = 1.00$ ).

Chromosome	Measurements (Replicates)										Mean $\pm$ SD	Range	Percentage Variation <sup>a</sup>
	1	2	3	4	5	6	7	8	9	10			
1	4.36	4.44	4.34	4.32	4.37	4.34	4.35	4.43	4.34	4.33	4.36 $\pm$ 0.04	0.12	2.75
2	4.09	4.14	3.96	4.02	4.14	4.10	4.00	4.02	4.15	4.12	4.07 $\pm$ 0.07	0.19	4.66
3	4.02	4.04	3.95	4.02	4.04	3.95	4.00	4.02	4.03	4.00	4.01 $\pm$ 0.03	0.09	2.24
4	3.86	3.93	3.89	3.91	3.88	3.89	3.86	3.92	3.89	3.90	3.89 $\pm$ 0.02	0.07	1.80
5	3.69	3.75	3.62	3.69	3.70	3.62	3.69	3.75	3.62	3.69	3.68 $\pm$ 0.05	0.13	3.53
6	3.65	3.67	3.65	3.65	3.67	3.65	3.66	3.65	3.67	3.65	3.66 $\pm$ 0.01	0.02	0.55
7	3.62	3.66	3.53	3.62	3.66	3.62	3.53	3.62	3.66	3.62	3.61 $\pm$ 0.05	0.13	3.60
8	3.53	3.59	3.56	3.55	3.55	3.53	3.56	3.55	3.55	3.53	3.55 $\pm$ 0.02	0.06	1.69
9	3.53	3.64	3.57	3.55	3.64	3.53	3.57	3.55	3.64	3.53	3.58 $\pm$ 0.05	0.11	3.07
10	3.46	3.52	3.42	3.46	3.47	3.42	3.46	3.51	3.42	3.43	3.46 $\pm$ 0.04	0.10	2.89
11	3.41	3.45	3.36	3.37	3.41	3.30	3.41	3.45	3.35	3.40	3.39 $\pm$ 0.07	0.15	4.42
12	3.25	3.24	3.22	3.22	3.25	3.22	3.27	3.24	3.22	3.22	3.24 $\pm$ 0.02	0.05	1.54
13	3.13	3.14	3.17	3.15	3.14	3.13	3.18	3.16	3.14	3.13	3.15 $\pm$ 0.02	0.05	1.59
14	3.00	3.03	3.00	3.01	3.03	3.00	3.01	3.01	3.03	3.02	3.01 $\pm$ 0.01	0.03	1.00
15	2.96	3.07	3.04	3.02	2.95	3.04	3.00	3.08	3.03	3.02	3.02 $\pm$ 0.04	0.13	4.30
16	2.88	2.91	2.83	2.86	2.89	2.83	2.89	2.91	2.85	2.86	2.87 $\pm$ 0.03	0.08	2.79
17	2.77	2.84	2.77	2.78	2.84	2.78	2.77	2.78	2.83	2.78	2.79 $\pm$ 0.03	0.07	2.51
18	2.69	2.72	2.59	2.63	2.70	2.69	2.60	2.63	2.68	2.69	2.66 $\pm$ 0.05	0.13	4.89
19	2.63	2.70	2.66	2.65	2.63	2.66	2.64	2.70	2.66	2.66	2.66 $\pm$ 0.02	0.07	2.63
20	2.53	2.57	2.51	2.51	2.53	2.51	2.51	2.53	2.53	2.52	2.53 $\pm$ 0.02	0.06	2.37
Average	3.35	3.40	3.32	3.35	3.37	3.34	3.35	3.38	3.36	3.35			
$\pm$ SD	$\pm$ 0.27	$\pm$ 0.27	$\pm$ 0.26	$\pm$ 0.27	$\pm$ 0.28	$\pm$ 0.26	$\pm$ 0.27	$\pm$ 0.27	$\pm$ 0.27	$\pm$ 0.27			

<sup>a</sup> Percentage variation = (range/mean)  $\times$  100.

TABLE 2.

Variation analysis of relative lengths of chromosomes derived from embryos of the eastern oyster; one-way ANOVA was used to compare data from five representative spreads with 10 repeated measurements for each chromosome; the measurements were expressed as percentage of total length of the entire chromosome complement.

Chromosome	Spread 1	Spread 2	Spread 3	Spread 4	Spread 5	Mean $\pm$ SE	p value	Range	Percentage Variation <sup>a</sup>
1	6.43 $\pm$ 0.00	6.29 $\pm$ 0.00	6.51 $\pm$ 0.00	6.48 $\pm$ 0.01	6.33 $\pm$ 0.01	6.43 $\pm$ 0.10	<0.001	0.22	3.42
2	6.13 $\pm$ 0.00	6.29 $\pm$ 0.00	6.16 $\pm$ 0.01	6.20 $\pm$ 0.01	6.18 $\pm$ 0.01	6.19 $\pm$ 0.07	0.001	0.16	2.58
3	6.06 $\pm$ 0.01	5.83 $\pm$ 0.00	6.06 $\pm$ 0.00	6.11 $\pm$ 0.00	6.16 $\pm$ 0.01	6.01 $\pm$ 0.12	<0.001	0.33	5.49
4	6.01 $\pm$ 0.01	5.66 $\pm$ 0.01	5.68 $\pm$ 0.00	5.95 $\pm$ 0.01	5.90 $\pm$ 0.00	5.83 $\pm$ 0.18	<0.001	0.35	6.00
5	5.57 $\pm$ 0.01	5.33 $\pm$ 0.01	5.49 $\pm$ 0.01	5.71 $\pm$ 0.01	5.67 $\pm$ 0.01	5.52 $\pm$ 0.16	<0.001	0.38	6.88
6	5.42 $\pm$ 0.00	5.33 $\pm$ 0.00	5.43 $\pm$ 0.01	5.64 $\pm$ 0.01	5.45 $\pm$ 0.01	5.46 $\pm$ 0.13	<0.001	0.31	5.68
7	5.50 $\pm$ 0.00	5.24 $\pm$ 0.00	5.40 $\pm$ 0.01	5.64 $\pm$ 0.01	5.45 $\pm$ 0.01	5.44 $\pm$ 0.17	<0.001	0.40	7.35
8	5.49 $\pm$ 0.00	5.17 $\pm$ 0.00	5.35 $\pm$ 0.01	5.29 $\pm$ 0.01	5.21 $\pm$ 0.01	5.33 $\pm$ 0.13	<0.001	0.32	6.00
9	5.36 $\pm$ 0.00	5.07 $\pm$ 0.00	5.32 $\pm$ 0.01	5.00 $\pm$ 0.01	5.15 $\pm$ 0.00	5.19 $\pm$ 0.18	<0.001	0.36	6.94
10	4.93 $\pm$ 0.01	5.05 $\pm$ 0.00	5.07 $\pm$ 0.01	4.82 $\pm$ 0.00	5.08 $\pm$ 0.01	5.02 $\pm$ 0.07	<0.001	0.26	5.18
11	4.82 $\pm$ 0.01	4.93 $\pm$ 0.00	4.86 $\pm$ 0.01	4.82 $\pm$ 0.00	4.91 $\pm$ 0.01	4.86 $\pm$ 0.05	0.001	0.11	2.26
12	4.75 $\pm$ 0.00	4.82 $\pm$ 0.00	4.78 $\pm$ 0.01	4.79 $\pm$ 0.00	4.75 $\pm$ 0.01	4.78 $\pm$ 0.03	0.020	0.07	1.46
13	4.74 $\pm$ 0.01	4.81 $\pm$ 0.00	4.66 $\pm$ 0.01	4.79 $\pm$ 0.01	4.72 $\pm$ 0.01	4.75 $\pm$ 0.07	0.001	0.15	3.16
14	4.33 $\pm$ 0.01	4.74 $\pm$ 0.01	4.48 $\pm$ 0.01	4.53 $\pm$ 0.01	4.57 $\pm$ 0.00	4.52 $\pm$ 0.17	<0.001	0.41	9.07
15	4.44 $\pm$ 0.00	4.68 $\pm$ 0.00	4.45 $\pm$ 0.00	4.38 $\pm$ 0.01	4.37 $\pm$ 0.00	4.49 $\pm$ 0.13	<0.001	0.31	6.90
16	4.28 $\pm$ 0.01	4.51 $\pm$ 0.00	4.39 $\pm$ 0.00	4.27 $\pm$ 0.01	4.24 $\pm$ 0.01	4.36 $\pm$ 0.11	<0.001	0.27	6.19
17	4.18 $\pm$ 0.01	4.36 $\pm$ 0.01	4.11 $\pm$ 0.00	4.05 $\pm$ 0.01	4.19 $\pm$ 0.01	4.17 $\pm$ 0.13	<0.001	0.31	7.43
18	3.93 $\pm$ 0.01	4.26 $\pm$ 0.00	3.95 $\pm$ 0.01	4.10 $\pm$ 0.01	4.09 $\pm$ 0.01	4.06 $\pm$ 0.15	<0.001	0.33	8.13
19	3.90 $\pm$ 0.01	4.02 $\pm$ 0.01	3.97 $\pm$ 0.01	3.87 $\pm$ 0.01	3.82 $\pm$ 0.01	3.94 $\pm$ 0.07	0.001	0.20	5.08
20	3.83 $\pm$ 0.01	3.62 $\pm$ 0.00	3.67 $\pm$ 0.00	3.71 $\pm$ 0.01	3.76 $\pm$ 0.01	3.71 $\pm$ 0.07	<0.001	0.21	5.66

<sup>a</sup> Percentage variation = (range/grand mean)  $\times$  100.

TABLE 3.

Relative lengths of individual chromosomes derived from gill, embryo, testis, and ovary of the eastern oyster were compared by one-way ANOVA ( $n = 10$  spreads for each tissue type); the measurements were expressed as percentage of total length of the entire chromosome complement.

Chromosome Number	Gill	Embryo	Testis (Diakinesis)	Ovary (Pachytene)	p value
1	12.70 ± 0.54	12.62 ± 0.01	16.84 ± 0.12	15.15 ± 0.32	0.001
2	12.20 ± 0.43	11.53 ± 0.03	11.63 ± 0.32	12.63 ± 0.07	0.001
3	11.10 ± 0.37	10.98 ± 0.02	11.20 ± 0.24	11.06 ± 0.08	0.09
4	10.87 ± 0.40	10.77 ± 0.01	10.83 ± 0.13	10.53 ± 0.11	0.07
5	10.45 ± 0.51	10.04 ± 0.03	9.64 ± 0.09	10.40 ± 0.10	0.001
6	9.83 ± 0.36	9.56 ± 0.01	9.30 ± 0.05	10.36 ± 0.07	0.001
7	9.65 ± 0.32	9.36 ± 0.02	8.42 ± 0.10	9.83 ± 0.08	0.001
8	8.10 ± 0.27	8.84 ± 0.04	7.84 ± 0.08	7.34 ± 0.12	0.001
9	8.00 ± 0.46	8.13 ± 0.03	7.04 ± 0.05	6.63 ± 0.10	0.001
10	6.42 ± 0.52	7.63 ± 0.02	6.48 ± 0.07	5.90 ± 0.05	0.001

based on measurements of 10 complete chromosome spreads for each source material.

### RESULTS

Ten pairs of metaphase chromosomes were observed in cells obtained from gill (Fig. 1a) and embryos (Fig. 2) with seven pairs of metacentrics and three pairs of submetacentrics. Embryonic chromosomes were arranged in descending order by size (Fig. 2) for comparison with the karyotypes prepared from meiotic spreads. Two pairs of chromosomes from the embryos typically had visible secondary constrictions (arrowheads in Fig. 2).

Chromosome spreads in different phases of meiotic division were found in gonadal tissues (Fig. 2), including diakinesis and pachytene bivalents, and a few mitotic (somatic) chromosome spreads were found (Fig. 1b). In ovary, most spreads (~45%) were pachytene chromosomes; whereas, in testis, most spreads (~70%) were in diakinesis. Haploid karyotypes (Fig. 2) were developed for chromosomes in diakinesis and pachytene stages, in which chromosomes were arranged by size in descending order. Chromomere bands were distinct on diakinesis chromosomes and less distinct on pachytene chromosomes, and were reproducible for each of the 10 chromosomes. Idiograms were prepared for each chromosome based on size, centromeric index (calculated from the chromosomes produced from embryonic tissues), and chromomere bands of diakinesis chromosomes (Fig. 2).

There was no difference ( $p = 1.0$ ) among 10 repeated measurements of total length for a given chromosome spread (Table 1). However, relative lengths of chromosomes were significantly different ( $p < .05$ ) among different spreads prepared from the same

tissue type (Table 2). The relative lengths of chromosomes were significantly different ( $p < .05$ ) among the four tissue types (gill, embryo, testis, and ovary) except for chromosomes 3 and 4 ( $p > .05$ ) (Table 3).

### DISCUSSION

In this study, we found that chromosomes derived from four tissue types had distinct morphological features providing different options for year-round genetic studies of the eastern oyster (Tables 4, 5). The mitotic activity of oyster somatic cells is low, and there are no methods available for stimulation of mitosis in oyster somatic cells *in vivo* or *in vitro* (Cornet 1993). Although an increase of colchicine concentration increased the number of spreads observed, the resultant chromosomes were short, less distinct in size, and not suitable for use in physical mapping. Chromosomes prepared from embryos were elongated and provided images of high resolution. Chromosome structures such as secondary constrictions were recognizable on these chromosomes. However, heavy background caused by adherent materials limited subsequent analysis. Techniques have been developed for removing yolk materials from preparations of oyster chromosomes derived from cleaving eggs (Longwell and Stiles 1968). In this study, we found that 50% acetic acid could eliminate most background materials (data not shown). Nevertheless, the effects of these treatments on the quality of chromosomes for use in physical gene mapping needs to be evaluated.

In this study, a new protocol was introduced for preparation of bivalent chromosomes from ripe gonad tissue of the eastern oyster, which omitted colchicine treatment and included a prolonged hy-

TABLE 4.

Ploidy level, availability, division stage, and spreads per slide of four tissue types used in this study; the number of spreads observed on each microscope slide was used to estimate mitotic index of each tissue type of eastern oyster.

Tissue	Ploidy	Availability	Division Stage	Spreads per Slide <sup>a</sup>
Gill	2N	Year-around	Mitotic metaphase	Low
Embryo	2N	Spawning season	Mitotic metaphase	High
Testis	1N, 2N	Spawning season	Mostly diakinesis	—
Ovary	1N, 2N	Spawning season	Mostly pachytene	—

<sup>a</sup> Low, <5 spreads per slide; high, >30 spreads per slide, and —, cell division dependent on season.



TABLE 5.

Suitability of chromosomes derived from different tissues of eastern oyster for physical genome mapping.

Tissue	Elongation	Dispersal	Resolution	Centromeres Identified	Chromomere Bands	Pairs Identified	Mapping
Gill	Short	Separated	Low	Yes	No	3-5	Not suitable
Embryo	Long	Separated	High	Yes	No	7	Suitable
Testis (diakinesis)	Intermediate	Less separated	High	No	Yes	10	Suitable
Ovary (pachytene)	Very long	Overlapping	Highest	No	Yes	10	Suitable

potonic treatment step. Bivalent haploid spreads yielded the advantages of reduced chromosome numbers, no need for pairing of homologous chromosomes, and identifiable chromomere bands on most chromosomes. However, these chromosomes were difficult to disperse, especially for the pachytene bivalents. Although incubation of gonad tissue with colchicine would facilitate dispersal of bivalent chromosomes, the chromomere bands on these chromosomes would become less identifiable (Yu et al. 1994). Further identification of the advantages and disadvantages of chromosomes from different source tissues is essential for analysis of oyster chromosomes and establishment of a representative karyotype in this species for such activities as physical mapping of genes.

In this study, the computer-based image analysis system provided a reproducible and objective method for measurement of chromosomes. The difference (percentage variation) among measurements of individual chromosomes was less than 5%, which accounted for technical variation including manual error. Variation caused by biological factors was analyzed by comparing different spreads of the same tissue type or spreads of different tissue types. The difference in average relative length was found to be as high as 9% among different spreads from individual embryos. These differences were largely derived from the continuous changes of chromosome morphology produced during the cell cycle. On the other hand, results of this study demonstrated that our measurement system is highly sensitive and capable of detecting minor differences among individual chromosomes. These results suggest that methods need to be developed (such as use of marker chromosomes) for standardization of the eastern oyster karyotype.

Pairing of homologous chromosomes based solely on size and centromeric index was difficult for oyster chromosomes prepared from embryonic cells. Several chromosome pairs were asymmetric in size, centromeric index, and secondary constrictions. A typical example was chromosome 1, the largest metacentric chromosome, which displayed differences in relative length between homologues of 0.2 to 0.4% and in presence or absence of secondary constriction. These asymmetric features could be attributable to normal development of embryos, abnormal development, or integration of features of the genomes of the parents. We did not observe such asymmetric features on chromosomes of other cells, although these chromosomes were highly condensed.

Some differences were found between the karyotype developed previously for embryos of *C. virginica* (Longwell and Stiles 1968) and the one developed in this study. Only three submetacentric chromosomes were found in this study, as compared with four submetacentrics described previously. Also, the second largest chromosome was found to be metacentric rather than submetacentric, as described previously. Reasons for these differences include differences in measurement techniques, variation in the contraction of chromosomes used in each study, and genetic polymorphism among populations of the eastern oyster. Development of such methods as C-banding (for identification of constitutive heterochromatin) would be helpful for resolving these differences by identifying the location of centromeres.

In summary, most chromosomes (seven or eight) prepared from embryos could be identified based on morphological measurements; however, the pairing of homologous chromosomes was difficult. Thus, embryos are a convenient source material for chromosome preparation, although their utility for high-resolution analysis is limited. Meiotic chromosomes, although less frequently studied, were useful, given their reduced numbers and the presence of unique, naturally occurring bands on each diakinesis chromosome.

This study provided information about the composition of the eastern oyster genome. Techniques developed for meiotic chromosomes and idiograms based on these chromosomes have proved to be valuable for physical genetic mapping of oysters in our laboratories (unpublished data). Integrative analysis of mitotic metaphase chromosomes and meiotic bivalent chromosomes will be a useful tool for specific identification of chromosomes in the oyster genome until other techniques become available for mollusks.

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## GENETIC DIVERSITY IN THE EASTERN OYSTER (*CRASSOSTREA VIRGINICA*) FROM MASSACHUSETTS USING THE RAPD TECHNIQUE

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**ABSTRACT** The random amplified polymorphic DNA (RAPD) technique was used to examine the genetic variability in eastern oysters (*Crassostrea virginica*) from four wild-naturalized stocks (Wellfleet, Wareham River, East Wareham/Onset and Barnstable Harbor) and one cultured stock (Cotuit) in Massachusetts. Initially, 20 oligonucleotide primers (Kits A, B, C, G, M, and Z; Operon Technologies Inc., Alameda, California) were screened and 10 were selected to amplify DNA from 79 samples representing these five sites. A total of 90 DNA bands ranging in size from 205 to 1,400 base pairs (bp) were scored. The highest level of polymorphisms were detected in samples from Barnstable Harbor (74%) followed by Wellfleet (71%), Wareham River (70%), East Wareham/Onset (62%), and Cotuit (54%). There were significant differences in polymorphisms between three wild-naturalized (Barnstable Harbor, Wellfleet, and Wareham River) and cultured samples. East Wareham/Onset samples did not show significant differences with the cultured population. Potential site-specific RAPD markers were identified with primers OPA10, OPA17, OPC10, OPG06, and OPM18. The frequency of these site-specific RAPD markers varied among the sample collection sites. In addition, four unique alleles (OPA17-900 and 875 bp; OPC10-450 bp, and OPG06-270 bp) were identified in samples from Barnstable Harbor, Wareham River, and East Wareham/Onset. Our data provide baseline information on the genetic variation in cultured and wild oyster stocks in Massachusetts and may be useful for future management of the resource.

**KEY WORDS:** Eastern oyster, *Crassostrea virginica*, RAPD, genetic diversity

### INTRODUCTION

The eastern oyster (*Crassostrea virginica*) is a benthic marine species with a planktonic larva and a sessile adult stage (Hedgecock 1995). The natural habitat ranges from St. Lawrence Bay, Nova Scotia, through the Gulf of Mexico to the Yucatan Peninsula, Mexico and into the West Indies (Galtsoff 1964). The genetic structure of *C. virginica* across its entire habitat has been examined using different molecular techniques (Buroker 1983, Reeb and Avise 1990, Karl and Avise 1992, King et al. 1994, McDonald et al. 1996, Small and Chapman 1997). Allozyme polymorphism analyses indicated a high gene flow in *C. virginica* populations from Cape Cod, Massachusetts (MA) to the Gulf of Mexico (Buroker 1983), although restriction fragment length polymorphisms (RFLPs) of whole mitochondrial DNA and some (but not all) nuclear DNA markers suggested a genetic break in eastern Florida separating Atlantic populations from those of the Gulf of Mexico (Reeb and Avise 1990, Karl and Avise 1992, King et al. 1994, McDonald et al. 1996). Oyster populations from Laguna Madre, Texas, constitute a genetically divergent group from other populations caused by differential selection operating in this hyperhaline environment (King et al. 1994). Mitochondrial RFLPs using 16S ribosomal RNA gene has been used to distinguish *C. virginica* from two closely related Asian oyster species (*C. gigas* and *C. ariakensis*) (O'Foighil et al. 1995). Sequence data revealed that Asian oyster species showed higher levels of similarity to each other (95%) than to *C. virginica* (84-86%). Recently, random amplified polymorphic DNA (RAPD) technique was used to distinguish species in the marine bivalve genus *Donax* and evaluate

its biogeography (Adamkewicz and Harasewych 1996). RAPD technique requires no prior knowledge of the genome, uses very little DNA, detects high levels of polymorphisms, analyzes a large portion of the genome in a short time, and is faster than such molecular techniques as RFLP and microsatellites. For these reasons, RAPD technique has become a powerful tool to assay the genetic variation of populations (Hadrys et al. 1992). Although the genetic variability of natural oyster populations has been evaluated in different studies, information on the genetic diversity of hatchery stocks and its comparison to native populations is limited.

In recent years, native and cultured populations of oysters in the northeastern United States have suffered periodic heavy losses from diseases such as Dermo (*Perkinsus marinus*), MSX (*Haplosporidium nelsoni*), and Juvenile Oyster Disease (Gaffney and Bushek 1996, Lewis et al. 1996). It is possible that the increase in disease incidence in oysters may be caused by reduced genetic diversity or the introduction or transfer of stocks (Ford 1992). In oyster hatcheries, broodstock are sometimes selected primarily on the basis of such morphological features as size, shell characteristics, color, and growth. In selected breeding programs, only a small number of broodstock are generally used, enhancing the possibility of decreased genetic variation in cultured, as compared to wild, stocks (Wilkins 1976). In this study, the RAPD technique was used to evaluate the genetic variability in one cultured and four wild populations of eastern oysters from different geographic locations in Cape Cod areas in Massachusetts.

### MATERIALS AND METHODS

#### Sample Collection

Oyster samples were collected from one cultured and four wild populations in Cape Cod, Massachusetts (Table 1, Fig. 1). Initially, 143 samples (30 each from Cotuit, Wellfleet, and Wareham River, 29 from Barnstable Harbor, and 24 from East Wareham/Onset)

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were collected. However, good quality DNA (undegraded/high molecular weight DNA) was obtained for only the 79 samples (Table 1) used for the RAPD assay. The cultured population (Cotuit) refers to hatchery oysters purchased by the growers (Cotuit Oyster Co., Inc.) and maintained for the entire lifespan of the oyster until sale. These oysters originated from Ocean Pond Corp. in Fishers Island, New York and were seeded in Cotuit Bay in 1995. Wild populations were represented by oysters from the areas of Wellfleet, Wareham River, East Wareham/Onset, and Barnstable Harbor. All four locations have received oyster transplants from other areas along the east coast over the last 50 years, which have naturalized with any previously existing oyster populations. The last imports were in the mid-1980s. Thus, the existing stocks are best described as wild naturalized stocks. Among these wild naturalized populations, Onset has been the least diluted by importation. There was no significant native population in Barnstable Harbor until oysters were imported for aquaculture and municipal purposes from Connecticut in the early 1980s. These oysters subsequently spawned, and the offspring survived to contribute the present-day population in this new area.

#### DNA Extraction and RAPD Analysis

Total DNA was extracted from the slow adductor muscle (0.5–1g) using a guanidine isothiocyanate or a lysis method (Garcia et al. 1994). The DNA quality was tested by running the samples in a 1% agarose gel, and only samples with good quality DNA were taken for a polymerase chain reaction (PCR).

Initially, 20 oligonucleotide primers from six different kits (Kits A, B, C, G, M, and Z; Operon Technologies Inc., Alameda, California) were screened using one animal from each population. Ten primers were then selected based on their ability to amplify easily scorable DNA bands. The details of the primers tested are given in Table 2. PCR amplification was performed in a 25  $\mu$ L reaction volume containing 100 ng DNA, 1X PCR buffer, 2.0 mM MgCl<sub>2</sub>, 0.2 mM dNTPs, 0.3  $\mu$ g 10-mer primer, and 2.5 U of *Taq* DNA polymerase (Promega), as described in Garcia et al. 1994. Amplifications were carried out in an M. J. Research Thermocycler PTC-100 at 92°C for 60 s, 35°C for 90 s, and 72°C for 60 s for 40 cycles. The amplification products were run in a 2% agarose gel containing ethidium bromide (0.3  $\mu$ g/mL) in TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM sodium EDTA). The gels were electrophoresed at 45 V for ~18 h and photographed.

PCR amplified DNA bands were scored on the basis of their presence or absence for each sample and each primer. A DNA

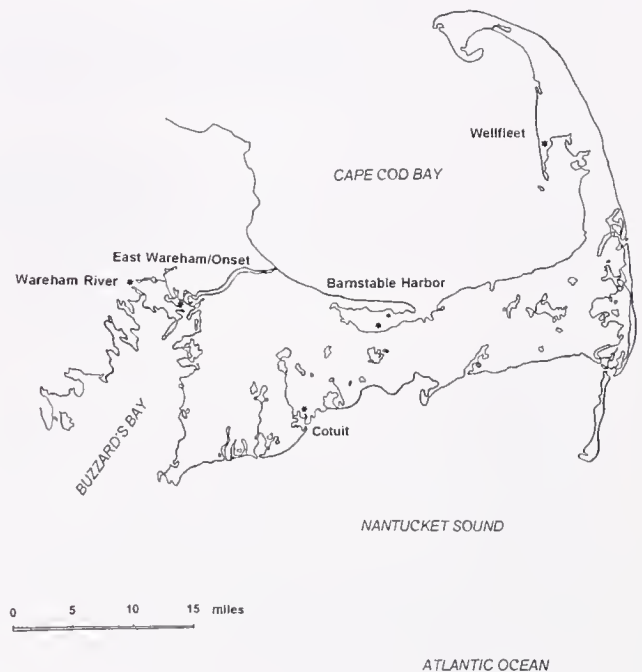


Figure 1. Geographic location of eastern oyster sample collection sites from Cotuit, Wellfleet, Wareham River, East Wareham/Onset, and Barnstable Harbor on Cape Cod, Massachusetts.

band was considered monomorphic when it was present in all of the individuals of a population; whereas, if a DNA band was absent in some or even one individual of a population, it was considered polymorphic. Only distinct DNA bands were scored, and, for consistency, bands were scored by three individuals. The percentage of polymorphic DNA bands was calculated for each site, and the chi-square test was used to determine the significance of variation among the five populations. Differences were considered significant at  $p < .05$ .

#### RESULTS AND DISCUSSION

A total of 90 DNA bands were amplified by PCR using 10 oligonucleotide primers and oyster samples from one cultured and four wild populations representing five different geographic locations in Cape Cod, Massachusetts (Table 2). The sizes of DNA bands scored ranged from 205 to 1,400 base pairs (bp), with an

TABLE 1.  
Oyster (*C. virginica*) collection sites from Cape Cod, Massachusetts.

Location	Sample Size	Harvest Date	Type of Stock	Origin
Cotuit	15	April 29, 1997	Cultured	Ocean Pond Corp., Fishers Island, NY; seeded in 1995 <sup>a</sup>
Wellfleet	16	April 28, 1997	Wild-naturalized	Native and imported populations <sup>b</sup>
Wareham River	16	June 17, 1997	Wild-naturalized	Native and imported populations <sup>b</sup>
East Wareham/Onset	16	June 17, 1997	Wild-naturalized	Native and imported populations <sup>b</sup>
Barnstable Harbor	16	June 23, 1997	Wild-naturalized	Imported from Hammonasset River, CT <sup>c</sup>

<sup>a</sup> No information is available about the origin of broodstock used to perform the crosses or the generation number. The owner of Cotuit Oyster Co. Inc. indicated that these oysters originated from Ocean Pond Corp. in Fishers Island, NY and were seeded in 1995 in Cotuit Bay.

<sup>b</sup> Received last import in mid-1980s.

<sup>c</sup> Last import in early 1980s.

TABLE 2.

Primer sequences, size ranges, total number of DNA bands scored, and number of polymorphic bands amplified by RAPD technique in oyster DNA from Cape Cod, Massachusetts.

Primers	Sequence (5' → 3')	Size Range of DNA Bands Scored (bp)	Total Bands Scored	Number of Polymorphic Bands				
				Cultured		Wild Stocks		
				Cotuit	Wellfleet	Wareham River	East Wareham/Onset	Barnstable Harbor
OPA10	GTGATCGCAG	205–800	10	7	8	8	3	8
OPA17	GACCGCTTGT	225–900	9	3	5	5	6	9
OPB12	CCTTGACGCA	275–1400	10	4	7	4	6	4
OPC04	CCGCATCTAC	390–800	8	4	5	5	6	6
OPC06	GAACGGACTC	450–900	7	4	5	6	4	4
OPC10	TCTCTGGGTG	225–800	8	4	6	7	6	7
OPC12	TGTCATCCCC	220–710	8	3	4	4	3	4
OPG06	GTGCCTAACC	270–900	9	4	7	8	7	7
OPG07	GAACCTGCGG	375–1100	7	5	5	5	3	6
OPM18	CACCATCCGT	225–1400	14	11	12	11	12	12
Total number of bands			90	49	64	63	56	67
% polymorphism				54	71 <sup>a</sup>	70 <sup>a</sup>	62	74 <sup>a</sup>

<sup>a</sup> Significantly different from cultured samples,  $p < .05$ . East Wareham/Onset samples approached significant differences from Barnstable Harbor samples ( $p < .07$ ).

average of nine bands amplified per primer (Table 2). A representative RAPD profile for oyster samples from all five regions using the primer OPA17 is shown in Figure 2.

The percentage polymorphism detected by RAPD technique varied among the five populations. The samples from Barnstable Harbor showed the highest level of polymorphism (74%), and the cultured samples from Cotuit were the least polymorphic (54%). The populations from Wellfleet, Wareham River, and East Wareham/Onset showed 71, 70, and 62% polymorphism, respectively. Although there was no significant difference among Barnstable Harbor, Wellfleet, and Wareham River populations, the East Wareham/Onset samples approached a significant difference from the Barnstable Harbor samples ( $p < .07$ ). It is interesting to note that the Barnstable Harbor population, which had no significant native population before importation, contained the highest level of polymorphisms. This suggests that the oyster stocks originally imported to Barnstable Harbor had a high genetic variation, or perhaps the imported stock came from diverse places. Alternatively, it may be possible that the environmental conditions were highly favorable for adaptation of the imported stocks. Among the four wild populations, East Wareham/Onset had the lowest level of polymorphism (62%). This site is located near the mouth of Cape Cod canal. Because of many anthropogenic activities and increased sedimentation in this area, there may be greater

selection pressure, which might have been reflected in the RAPD assays.

RAPD analysis revealed that the cultured samples from Cotuit were not significantly different ( $p > .29$ ) from the East Wareham/Onset wild population but differed significantly from the Wellfleet ( $p < .02$ ), Wareham River ( $p < .03$ ), and Barnstable Harbor ( $p < .01$ ) populations. Overall, the cultured population was the least polymorphic among the five populations. In a study involving allozyme polymorphisms, Wilkins (1976) reported that cultured samples exhibited fewer polymorphisms as compared to wild stocks. However, English et al. (1997) reported high genetic variation in *C. gigas* (mean polymorphism: 0.70) both farmed and established Tasmanian populations. The discrepancy in polymorphism levels in hatchery stock reported in the present study, as compared to earlier reports, could be attributable to the difference in the number of parental oysters used for spawning. Nevertheless, our data suggest the need to use a large number of broodstock for spawning to enhance the diversity of cultured stocks.

Minimal genetic differentiation has been reported for many benthic marine species with planktonic larval stages (Buroker 1983, Palumbi and Wilson 1990). Analysis of the genetic structure of *C. virginica* from Cape Cod, Massachusetts through Brownsville, Texas using allozyme markers revealed very high similarities (99%) among geographically separated populations, suggesting a

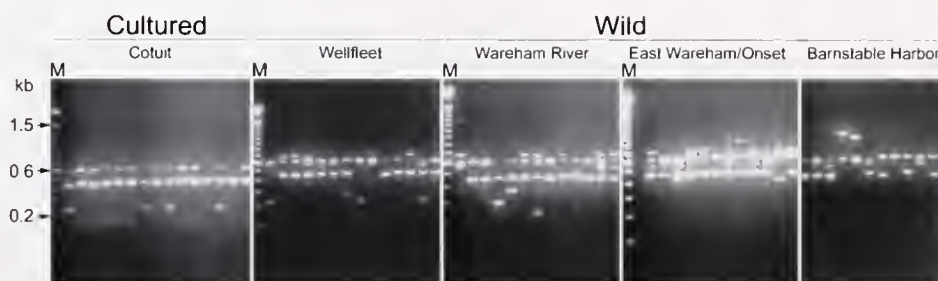


Figure 2. Composite figure of RAPD fingerprints of oyster samples obtained from five different sites in Cape Cod, Massachusetts analyzed with primer OPA17. M = 100 bp molecular weight marker of Gibco BRL.

TABLE 3.

The frequency of region-specific potential RAPD markers for oyster samples collected from five different locations in Cape Cod Massachusetts.

RAPD Marker <sup>a</sup>	Cotuit (n = 15)	Wellfleet (n = 16)	Wareham River (n = 16)	East Wareham/ Onset (n = 16)	Barnstable Harbor (n = 16)
OPA10-250	0.60	0.50	0.31	0.00	0.75
OPA17-675	0.00	0.31	0.38	0.69	0.56
OPA17-575	0.00	0.38	0.50	0.44	0.56
OPA17-550	0.00	0.25	0.19	0.38	0.19
OPA17-435	0.67	0.00	0.50	0.31	0.75
OPC10-800	0.20	0.56	0.56	0.88	0.56
OPG06-350	1.00	0.38	0.31	0.50	0.56
OPM18-350	0.93	0.69	0.50	0.69	0.20
OPM18-310	0.80	0.50	0.69	0.19	0.33

<sup>a</sup> Primer name followed by the size of DNA band amplified.

high gene flow (Buroker 1983). However, Reeb and Avise (1990) reported a distinct genetic break in oyster populations from the Atlantic coast and Gulf of Mexico while examining the RFLP pattern of whole mitochondrial DNA. The genetic discontinuity between oyster populations from the Atlantic Coast and the Gulf of Mexico was further confirmed by Karl and Avise (1992), using single-copy nuclear DNA markers. The discordance between the allozyme, mitochondrial, and single-copy nuclear DNA markers was attributed to balancing selection that prevented the accumulation of allozyme differences (Karl and Avise 1992). In contrast, using anonymous nuclear DNA polymorphisms, McDonald et al. (1996) failed to identify any genetic discontinuity between oysters from Panama, Florida and Charleston, South Carolina on the Gulf and Atlantic Coasts, respectively. Nevertheless, all these studies indicated a high within-population homogeneity in Atlantic Coasts including Cape Cod, Massachusetts regardless of the genetic markers used for the study (Buroker 1983, Reeb and Avise 1990, Karl and Avise 1992). Our RAPD data are, therefore, concordant with earlier studies indicating high genetic similarity within oyster populations in Massachusetts. The number of samples collected from each location and the number of primers screened in this study were limited. Therefore, more samples should be tested with additional primers to confirm the genetic homogeneity of oyster samples from Cape Cod, Massachusetts.

Nine RAPD markers showed differences in their frequency among populations (Table 3). Five of these markers (OPA10<sub>250</sub>, OPA17<sub>675</sub>, OPA17<sub>575</sub>, OPA17<sub>550</sub>, and OPA17<sub>435</sub>) could serve as

potential region-specific markers. A 250 bp DNA band amplified by the primer OPA10 was absent in East Wareham/Onset samples and present in the remaining populations at different frequencies (0.31–0.75). Similarly, the 675 bp, 575 bp, and 550 bp DNA bands amplified by OPA17 were absent in the cultured Cotuit samples, and a 435 bp band amplified by OPA-17 was absent in the Wellfleet samples. RAPD also identified four unique alleles that include a 900 bp and a 875 bp DNA bands amplified by the primer OPA17, a 270 bp band amplified by OPG06 primer, and a 450 bp band amplified by OPC10 primer (Table 4). Because we tested limited numbers of samples from each of the five geographic locations, it would be interesting to see if these region-specific as well as the unique markers can still be reproduced when a large number of samples are tested from all of these locations.

In recent years, RAPD markers have been used to assess the population genetic variation in many plant and animal species (Hadrys et al. 1992). However, its application in population genetic studies in aquaculture species is still limited (Gomes et al. 1998). The present study confirms the potential of RAPD markers for population genetic studies in shellfish and provides baseline information on the genetic diversity of wild and cultured populations of oysters in areas of Cape Cod, Massachusetts. Follow-up studies should be performed to determine if, indeed, all cultured stocks are low in genetic diversity or if environmental disturbance leads to lower levels of variation. Future efforts should also be made to examine the relationship between population genetic variation and the prevalence of diseases in oysters in these areas.

TABLE 4.

Unique alleles identified by RAPD technique and their frequency (in parenthesis) in oyster populations from five different locations in Cape Cod Massachusetts.

Primer	DNA Band (bp)	Cotuit (n = 15)	Wellfleet (n = 16)	Wareham River (n = 16)	East Wareham/ Onset (n = 16)	Barustable Harbor (n = 16)
OPA17	900	—	—	—	—	1 (0.06)
OPA17	875	—	—	—	1 (0.06)	1 (0.06)
OPC10	450	—	—	2 (0.13)	—	2 (0.13)
OPG06	270	—	—	1 (0.06)	2 (0.13)	2 (0.13)

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## EFFECTS OF AGE, SIZE, AND SEASON ON GROWTH OF SOFT TISSUE IN THE OYSTER *CRASSOSTREA GIGAS* (THUNBERG, 1793)

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**ABSTRACT** This study describes potential effects of age, size, and season on the relative allocation of resources to meat and shell growth in *Crassostrea gigas*. Two oyster size groups (slow- and fast-growing oysters) of the same cohort were followed over 2 years. Analysis of covariance detected significant differences in meat weight between groups, seasons, and years. Fast-growing oysters have more meat per gram of shell than do slow-growing, suggesting greater efficiency of the conversion of consumed energy to tissue in the former. Seasonal changes in both sets in meat were related to productivity pattern of phytoplankton, with the highest values of chlorophyll *a* in early summer and minimum in early winter. Differences observed between years in meat content of oysters are attributable both to variability in environmental conditions and to the effect of age.

**KEY WORDS:** *Crassostrea gigas*, Japanese oyster, growth, size, age, Eo estuary

### INTRODUCTION

The rapid growth, high meat yield, and relative robustness of the Pacific oyster, *Crassostrea gigas* (Thunberg 1793) has led to its introduction into many parts of the world as a successful species for aquaculture (Brown 1988). Of the nearly one million metric tons of oysters (genus *Ostrea* and *Crassostrea*) landed in the world in 1990 (7.2% of the total aquaculture production), 87% were *C. gigas* (FAO 1992). Oysters are often marketed in their shells, but meat is the primary marketable component, so any oyster culture operation should aim to maximize the energy allocated to meat growth and to obtain an optimum meat/shell ratio. Precise knowledge of the temporal relationship between shell and tissue production is of great importance identifying optimal harvesting times of cultured bivalves (Lucas and Beninger 1985). It is important in the management of oyster culture operations to understand the genetic and environmental factors influencing energy resource allocation (Raubenheimer and Cook 1990).

The performance of Pacific oysters under different environmental conditions has been assessed through measurements of growth rate, condition index, and survival rate (Quayle 1969, Askew 1972, Brown and Hartwick 1988a, Brown and Hartwick 1988b); however, no study dealing with adult oysters has explored how the relationship of meat-shell weight changes with the combined effects of age, season, and size. Age-specific responses may reflect variations in the allocation of assimilated energy into the such metabolic compartments as reproduction, storage, shell, or somatic tissues (Zandee et al. 1980). Seasonal changes in meat weight result from the storage and utilization of food reserves in relation to the complex interactions of food availability and temperature with growth and reproductive processes (Dare and Edwards 1975, Bayne and Newell 1983). The size effect represents changes in the relation of meat-shell depending upon the weight of the group. Usually, samples from different seasons contain individuals of similar size, and no information is obtained from size-class differences, assuming that individuals of different sizes respond similarly to changing environmental conditions (Mallet and Carver 1993). In addition, the timing of shell and tissue growth may vary substantially among different size or age classes (Peterson and Fegley 1986). Therefore, the objectives of this study were to determine temporal trends in meat production, and developing size, age, and season-specific relationships for shell and meat

growth by monitoring growth rates in slow- and fast-growing oysters belonging to the same cohort over 2 years.

### MATERIAL AND METHODS

One set of Japanese oysters (two million individuals, 0.6 g mean live weight) from a Spanish hatchery was planted in September 1992 in the intertidal zone of the Eo estuary (Fig. 1). In January 1994, size sorting was carried out using a commercial oyster grader. Then, the oyster set was separated into three groups depending upon whole oyster weight: the slow-growing oysters, medium-growing oysters, and the fast-growing oysters of the cohort (Fig. 2). Only the slow-and the fast-growing oysters were sampled in the subsequent analyses.

Both groups were reared for 2 years in standard oyster bags (1 × 0.5 m, 14 mm diameter) at densities of approximately 100 oysters per bag, arranged in a line on metal tables (60-cm high and 3-m length), in the middle of the commercial oyster culture area. The bags were turned over every 4 months to prevent development of fouling organisms. At the same time, they were rotated to avoid the biased effect of some food concentration gradient. The sampling procedures were as follows: 15 oysters from each set were randomly collected every 10 weeks during a 2-year period. Oysters were washed, weighed, and the meat was removed and immediately dried at 60°C to a constant weight (48–72 h) and reweighed to the nearest 0.01 g. The shell was air dried for 48 h and weighed to the nearest 0.1 g. Seawater temperature and salinity were measured at 1 to 3-m depth every day at the culture site. Daily temperature and salinity measurements were recorded as monthly means (Fig. 3) in order to make the representation clearer.

### Statistical Analysis

A three-way analysis of covariance (ANCOVA) was used to determinate the effect of age, size, and season on dry meat weight, with shell weight as a changing covariate, following Walne and Mann (1975), who recommended the use of weight rather than linear dimensions in the analysis of seasonal growth of oysters, because they are irregular in shape. In ANCOVA, the assumption of equal slopes was first tested, and without-slope heterogeneity intercepts (adjusted means) were tested. The samples were collected each year in January, March, June, August, and November, so this factor was treated as a fixed effect. Size (slowest- and

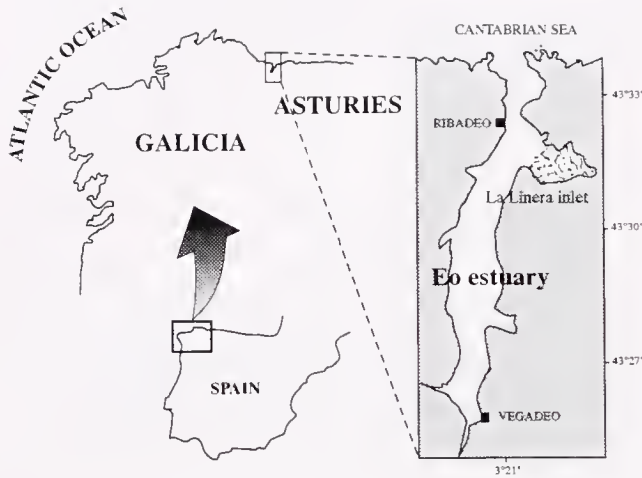


Figure 1. Eo estuary: culture takes place in the Linera inlet.

fastest-growing oysters) and age (2 and 3 years old) were also treated as fixed effects. The appropriate F statistics were calculated following Zar (1984). At the beginning of the experiment, both the covariate and dependent variable of the two sets were clearly different. Obviously, a comparison of treatment effects using the unadjusted weight means would, therefore, be invalid, because the weight means were already different before the effects were studied. By comparing adjusted means, this problem was overcome (Huitema 1980).

Data were logarithmically transformed and tested for normality (Lilliefors test,  $p > .1$ ) and homogeneity of sample variances (Bartlett test,  $p > .05$ ). An allometric regression equation using the least-squares method, was then used to relate dry meat weight  $M$  (g) to shell weight  $S$  (g) for each sample:  $\text{Ln}(100 \cdot M) = \text{Ln} a + b \text{Ln}(100 \cdot S)$ .

Estimates of adjusted dry meat weight were detransformed for clarity. These were obtained by taking antilogs of the previously log-transformed data. To reduce the bias inherent in the logarithmic transformation, the antilogs were multiplied by a correction factor (CF) calculated from the standard error of the estimate (SEE) (Sprugel 1983) as simply taking the antilog of a log-normal mean will underestimate the true mean value:  $\text{CF} = \exp(\text{SEE}^2/2)$ .

## RESULTS

The meat/shell regression slopes were not significantly different (all  $ps > .05$ ) for the two oyster classes. The adjusted means for the dry meat differed between the two size groups (ANCOVA,  $F_{1, 279} = 7.65$ ,  $p < .01$ ), among seasons (ANCOVA,  $F_{4, 279} = 42.18$ ,  $p < .001$ ) and between years (ANCOVA,  $F_{1, 279} = 8.28$ ,  $p < .01$ ). None of the interactions was significant (ANCOVA, all  $ps > .05$ ).

The adjusted dry meat weight of a standard oyster with a shell weight of 39.3 g (the grand covariate mean) showed a similar pattern in the 2 years (Fig. 4). The minimum dry meat weight was found in winter in both years. Thereafter, both size groups rose steadily to reach maximum dry meat values in July, after which time, tissue weight exhibited a continuous decline until March. The increase in adjusted meat weight was coincident with rising water temperatures (Fig. 3), although oysters achieved their maximum meat weight before the peak of water temperature (August).

Slow- and fast-growing oysters showed a similar pattern of

change in dry meat weight throughout the 2 years, although the fast-growing oysters had significantly more meat than did the slow-growing oysters. An age effect was also significant. The adjusted meat weight of 2- and 3-year-old oysters are calculated on the basis of the grand covariate mean (39.3 g), which allows examination of differences in age without the influence of weight. The linear regression equations of each group were used to obtain adjusted means of dry meat weight for each date (Fig. 4), using the common within-group slope (1.006) obtained from the ANCOVA.

Uncoupled rates of growth of shell and tissue may severely affect analysis of seasonal variation in growth in bivalve populations, resulting in apparent changes of adjusted weight that do not necessarily correspond to real losses or gains of weight by the animals (Hilbish 1986, Borrero and Hilbish 1988). In this experiment, the growth rates of shell and meat for the two size groups were highly correlated (Set Slow:  $r = 0.9437$ ,  $p < .001$ ; Set Fast:  $r = 0.8474$ ,  $p < .001$ ).

## DISCUSSION

In allometric studies, it is assumed that a linear log-log relationship exists between two body parts, and that this relationship persists throughout stable growth periods. A structural shift (i.e., a change in the value of the slope) may, however, occur in this log-linear relationship, as the organism enters a new phase in its life history (Vahl 1984). No differences among slopes of the dry meat-shell weight relationship were found in the studied weight range (from 7 to 160 g, live weight), which means that no changes were detected in the relative allocation of available resources away from meat to shell growth or vice versa.

### Season Effect

Changes in soft tissue weight are typically associated with seasonal variation in food availability (Bayne and Newell 1983), the reproductive cycle (Bayne and Worrall 1980) and patterns of energy storage and mobilization (Barber and Blake 1981). In this experiment, both size groups (slow- and fast-growing oysters) showed similar seasonal changes in adjusted dry meat weight. This pattern resembles the productivity pattern of phytoplankton in the study area, with a major peak of chlorophyll  $a$  in early summer (2.4  $\text{mg}/\text{m}^3$ ) and minimum values in early winter (0.46  $\text{mg}/\text{m}^3$ ) (Fundación Torres-Quevedo 1990). Therefore, the period of meat increase seems to result from increased feeding activity in response to the spring increase in phytoplankton and temperature, a finding that is in agreement with previous descriptions for the species (e.g., Malouf and Breese 1977, Heral et al. 1983, Ruiz et

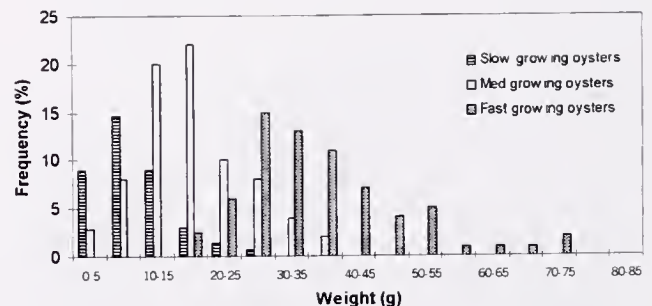


Figure 2. The weight distribution of the oyster population after subdivision into three groups: slow-, medium-, and fast-growing oysters in January 1994.

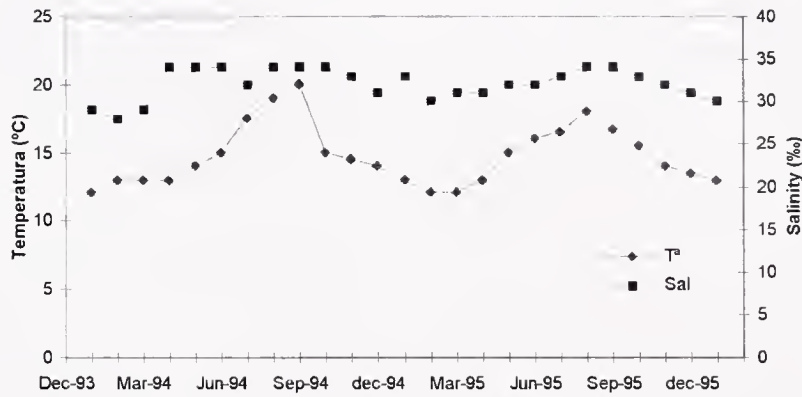


Figure 3. Temperature (°C) and salinity (‰) in the Eo estuary.

al. 1992). The meat increase in adult oysters, in contrast to the increase in young oysters, seems to be related mainly to the formation of reproductive tissue (Berthome et al. 1986, Heral 1989), thus the seasonal pattern of meat weight previously described may be primarily attributable to gamete production.

Uncoupled rates of meat and shell growth affect "adjusted-weight cycles", where soft tissues are statistically adjusted to a covariate using ANCOVA (Hilbish 1986). In the present study, rates of shell and meat growth were highly correlated; therefore, growth of meat and shell were coincident in both size groups. Seasonal variation in available resources creates a large degree of concordance among growth rates of various body components, even if allocation among components changes seasonally (Peterson and Fegley 1986). However, seasonal shifts in allocation of resources may prove to be general among bivalve mollusks, although different studies on bivalve mollusks have provided conflicting results (Borrero and Hilbish 1988). In any case, the bias introduced in adjusted means attributable to uncoupled rates of growth (Hilbish 1986) has a substantial effect when somatic growth is the main cause of meat weight fluctuations, but not when variations attributable to gametogenesis widely exceed somatic growth (Larouelle et al. 1994).

#### Interannual Variability (Age Effect)

Differences observed between years are attributable both to interannual variability in environmental conditions and to the ef-

fect of age. Therefore, comparisons between years can only be referred to as interannual variability. Variation in meat production (mainly variation in gonad production in adult oysters) between years may originate from variable environmental conditions more than the effect of age.

Variability may reflect the ability of this species to adapt the reproductive investment according to environmental conditions, because gamete production is strongly influenced by minor changes in the available food supply and the yearly temperature regime (MacDonald and Thompson 1985, Shumway 1996). Therefore, it would be an appropriate reproductive strategy to invest any surplus energy in gametes when more favorable conditions appear (Hofmann et al. 1992, Ruiz et al. 1992). In this sense, Brown and Hartwick (1988a, Brown and Hartwick 1988b) found that, in comparison to high-growth sites, oysters at medium-growth sites have lower dry meat weight to shell weight ratios, which suggests that preferential shell thickening may be more energetically efficient under conditions of long-term low food availability.

#### Size Effect

Meat weight differed between the two size-groups, although no differences appeared in the timing of growth in shell or meat. This means that, for standard oyster (39.3 g shell weight), the fast-growing oyster has more meat per gram of shell than the slow-growing oyster. It is well known that pronounced differences in

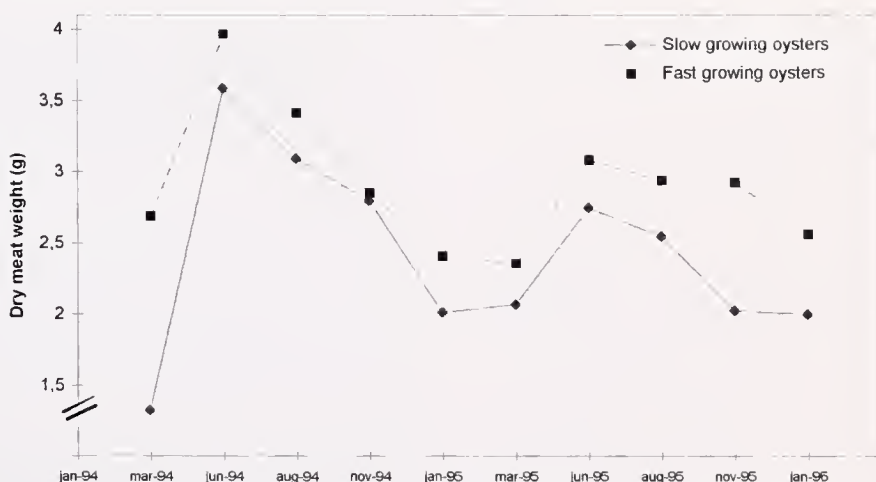


Figure 4. Temporal variation of dry meat weight (g) for slow- and fast-growing oysters during 2 years.

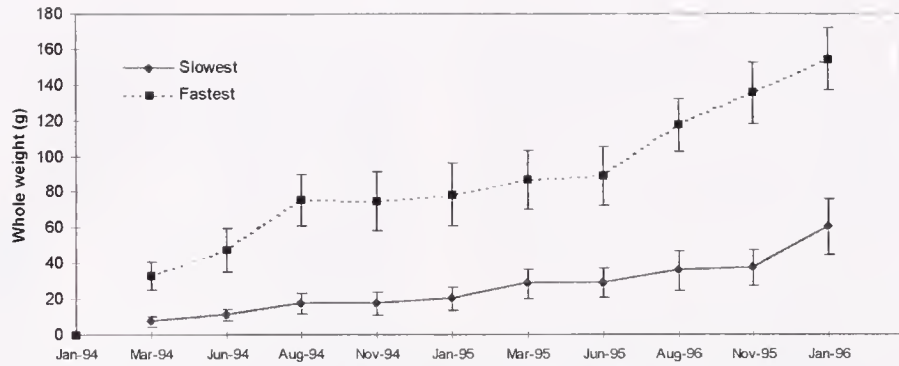


Figure 5. Temporal variation of whole weight ( $\pm 1$  SE) of the two size groups during 2 years.

size appear in oysters of the same cohort reared under uniform conditions (Walne 1958). These differences were found to be correlated with the degree of heterozygosity (e.g., Sigh and Zouros 1978, Koehn and Shumway 1982, Alvarez et al. 1989); whereas, others suggest that physiological variation in growth rate (i.e., the variation not attributable to the environmental factors) may be attributable as much to variation in growth stimulating peptides as to genetic variation at metabolically important loci or multilocus heterozygosity (Painter and DiMichelle 1990, Hedgecock 1995). Individual variation in growth rates can be attributed to variation in available food and/or variation in efficiencies with which food derived from available resources can be used for growth. The two size groups in this experiment were cultured at the same density (as number of oysters per bag) and location; thus, fast growing oysters may reflect a greater efficiency of the conversion of consumed energy to tissue, faster rates of feeding, or reduced energy requirements for maintenance metabolism (Fig. 5) (Hawkins and Day 1996). For example, in *Triostrea chilensis*, the net absorption efficiency increases with individual size, explaining at least part of the size differences found within the same cohort (Vergara et al. 1992). On balance, the relation between heterozygosity and growth rate is usually a modest one ( $r^2 = 0.05$ ); therefore, the association between heterozygosity and fitness traits remain to be determined (Gaffney 1996).

The proportion of total production expended on gamete output

in the Japanese oyster (*C. gigas*) rose from 18% in 1-year-old oysters to 84% in the adults (Heral 1989). Hence, as the oyster grows, most of the energy derived from ingestion is partitioned to reproductive processes (Dame 1976, Heral 1989, Thompson et al. 1996), so differences in meat contents are mainly related to differences in reproductive tissue production. This leads logically to the conclusion that fast-growing oysters are able to invest more in reproductive activity and that this may confer fitness advantages. Because fitness can be broken into two major components, the total number of offspring produced and the quality of these offspring (Falconer and Mackay 1996), it seems that large size oysters have greater reproductive success.

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## USE OF L-DOPA AND SOLUBLE BACTERIAL PRODUCTS TO IMPROVE SET OF *CRASSOSTREA VIRGINICA* (GMELIN, 1791) AND *C. GIGAS* (THUNBERG, 1793)

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**ABSTRACT** Solutions of L-dihydroxyphenylalanine (L-DOPA) and culture supernatants of the marine bacterium *Shewanella colwelliana* were used to treat competent *Crassostrea* larvae before transferring them to setting tanks. Setting trials were done in laboratory containers and on a larger scale in a Chesapeake Bay hatchery. Hatchery results showed a consistent and repeatable improvement in set of *C. virginica* after treatment with either L-DOPA or bacterial supernatants. Laboratory results were more variable, and differences between treatments were less pronounced. Pretreatment of larvae with theophylline led to a more rapid onset of settlement behavior in L-DOPA treatments but not in supernatant treatments, and had no clear effect on over-all set on cultch.

**KEY WORDS:** *Crassostrea gigas*, *Crassostrea virginica*, oyster set, set cues, bacteria, *Shewanella colwelliana*, L-DOPA, theophylline

### INTRODUCTION

Microbial surface films and their products are known to mediate the settlement and metamorphosis of a variety of marine invertebrates (Mitchell 1984, Maki and Mitchell 1986). Specific bacterial products have been implicated in the induction of settlement and morphogenesis of scyphozoans (Neumann 1979), polychaete worms (Kirchman et al. 1982a, Kirchman et al. 1982b), bryozoans (Brancato and Woollacott 1982), sea urchins (Cameron and Hinegardner 1974), and oysters (Weiner et al. 1985, Weiner et al. 1989). Metamorphosis of the red abalone, *Haliotis rubescens*, is induced by metabolic products of the crustose coralline algae on which they set (Morse et al. 1979). Inductive factors may be such soluble compounds as peptides or protein conjugates (Fitt et al. 1987, Siddall 1982) or fatty acids (Pawlik 1986), or they may be such insoluble components of the microbial film itself as polysaccharides or glycoproteins (Kirchman et al. 1982b). Whether larvae that respond to bacterial products require direct contact with microbial films or sense soluble cues released from them is not known for most species.

Previous studies have shown that larvae of *Crassostrea virginica* and *C. gigas* undergo settlement behavior and metamorphosis in response to the presence of the neurotransmitter precursor L-3,4-dihydroxyphenylalanine (L-DOPA) (Coon et al. 1985, Coon et al. 1990a). Settlement and metamorphosis of these oysters also is enhanced by compounds produced by the marine bacterium,

initially classified as *Alteromonas colwelliana* (Weiner et al. 1988) and now classified as *Shewanella colwelliana* (Coyne et al. 1989), which was isolated from oyster setting tanks (Weiner et al. 1985, Weiner et al. 1989). One or more soluble cues, as well as an extracellular polysaccharide component of *S. colwelliana* films seem to be involved in this process, but the exact mechanism of induction by bacterial products is not yet clear (Weiner et al. 1989, Bonar et al. 1990). Fitt et al. (1989) demonstrated that oyster larvae are able to respond behaviorally and complete metamorphosis after exposure to culture media supernatants of several species of bacteria, including *S. colwelliana*.

There has been much speculation about the biotechnological potential of controlling specific microbial-invertebrate interactions in aquaculture (Bonar et al. 1986). Use of exogenous microbial or "natural" cues to enhance settlement or growth of such economically valuable species as oysters could contribute significantly to increased fisheries production. Until now, however, successful use of microbial products to improve shellfish yields has not been commercially utilized.

In this report, we extend laboratory findings (Coon et al. 1985, Walch et al. 1989, Bonar et al. 1990, Fitt et al. 1990) to the hatchery scale and demonstrate that exogenous inducers of oyster settlement behavior can, in fact, be used to increase spat set. We have used solutions of L-DOPA or *S. colwelliana* supernatants to treat competent larvae before transferring them to setting tanks. Setting trials were done on a small scale in laboratory containers as well as on a larger scale in a Chesapeake Bay hatchery. The potential and limitations of this technology for use in oyster hatcheries is discussed.

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## MATERIALS AND METHODS

### Oyster Larvae

Competent larvae of the Eastern oyster, *Crassostrea virginica*, were obtained from the Maryland Department of Natural Resources (DNR) oyster hatchery at Deal Island, Maryland. Larvae of the Pacific oyster, *Crassostrea gigas*, were obtained from the Coast Oyster Company in Quilcene, Washington. Larvae used in the *in vitro* experiments were maintained in the laboratory, employing procedures described by Coon et al. (1990a).

### Chemicals

L-3,4-dihydroxyphenylalanine (L-DOPA) and theophylline were obtained from Sigma Chemical Co. (St. Louis, Missouri). L-DOPA was dissolved in 0.005 N HCl at a concentration of  $10^{-3}$  M prior to use, then was diluted with 0.2- $\mu$ m filtered seawater to  $10^{-4}$  M or  $10^{-5}$  M for treatment of the larvae. Theophylline ( $10^{-3}$  M) was dissolved directly in seawater.

### Bacterial Supernatants

The bacterial species *Shewanella colwelliana*, strain LST-D (Weiner et al. 1985, Weiner et al. 1989b), was grown at 25°C in 500 mL Marine Broth 2216 (Difco) in 2-L baffled flasks on a rotary shaker. Cultures were harvested in the late logarithmic stage of growth (2 days), centrifuged at 10,000 g for 15 min, and the supernatants were frozen for use in larval treatments and behavioral assays.

### Behavioral Assays

Assays of settlement "search" behavior (Coon et al. 1985) were performed in Costar 24-well tissue culture plates as described by Coon et al. (1990a). Twenty to 40 larvae were placed in each well with 500  $\mu$ l of 0.45  $\mu$ m filtered seawater (15‰ salinity for *C. virginica*, 30‰ for *C. gigas*). Bacterial supernatants or L-DOPA were added to the wells in varying concentrations, and the number of larvae swimming or crawling with the foot extended during a 30-s period was recorded at 5 to 10-min intervals for up to 1 h. Results are expressed as the mean percentage behavior of three replicates.

### In Vitro Setting Experiments

Clean, 8-inch diameter glass bowls were prepared with 1 liter of filtered seawater, at the appropriate salinity, and equal numbers of aged *C. virginica* shells were selected to be approximately equal in size (5  $\times$  8 cm) and relatively flat. They were washed lightly with a clean brush and tap water before use.

Competent larvae were treated in beakers containing 500 to 1,000 larvae in 500 mL of seawater with the following additions: (1) control, no additions; (2) L-DOPA,  $10^{-4}$  M (10 min); (3) L-DOPA,  $10^{-5}$  M (30 min); (4) theophylline,  $10^{-3}$  M (5 min), and L-DOPA,  $10^{-4}$  M (additional 10 min); (5) *S. colwelliana* supernatant, 25% (10 min); and (6) theophylline,  $10^{-3}$  M (5 min), and *S. colwelliana* supernatant, 25% (additional 10 min).

After treatment for the specified length of time, the larvae were sieved out of the beakers, rinsed with seawater, and distributed as evenly as possible into the prepared bowls. The bowls were covered and left at 20°C for 4 days. The number of spat set on each shell was counted with a dissecting microscope.

In another experiment, four 5-gallon glass aquaria were prepared containing 1- $\mu$ m filtered seawater and clean oyster shell cultch in nylon mesh bags (100 similarly sized and shaped shells per tank). *C. gigas* larvae were treated, as described above, in beakers containing 500 mL seawater, with the following additions: (1) control, no additions; (2) L-DOPA,  $10^{-4}$  M (10 min); (3) theophylline,  $10^{-3}$  M (5 min), and L-DOPA,  $10^{-4}$  M (additional 10 min); and (4) L-DOPA,  $10^{-5}$  M (30 min). Treated larvae were transferred to the aquaria (final density 3–4 larvae per mL). Tanks were aerated gently, and larvae were fed during set. The number of spat on each shell was counted after 4 days.

### Hatchery Setting Experiments

Hatchery trials were conducted at the Maryland DNR Oyster Hatchery at Deal Island, Maryland. Setting was done in 2.4  $\times$  1.2  $\times$  0.3-meter wooden trays containing coarse-filtered, aerated bay water. *C. virginica* larvae were treated before set in the manner described above and distributed as evenly as possible into prepared setting tanks. Approximately 500,000 larvae were added to each tank, giving a concentration of about 40 larvae per Liter. Larvae were fed cultured *Isochrysis galbana* regularly during set.

In the first set of hatchery trials, 300–400 clean, similarly sized and shaped aged shells were arranged interior side up in a monolayer on the bottom of each setting tray. Larvae were treated as follows and distributed into separate tanks.

#### Experiment 1

- (1) control, no treatment
- (2) theophylline,  $10^{-3}$  M (5 min), and L-DOPA,  $10^{-4}$  M (additional 10 min)

#### Experiment 2

- (1) Control, no treatment
- (2) Theophylline,  $10^{-3}$  M (5 min), and L-DOPA,  $10^{-4}$  M (additional 10 min)
- (3) *S. colwelliana* supernatant, 25% (10 min)

After 4 days, the shells were removed from the tanks and washed gently under a stream of seawater to remove unattached larvae. The number of spat on all shells was counted.

The following summer, an additional experiment was conducted using clean, aged oyster shell cultch in nylon mesh bags (100–150 shells per bag), which more nearly mimicks routine hatchery practices. Larvae were treated with  $10^{-4}$  M L-DOPA for 10 min and distributed in one of the setting tanks. Untreated larvae were added to the second tank as a control. After set, the cultch were placed in a growout raceway. Spat on both sides of 120 random shells from three bags in each tank were counted after about 4 weeks.

## RESULTS

### Behavioral Assays

$10^{-4}$  M L-DOPA induced settlement behavior within 20 min in nearly 100% of *Crassostrea* larvae.  $10^{-5}$  M L-DOPA also induced behavior, but more slowly and at a lower level (70% of larvae behaving after 30 min) (Fig. 1A). Treatment of larvae for 10 min with various concentrations of bacterial supernatants also induced a high level of settlement behavior (Fig. 1B). Behavior was sus-



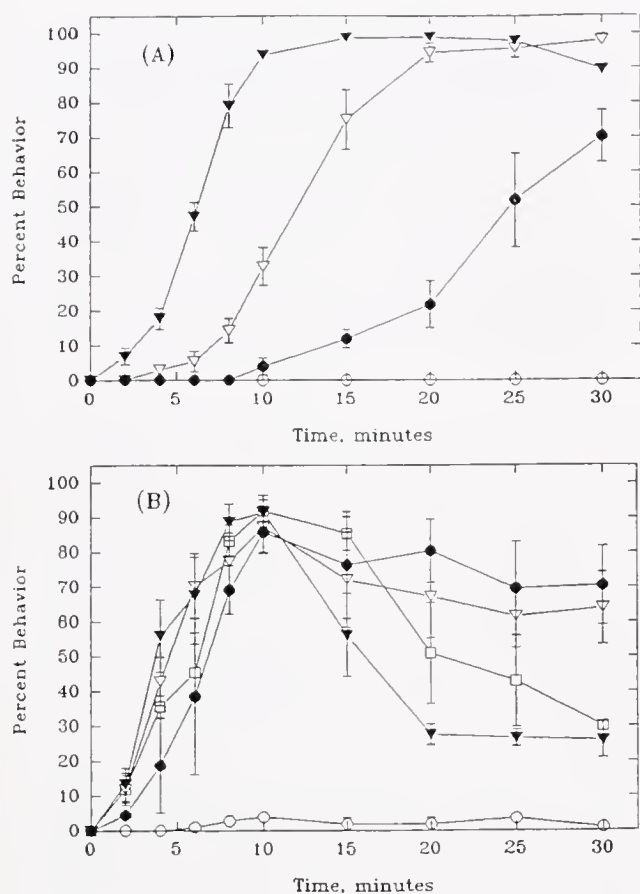


Figure 1. Induction of settlement behavior in *Crassostrea gigas* larvae by L-DOPA and bacterial supernatants. (A) L-DOPA treatments: seawater control (open circles),  $10^{-5}$  M L-DOPA (filled circles),  $10^{-4}$  M L-DOPA (open triangles), theophylline +  $10^{-4}$  M L-DOPA (filled triangles). (B) Bacterial supernatant treatments: theophylline control (open circles), 12.5% supernatant (filled circles), 25% supernatant (open triangles), 50% supernatant (filled triangles), theophylline + 25% supernatant (open squares).

tained over the longest period when the supernatants were diluted to 25 or 12.5%.

Pretreatment of larvae for 5 min with  $10^{-3}$  M theophylline caused them to respond faster to L-DOPA. Maximum settlement behavior was observed in theophylline-treated larvae at least 10 min sooner than in those treated with  $10^{-4}$  M L-DOPA alone (Fig. 1A). Theophylline pretreatment had no significant effect on settlement behavior induction by bacterial supernatants (Fig. 1B).

#### In Vitro Setting Experiments

Results of small-scale setting experiments in glass bowls are shown in Table 1. For both *C. virginica* and *C. gigas*, the mean spat set for all treatments were higher than the controls. In the case of *C. gigas*, these means were found not to be significantly different [ $p > .05$ , single classification analysis of variance (ANOVA)]. Treatment of *C. virginica* larvae, however, did result in significant differences in spat set ( $p < .001$ ). The largest increase in spat set was obtained by treatment with  $10^{-5}$  M L-DOPA for 30 min.

When *C. gigas* larvae were set on cultch in glass aquaria, again, the means for spat set were higher in all treatments than in the

TABLE 1.

Results of *in vitro* setting experiments in glass bowls.

Larval Treatment	Time	Spat per Shell (mean $\pm$ SE, n = 6)	
		<i>C. virginica</i> *	<i>C. gigas</i> †
Control	10 min.	2.0 $\pm$ 0.8 <sup>a,b</sup>	68.0 $\pm$ 23.1
$10^{-4}$ M L-DOPA	10 min.	14.0 $\pm$ 3.3 <sup>a</sup>	102.3 $\pm$ 19.8
$10^{-5}$ M L-DOPA	30 min.	70.3 $\pm$ 19.7	120.0 $\pm$ 41.8
Theophylline + $10^{-4}$ M L-DOPA	5 min. + 10 min.	6.8 $\pm$ 4.0 <sup>a,b</sup>	122.3 $\pm$ 29.6
<i>S. colwelliana</i> supernatant	10 min.	15.5 $\pm$ 3.8 <sup>a</sup>	69.5 $\pm$ 16.5
Theophylline + <i>S. colwelliana</i> supernatant	5 min. + 10 min.	24.2 $\pm$ 6.0 <sup>a,b</sup>	99.5 $\pm$ 26.7

\* Treatment means with the same superscript letter are not significantly different.

† No treatment means for *C. gigas* are different from the control.

control (Table 2). ANOVA showed only the  $10^{-4}$  m DOPA treatment to be significantly better than the control ( $p < .05$ ). The three larval treatments were not significantly different from one another.

#### Hatchery Setting Experiments

Results of hatchery setting trials conducted during both seasons demonstrated that treatment of larvae with L-DOPA, with theophylline and L-DOPA, or with *S. colwelliana* supernatants significantly improved set of *C. virginica* on cultch (Fig. 2, Table 3). In the experiment done the second summer, in which larvae were treated for 10 min with  $10^{-4}$  M L-DOPA and set on bagged cultch, the treatment increased set by about 30% over untreated controls ( $p < .05$ , Mann-Whitney U-test) (Fig. 2). This figure also shows that the outside surfaces of the cultch consistently had more spat on them than the interior surfaces. This was true in nearly all of our setting experiments.

Setting trials conducted the first season demonstrated very significant increases in spat set. Normal spat set that summer was quite low, with cultch in control tanks (no larval treatment) having means of less than one spat per shell and a maximum of 10 per shell (Table 3). In other tanks containing the same cohorts of larvae treated with theophylline and  $10^{-4}$  m L-DOPA for 10 min, mean spat set was  $\geq 12$  per shell, with some shells having more than 100 spat (Table 3, expt. 2). Treatment of larvae with a 1:3 dilution of 2-day-old *S. colwelliana* culture supernatant led to an increase in spat set nearly identical to that of the L-DOPA treat-

TABLE 2.

Results of *in vitro* *C. gigas* set on cultch bags in aquaria.

Larval Treatment	Spat per Shell (Mean $\pm$ SE, n = 100)*
Control	19.3 $\pm$ 1.9 <sup>a</sup>
$10^{-4}$ M L-DOPA	28.0 $\pm$ 2.2 <sup>b</sup>
$10^{-5}$ M L-DOPA	24.6 $\pm$ 2.3 <sup>a,b</sup>
Theophylline + $10^{-4}$ M L-DOPA	23.5 $\pm$ 2.2 <sup>a,b</sup>

\* Treatment means with the same superscript letter are not significantly different.

ment (Table 3, expt. 2). These increases were statistically significant ( $p < .01$ , Mann-Whitney U-test).

### DISCUSSION

Many environmental factors have been shown to influence oyster settlement and metamorphosis, including naturally occurring chemicals isolated from oyster habitats or from oyster tissues themselves (Crisp 1967, Degens et al. 1967, Veitch and Hidu 1971, Waite and Anderson 1980, Bonar et al. 1989). A distinction can be made between factors that promote settlement (a reversible behavior) and those that induce metamorphosis (irreversible morphogenesis) (Bonar et al. 1990). Coon et al. (1985) have shown that L-DOPA induces settlement behavior in oyster larvae. The response consists of swimming with the foot extended and crawling on the substratum. The relationship of L-DOPA to the presumed natural inducer is unknown, but it consistently induces a sequence of settlement behaviors in >90% of competent *Crassostrea* larvae (Coon et al. 1985, Coon et al. 1990a, and Table 1).

It also has been demonstrated that one or more soluble factors produced by certain marine bacteria cue oyster larval search behavior (Fitt et al. 1989, Weiner et al. 1989b, Fitt et al. 1990). Surface films of one particular bacterium, *S. colwelliana*, are especially active in promoting set on a variety of substrata (Weiner et al. 1985, Weiner et al. 1989b). Studies in our laboratories have shown that a number of metabolites produced by *S. colwelliana*; that is, ammonia and L-DOPA, induce settlement behavior in *Crassostrea* larvae (Dagasan and Weiner 1988, Walch et al. 1989, Weiner et al. 1989b). The major inducer present in *S. colwelliana* laboratory culture supernatants has been shown to be ammonia (Coon et al. 1990b, Walch et al. in preparation). A model of oyster set proposes that these soluble cues induce competent larvae to drop to the substratum and begin sampling the surface, looking for an appropriate settlement site. Secondary cues associated with the surface, such as specific exopolysaccharides, are required for ce-

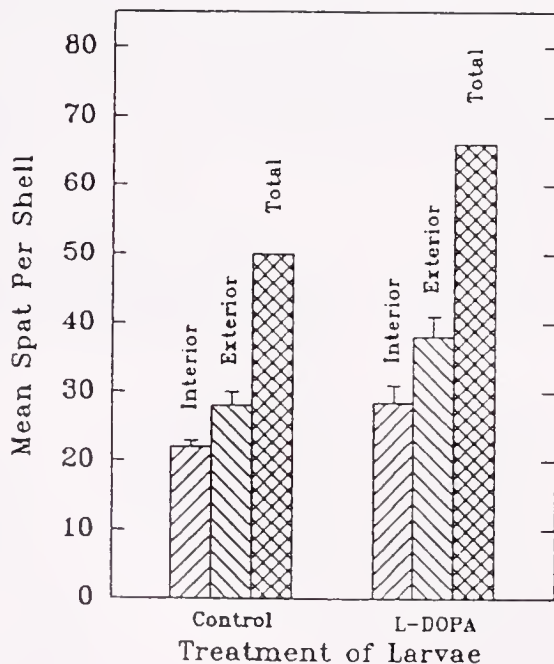


Figure 2. Results of the hatchery setting experiment evaluating the effect of L-DOPA treatment on spat set using shell cultch in bags. Data represent mean no. spat per shell  $\pm$  SE ( $n = 120$ ).

TABLE 3.

Results of hatchery setting experiments evaluating set of *C. virginica* on cultch after pretreatment of larvae, numbers represent mean no. spat per shell  $\pm$  SE ( $n$ ).

	Experiment #1*	Experiment #2*
Control	0.2 $\pm$ 0.04 (380) <sup>a</sup>	0.6 $\pm$ 0.08 (343) <sup>a</sup>
Theophylline + 10 <sup>-4</sup> M DOPA	2.1 $\pm$ 0.8 (377) <sup>b</sup>	12.2 $\pm$ 1.2 (345) <sup>b</sup>
<i>S. colwelliana</i> supernatant	—	16.0 $\pm$ 2.1 (309) <sup>b</sup>

\* Treatment means with the same superscript letter within an experiment are not significantly different.

mentation and metamorphosis (Bonar et al. 1990, Weiner et al. 1989a, Weiner et al. 1989b). The effectiveness of such surface cues may be enhanced by such additional bacterial products as homogentisic acid (Coon et al. 1994, Weiner et al. in preparation).

Given that L-DOPA and soluble bacterial metabolites induce settlement behavior in oyster larvae and that this behavior normally is a prerequisite for cementation and metamorphosis, it was hypothesized that exposure of larvae to these chemicals would increase total spat set. Our results confirm this, at least for the conditions described here. Other studies have shown that L-DOPA induces metamorphosis in *C. gigas* (Henderson 1981, Cooper 1983, Coon et al. 1985). In general, however, they have failed to demonstrate increases in percentage set on cultch surfaces. Cooper (1983), for example, found that, although L-DOPA treatments consistently increased the percentage of larvae completing metamorphosis (20–30% increase relative to controls), many of these larvae underwent morphogenesis without attachment, sometimes resulting in a net decrease in total spat set on the cultch.

Results of hatchery experiments described here show a consistent and repeatable improvement in set of *C. virginica* after treatment with either L-DOPA or bacterial culture supernatants. Laboratory results were more variable, and differences between treatments were, in general, less pronounced. Differences between treatments also tended to be greater for *C. virginica* than for *C. gigas*; this may be related to our experience that *C. gigas* normally has a higher percentage set than *C. virginica*. The response of larvae to soluble inducers has been shown to vary considerably with larval age and condition as well as a number of environmental factors (Cooper 1983, Coon et al. 1990a, Fitt et al. 1990). This undoubtedly contributed to the variability inherent in our own experiments, as well as differences between our results and those of other researchers. In general, improvement of set was most dramatic when the larvae were otherwise setting poorly, as was the case with *C. virginica* during the summer in which the first set of trials was conducted (Table 3). Although *C. virginica* and *C. gigas* react qualitatively the same to soluble cues (Coon et al. 1990a), our studies have consistently shown that *C. virginica* did not set as well as *C. gigas* in any experimental regime (Weiner et al. 1989b).

In the hatchery trials described here, a single concentration of L-DOPA (10<sup>-4</sup> M) was used for larval treatments. Laboratory studies have indicated that, although larvae respond to 10<sup>-5</sup> M DOPA more slowly and in somewhat lower numbers (Fig. 1A), they tend to spend more time exhibiting settlement behavior than those exposed to higher concentrations. This may be an advantage in hatchery setting systems, resulting in more even and consistent set throughout the cultch bags.

Behavioral assays (Fig. 1A) demonstrate the rationale for pretreating larvae with theophylline before exposing them to L-

DOPA. Theophylline is a caffeine analog known to inhibit the enzyme adenylate cyclase, resulting in higher intracellular pools of cyclic AMP. Cyclic AMP may be involved in mediating the L-DOPA-induced behavioral response in *Crassostrea*, but this has not been proved (Bonar et al. 1990). Our results showed that pre-exposure of larvae to theophylline caused a much faster behavioral response relative to L-DOPA treatments alone (Fig. 1A). Furthermore, we observed that the theophylline-treated larvae were "stickier"; they spent much more time crawling than those treated only with L-DOPA. Despite the positive effect on settlement behavior, we were surprised that comparisons of theophylline treatments and L-DOPA controls failed to demonstrate any significant differences in total set (Tables 1 and 2), although casual observations revealed substantially more nonspecific set on the bottoms of the tanks when larvae were treated with theophylline before L-DOPA.

It is interesting to note that theophylline did not have a significant effect on the behavior of larvae exposed to *S. colwelliana* supernatants (Fig. 1B). The behavioral response of *Crassostrea* larvae to bacterial culture supernatants and to L-DOPA seem to be similar, but the primary inducer in the supernatants away from the biofilms has been shown to be ammonia rather than L-DOPA. Whereas L-DOPA-induced settlement behavior is a receptor-mediated response and may involve cAMP as a second messenger,

ammonia-induced behavior seems to be a nonspecific response to intracellular pH changes (Bonar et al. 1990, Coon et al. 1990b) that would not be sensitive to cAMP levels.

The results reported here indicate that treatment of competent oyster larvae with exogenous inducers of settlement behavior may be a useful technology for improving spat yields in hatcheries. This is the first report of the use of bacterial metabolites to increase set in the field. Current work focuses on optimization of treatment conditions and evaluation of growth and survival of the treated spat. Work is also in progress to improve the effectiveness and yield of soluble bacterial inducers through biotechnological approaches.

#### ACKNOWLEDGMENTS

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## GROWTH AND BIOCHEMICAL COMPOSITION OF *CRASSOSTREA GIGAS* (THUNBERG) AND *OSTREA EDULIS* (LINNÉ) IN TWO ESTUARIES FROM THE NORTH OF PORTUGAL

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**ABSTRACT** Oysters *C. gigas* and *O. edulis* were grown in hanging cultures at river Lima estuary and Ria of Aveiro, in the north of Portugal. Growth and biochemical composition were recorded between May 1990 and October 1991. Temperature, salinity, and particulate matter in the water, at both stations, were monitored at the same time. No differences were found in the environmental parameters between stations, although the oysters presented some differences, mainly in biochemical composition. *C. gigas* at both locations showed some shell thickening, evaluated through the shell thickness index (STI). The presence of butyltin compounds in the water seems to be the most reasonable explanation for the anomalous thickening and the highest constraint to the development of commercial oyster farms. Both stations have good trophic conditions for culturing the two species.

**KEY WORDS:** biochemical composition, *Crassostrea gigas*, Lima estuary, *Ostrea edulis*, oyster culture, Ria of Aveiro, TBT

### INTRODUCTION

Oyster production in Portugal was very important at the end of 1960s, especially that of the *Crassostrea angulata* species, known as the Portuguese oyster. This species, which is very similar to the Japanese oyster *Crassostrea gigas* (their hybrids originate from similar progeny) (see review by Gaffney and Allen 1993), was intensively exploited, mainly at the river Sado estuary, reaching a record number of exports at 10,448 tons in 1968 (Vilela 1975). In the 1970s, sanitary problems and an increase in industrial pollution at the river Sado estuary resulted in a dramatic decrease in Portuguese oyster stocks. Some years before, similar condition affected oysters from river Tejo estuary.

At the same time, in France, high mortalities in the Portuguese oysters, caused by gill disease (which still persists), led to a crisis in the French oyster industry (the largest in Europe). In Portugal, oyster production practically disappeared, but in France, oyster culture quickly recovered, with the introduction of *C. gigas* from Japan.

*C. gigas* is being cultured in increasing numbers worldwide. The attraction of this species is its efficiency as a filter feeder, its fast growth rate, and its tolerance of a wide range of physical conditions, such as temperature, salinity, and silt load in the water (Quayle 1969, Bardach et al. 1972, Shpigel and Blaylock 1991).

On the north coast of Portugal, natural stocks of the European oyster (*O. edulis*, Linné) can be found in some areas where there is potential for cultivation of both *O. edulis* and *C. gigas*. Sheltered coastal areas allow aquaculture development; however, at these sites, several antropogenic substances are discharged. Tributyltin (TBT) is one of the most toxic substances introduced into the marine environment. It is the main active component in organotin-based antifouling ship paints (Smith and Smith 1975, Zuckerman et al. 1978). Organotins are primary cause of oyster shell deformations (chambering and thickening), first reported in *C. gigas* at the Arcachon bay, France by Alzieu et al. (1980) and Alzieu et al. (1982). The same deformations were also observed by Waldock and Thain (1983) along the east coast of Britain.

Organotin compounds were suspected as being responsible for

anomalous shell thickening in the oysters from the Tejo estuary in Portugal (Andrea et al. 1983). Phelps (1993) studied TBT impact on *C. angulata* in some Portuguese estuaries, using the shell thickness index (STI) as indicator and noticed that oysters from the Sado estuary had high levels of TBT contamination.

The present study describes *C. gigas* and *O. edulis* growing performances, biochemical composition, and STI at two estuaries on the northern coast of Portugal between June 1990 and October 1991. Because local variations in water quality can significantly affect the life cycle and reproduction of oysters in coastal areas (Héral et al. 1984, Héral et al. 1987, Brown and Hartwick 1988), water temperature, salinity, and total particulate matter were also measured.

### MATERIALS AND METHODS

This experiment occurred at the Lima estuary and Ria of Aveiro in northern Portugal (Fig. 1). The Lima River has 2,480 km<sup>2</sup> hydrological basin. Its estuary has substantial ship repairing industries. The experimental system located at Lima estuary was a 15-m longline, stainless-steel cable hanging near the surface. Floatation was achieved by plastic barrels tied along the cable, which was anchored by cement blocks.

Ria of Aveiro is a seawater lagoon with a total area of 47 km<sup>2</sup> and a freshwater inflow of 3 to 60 m<sup>3</sup>/s<sup>-1</sup>, caused by seasonal precipitation and runoff phenomena. The experimental system at Aveiro was a small raft (2 × 2 m) of galvanized tubing with two plastic floats. The raft, anchored by cement blocks, was placed near the sea entrance, at Ria de Aveiro's main channel (Fig. 1).

At both stations, oysters were placed in pill-shaped baskets, as described elsewhere (Almeida et al. 1997). The experiment at Lima estuary was destroyed by a storm in September 1991.

Seawater was sampled every 2 weeks at each site. Temperature, salinity, and particulate matter were measured as described elsewhere (Almeida et al. 1997). Water current was measured with a current flowmeter BFM001.

*Crassostrea gigas* juveniles with a mean shell height of 20.1 ± 6.4 mm came from Marennes-Oleron (France) and were obtained from natural spatfall. *Ostrea edulis* juveniles with a mean of 33.1

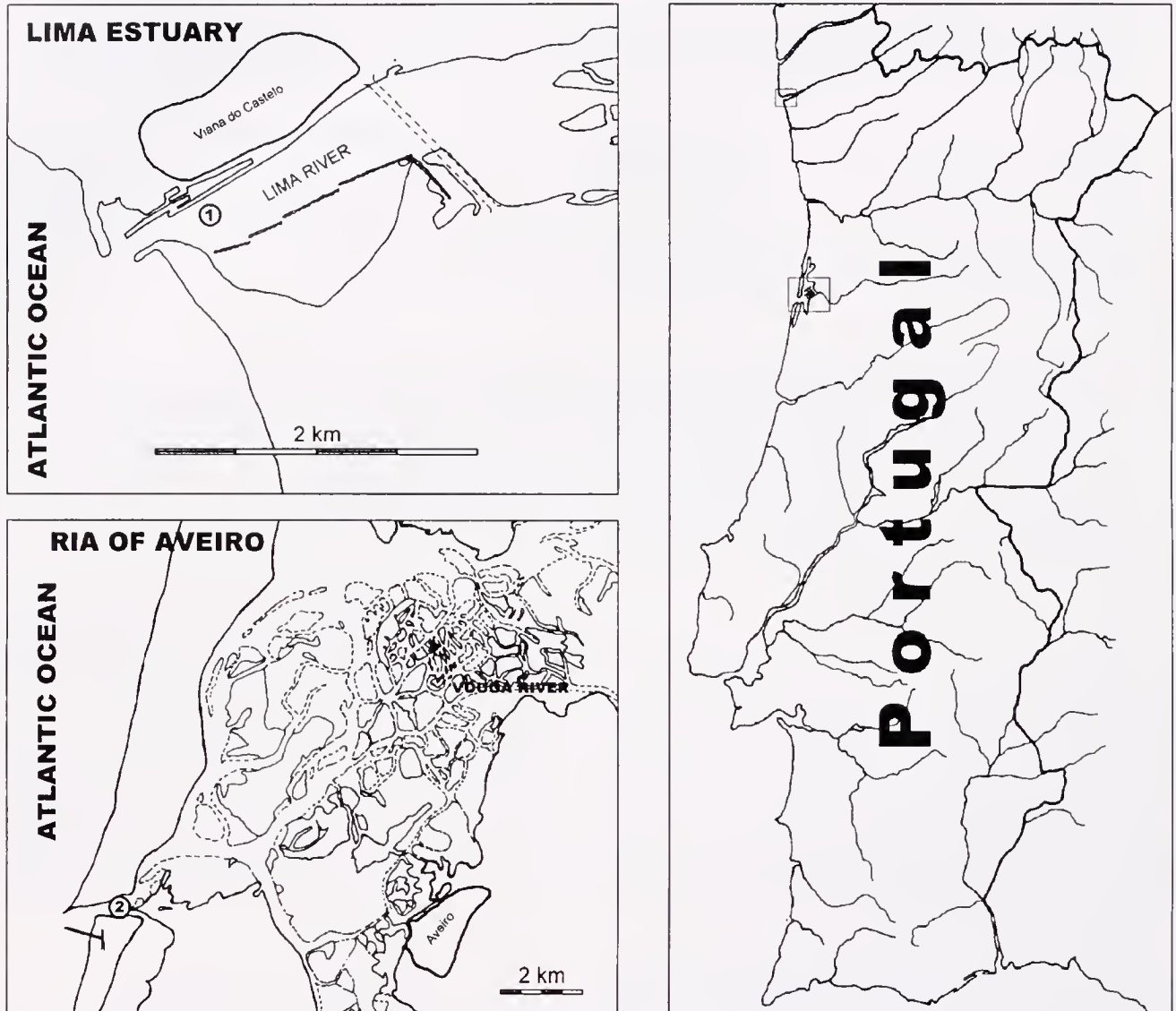


Figure 1. Location of the two stations: (1) Lima estuary; and (2) Ria of Aveiro.

$\pm 5.2$  mm length came from "Ostreira" a galician (Spain) hatchery. About 1,000 oysters, all from the same original stock, were placed at each site. Twice a month, all the baskets were agitated in the water to remove accumulated silt and feces. Every 45 days, a sample of 30 oysters were picked at random from each station for further analyses.

In the laboratory, oysters were scrubbed under running tap water to remove encrusting organisms. Linear dimensions of individuals were measured, and mean live weight was determined. Oysters were then opened and, tissues were excised. Wet meat weight was determined after superficial drying the extracted meat with absorbent paper. The flesh of 15 oysters were pooled and homogenized. Marine invertebrates in the field frequently have variable biochemical composition, and the use of pooled tissues from many individuals to determine the average composition may provide useful information (Giese 1967). Dry meat weight, ash, proteins, glycogen, and lipids were determined as described elsewhere (Almeida et al. 1997).

Monthly instantaneous growth rate ( $G_{30}$ ) was calculated as  $G_{30}$

$= \left( \frac{\log e(Z^{t+1}/Z^t)}{\log eD} \right) \times 30$ , where  $Z^{t+1}$  is the mean shell height (cm) of the current month,  $Z^t$  is the mean shell height of the previous month, and  $D$  is the number of days between observations (Ricker 1975). Condition index (CI) was calculated from the dry weights of meat and shell according to the formula  $CI = \text{dry meat weight (mg)}/\text{dry shell weight (g)}$  (Walne and Mann 1975).

Thickness of the superior valve in *C. gigas* was measured with vernier callipers, after being cut at its longest axis with an esmeril disk adapted to a minidriller. Shell thickness index was determined according to the formula  $STI = L/T$ , where  $L$  is the length of the superior valve, and  $T$  is its thickness. Dyrinda (1992) compared the three most common methods to determine the STI and suggested that STI reflects abnormal thickening with greater accuracy and is easier to use.

Analysis of variance (ANOVA) was performed using the STATISTICA 4.5 (Windows 95) statistical package. ANOVA assumptions were tested with Levene and Kruskal-Wallis tests. Particulate inorganic material (PIM) and particulate organic material (POM) data were transformed ( $\log x$ ). Arcsine transformation was

carried out on biochemical data, which were compared as percentages.

## RESULTS

### Environmental Conditions

Figure 2b shows seawater temperatures at both stations from May 1990 to October 1991. At station Lima, temperatures ranged from a minimum of 8°C in February to a maximum of 23°C in September of 1991. At station Aveiro, temperatures ranged from a minimum of 9.5°C in January of 1991 to a maximum of 21.5°C in May 1990. Mean temperatures at stations Lima and Aveiro during the experimental period were  $15.1 \pm 3.4$  and  $14.9 \pm 3.2$ °C, respectively. Salinity values also showed minor differences between stations (Fig. 2a). The mean salinity was  $33.1 \pm 3.6$  and  $33.1 \pm 3.9$  ppt ( $n = 35$ ) for Lima and Aveiro, respectively. The current flow varied between  $0.12\text{--}0.40\text{ m/s}^{-1}$  and  $0.07\text{--}0.84\text{ m/s}^{-1}$  at Lima and Aveiro, respectively.

POM values varied within  $1.0\text{--}9.5$  and  $1.0\text{--}10.0\text{ mg/l}^{-1}$  at stations Lima and Aveiro, respectively (Fig. 2c). The mean POM content was  $3.3 \pm 1.8$  and  $3.2 \pm 1.6\text{ mg/l}^{-1}$  ( $n = 35$ ) at Lima and Aveiro.

PIM values varied within  $2.0\text{--}51.0$  and  $4.3\text{--}70.3\text{ mg/l}^{-1}$  at Lima and Aveiro, respectively (Fig. 2d). Extreme seasonal maxima were recorded in October of 1991 at Lima and in May of 1991 at Aveiro. The mean PIM content was  $13.2 \pm 8.9$  and  $14.6 \pm 11.6\text{ mg/l}^{-1}$  at Lima and Aveiro.

### Oyster Growth

#### *Crassostrea gigas*

Mean live weight (g) for *C. gigas* at both stations, from May 1990 to October 1991 is plotted in Figure 3a. At the end of the experimental period, oysters had a mean weight of  $75 \pm 16$  and  $83 \pm 21$  g at stations Lima and Aveiro. Figure 3b presents the height

instantaneous growth rate for both stations and shows some fluctuations over the study period. Growth rate was higher during the first 4 months of culture. From November to April, the growing rate was zero, after which, the growth showed a small recovery.

Oysters at stations Lima and Aveiro reached a maximum dry tissue weight of 3.0 and 4.2 g, respectively, in July of 1991. After July and until the end of the experimental period, there was a decrease in dry tissue weight at both stations (Fig. 3c). Condition index values were very high in the summer of 1991 at station Aveiro, with a maximum of 85. At station Lima, the highest values were registered in June and July 1991, with a maximum of 103 (Fig. 3d).

Seasonal variations on ash content are similar in both stations, with values ranging from 9.4 to 15.0%. Meat water content is significantly lower ( $p < .05$ ) at station Lima than at station Aveiro (Fig. 3e). Mean percentages of meat water content are  $80.0 \pm 3.5$  and  $85.8 \pm 4.0$  at Lima and Aveiro.

Meat protein, carbohydrate, and lipid, as a percentage of the ash-free dry weight (% AFDW), are shown in Fig. 3, from January to October 1991. Lipid values at station Lima were more or less constant throughout the year. At station Aveiro, lipid percentage decreased from January to May with a minimum value of 1.0%, recovering to 8.5% in July (Fig. 3f). Mean lipid content at station Aveiro was significantly lower ( $p < .05$ ) than at station Lima. Carbohydrate content was also different throughout the summer, when comparing oysters from both stations. Although at station Lima, carbohydrate levels were high during the summer, with a maximum value of 28.8% in July; at station Aveiro, carbohydrate content was lower during the summer, with values around 6.4% (Fig. 3g). Protein content was more or less constant throughout the year, being significantly lower ( $p < .01$ ) at Lima. Mean protein content was  $62.3 \pm 3.8$  and  $70.0 \pm 3.4\%$  at stations Lima and Aveiro, respectively (Fig. 3h).

An STI of mean gravity (between 5 and 10) (Alzieu and Portmann 1984) was recorded in 30% of the oysters sampled at both

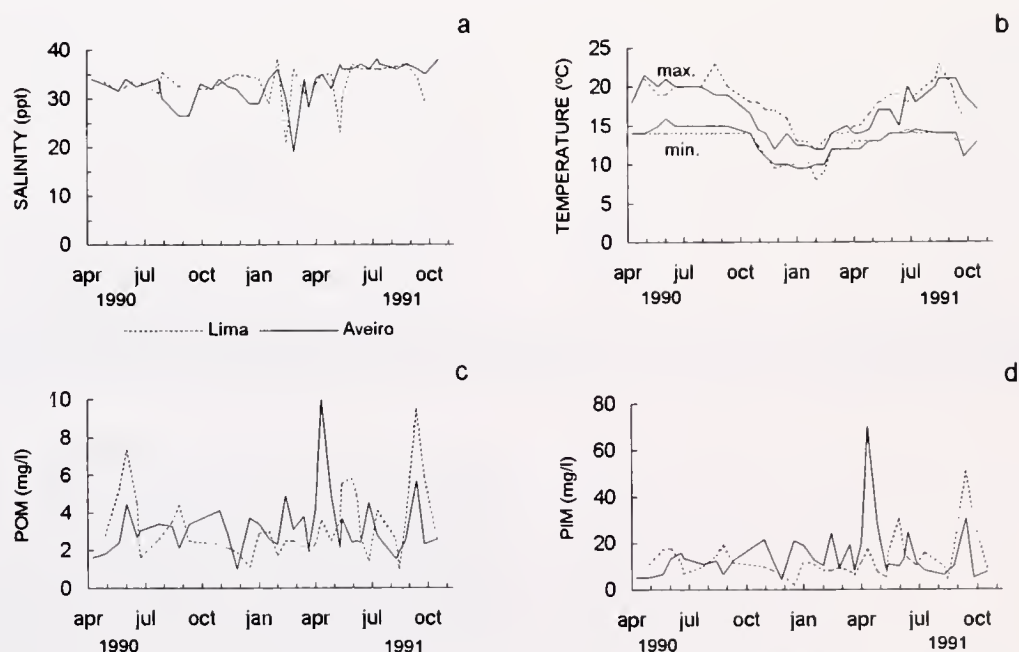


Figure 2. Seasonal variation in salinity: (a); maximum and minimum temperatures (b); particulate organic matter (POM) (c); and particulate inorganic matter (PIM) (d) at the two stations from May 1990 to October 1991.

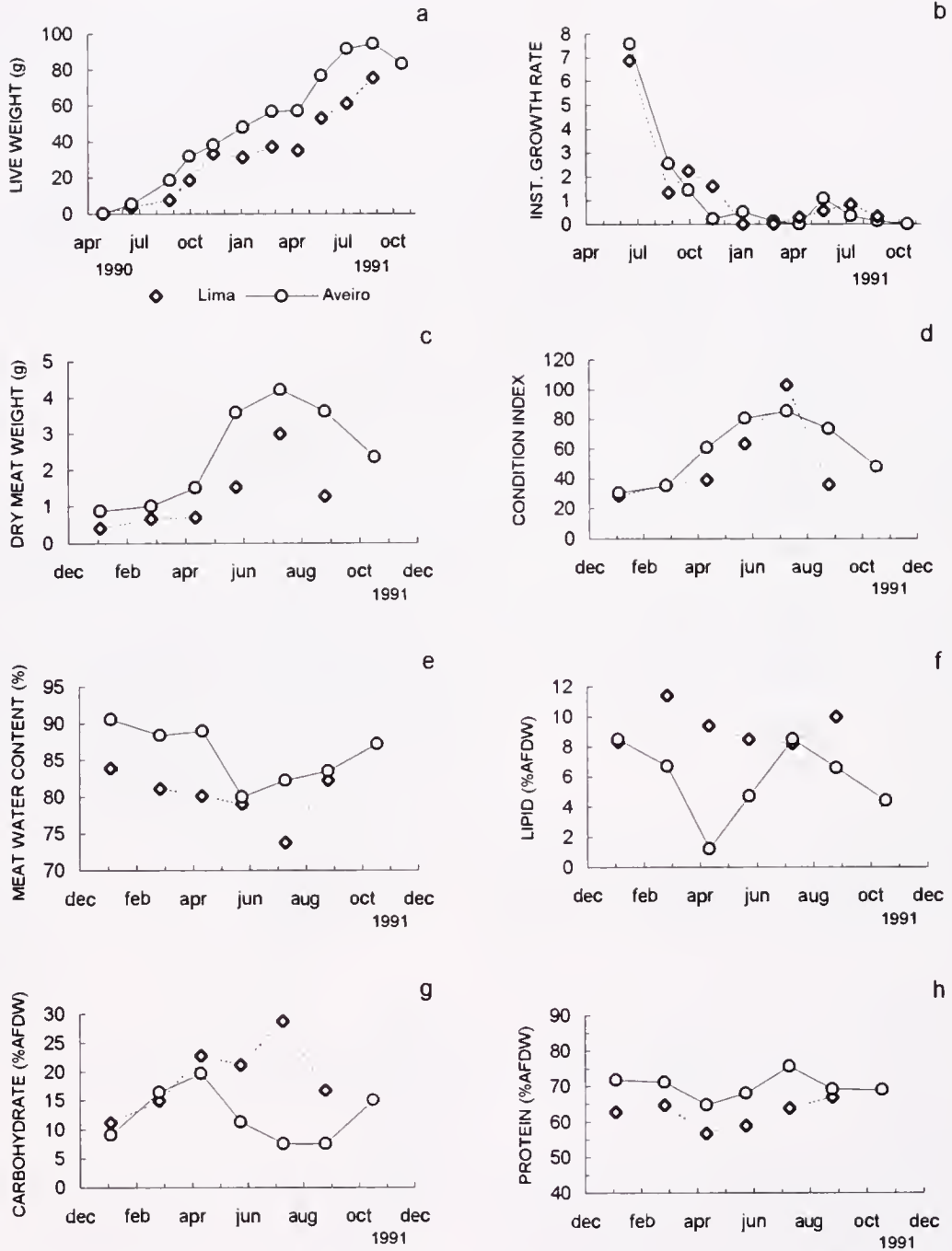


Figure 3. Seasonal variation on *C. gigas* live weight: (a): instantaneous growth rate in height ( $G_{30}$ ) (b); dry meat weight (c); condition index (d); meat water content (e); lipid as a percentage of the ash-free dry weight (AFDW) (f); carbohydrate (g); and protein (h) reared at Lima estuary and Ria of Aveiro.

stations. The remaining 70% showed an STI with low gravity or without gravity.

**Ostrea edulis**

Mean live weight for *O. edulis* at both stations is plotted in Figure 4a. At the end of the experimental period, oysters had a mean live weight of  $47 \pm 9$  and  $45 \pm 8$  g at stations Lima and Aveiro, respectively. Figure 4b presents the height instantaneous growth rate ( $G_{30}$ ) for both stations. Growth rate was high at station

Aveiro during the first 7 months. Between January and April, the growth rate was zero, showing a recovery between May and July of the second growing season. At station Lima, the period of no growth was between December and June.

Dry meat weight increased almost constantly between January and the end of the study at both stations (Fig. 4c). CI showed some differences between June and August 1991. At station Lima, values were high during this period, with a maximum of 66 in July. At station Aveiro, values were low, with a minimum of 25 in July. The maximum CI value reached at this station was 46 in April (Fig. 4d).



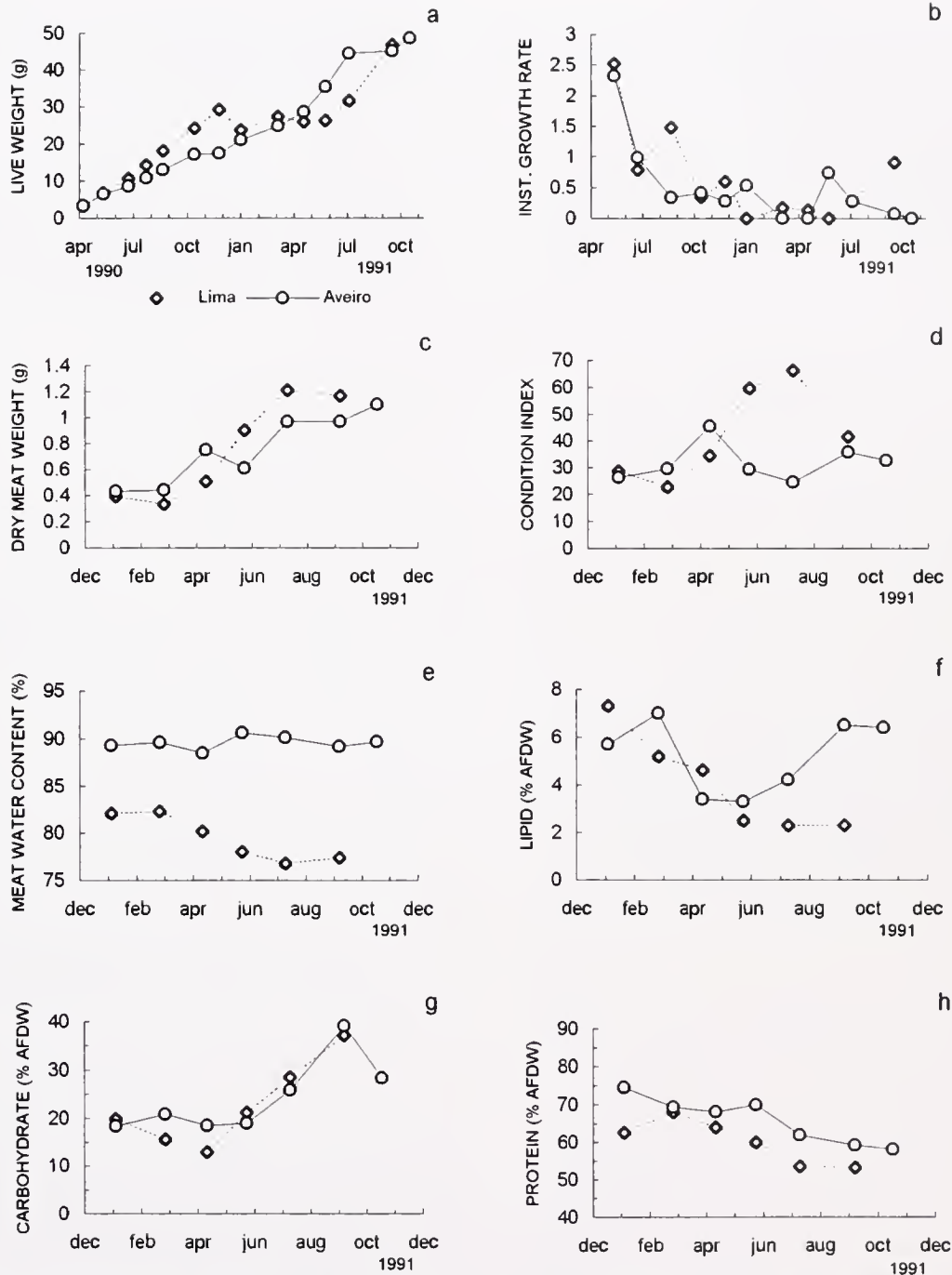


Figure 4. Seasonal variation on *O. edulis* live weight (a); instantaneous growth rate in height ( $G_{30}$ ) (b); dry meat weight (c); condition index (d); meat water content (e); lipid as a percentage of the ash-free dry weight (AFDW) (f); carbohydrate (g); and protein (h) reared at Lima estuary and Ria of Aveiro.

Seasonal variations on ash content were similar in both stations, except in June, when ash values were 9.2 and 16.7% at stations Lima and Aveiro, respectively. These were, at the same time, the lowest and highest values registered at both stations. Meat water content was significantly lower ( $p < .01$ ) at station Lima. Mean percentages of meat water content were  $79.5 \pm 2.4$  and  $89.6 \pm 0.7$  at Lima and Aveiro, respectively (Fig. 4e). Lipid values at station Lima decreased from January, with a mean of 7.3%, to the end of the experimental period, with 2.3%. At Aveiro, there was a de-

crease in lipid content in the spring and the beginning of summer, with a recovery at the end of summer and autumn (Fig. 4f). Carbohydrate values were very similar between stations, being more or less constant from January until June, with a maximum value of 37% in September (Fig. 4g).

Protein seasonal variation was similar between stations, decreasing from January to the end of the study. Mean protein content was  $60.2 \pm 5.9$  and  $65.9 \pm 6.1\%$  at stations Lima and Aveiro, respectively (Fig. 4h).

## DISCUSSION

None of the environmental parameters evaluated showed differences between stations. Nevertheless, some differences were observed between the oysters at Lima and Aveiro at the biochemical composition level. These differences were more accentuated in *C. gigas* than in *O. edulis*. One explanation for this could be the phytoplankton composition. The food value of different algal species has been evaluated for spat and juveniles of several bivalves (Enright et al. 1986, Laing and Millican 1986, Whyte et al. 1989, Whyte et al. 1990, Ferreiro et al. 1990). However, little is known about the effects of different food quality on growth of adult oysters. The relationship of bivalves to their food sources in the field is difficult to demonstrate, but seston quality and quantity have been related to growth and reproduction (Page and Hubbard 1987, Thompson and Nichols 1988, Utting 1988). Deslous-Paoli and Héral (1988) state that for *C. gigas*, stored energy, mainly as glycogen, is linked with the quality of food available during phytoplanktonic blooms in spring and autumn.

Bacteria content in the water could be another explanation for these differences. Oysters from the Lima estuary have a significantly higher bacterial content than oysters from Aveiro (unpublished data). This must be related to the lack of treatment of urban and industrial waters in this area. Bacteria are known to be one of the sources of bivalve diet (Brown et al. 1996).

*C. gigas* at station Lima has higher lipid and lower protein content than oysters from Aveiro. *C. gigas* from Aveiro show a typical pattern of lipid and carbohydrate variation related to the reproductive cycle. This pattern, in which transformation of glycogen reserves into lipids intervened for the formation of gametes (Gabbott 1976, Lubet 1976), could be seen during the spring. Such a definite pattern was not observed in oysters from Lima.

The variation in the meat water content was characterized by a decrease simultaneous with the build up of gametes and the accumulation of biochemical reserves. After the spawning period, meat water content began to increase. Curiously, meat water content was significantly lower in oysters from the Lima estuary, for both species. High water content is frequently associated with a low quality meat (Haven 1962, Shaw et al. 1967, Deslous-Paoli and Héral 1988), which is not the case. A minimum commercial weight of 60 g was attained in May and July 1991, more or less in 1 year of growth, in *C. gigas* from Aveiro and Lima, respectively.

Dry meat weight values at station Lima are similar to those presented by Deslous-Paoli and Héral (1988) at Marennes-Oleron (France), concerning 2-year-old oysters. Data from Ruiz et al. (1992a) at Ria de Arosa (Galicia, Spain) indicate similar environmental conditions to those found in this study. These authors observed, in oysters of the same species, a first cycle of gonad growth in spring, with spawning in June to July. After this first spawning season, there was a rapid recovery during summer, with a second gametogenic cycle. They found oysters with completely developed gonad in mid-October. This second gametogenic cycle was associated with the greatest annual phytoplankton blooms, at the end of summer and the beginning of autumn. At Aveiro, we noticed that chlorophyll *a* levels are high throughout the summer (unpublished data). This does not happen at Lima estuary. The second gametogenic cycle seems to reflect *C. gigas*' capacity to respond to the influence of seasonal phytoplankton blooms, as described by Lubet (1976). Data from Marennes-Oléron Bay (Atlantic French coast) indicate that oysters show only one dry weight maximum in July to August (Deslous-Paoli and Héral 1988), as observed in the oysters from Lima estuary.

*C. gigas* growth, in the two locations described here, was significantly higher than the growth of oysters in earthen ponds (Almeida et al. 1997). Open sites allow the oysters to filter greater water volumes and, consequently, to ingest greater amounts of food.

Shell thickness in *C. gigas* from both stations was probably caused by organotin compounds from antifouling paints used in boats. Both stations are located in channels with high maritime traffic, and near station Lima, there is also a ship-building yard. First indications of tributyltin effects in mollusks were from Pacific oysters, in the Atlantic French coast, near to recreational harbors (Alzieu et al. 1980). Studies on the effect of organotins in oysters indicated TBT as the agent responsible for shell thickening. This led to prohibition of the use of organotin compounds in boats smaller than 25 m in France and other European countries. *C. gigas* shell thickening is a readily and distinctly identifiable effect, which has been used with success in the biomonitoring of TBT in the UK, Ireland, France, the USA, Australia, and New Zealand (Alzieu et al. 1986, Stephenson et al. 1986, Wolniakowski et al. 1987, Batley et al. 1989, Ebdon et al. 1989, King et al. 1989, Minchin et al. 1996). Cortez et al. (1993) measured sediment contamination in butyltin compounds at Ria of Aveiro and found a degree of contamination considered to be a medium-high level at some sites. Butyltins were measured in the fine-grain fraction (<60  $\mu\text{m}$ ), which can be ingested by filter-feeding organisms and is easily resuspended and transported away.

Temperature and salinity levels, at both locations, prevalent during this study were compatible with *O. edulis* physiological requirements (Walne 1974, Mann 1979, Spencer 1988). Our growth rates on *O. edulis* were higher than those reported by Perez-Camacho and Beiras (1989) at Ria de Arosa and Cano and Rocamora (1996) at Mar Menor, in Spain, using spat with the same initial size.

A few *O. edulis* specimens collected in the summer were carrying larvae. The developmental zero value (the temperature below which no evidence of gonad development is found) is set around 7°C (Mann 1979, Wilson and Simons 1985). Both in Ria of Aveiro and the Lima estuary, the temperature never falls to a level low enough to interrupt gametogenesis. Similar observations were made by Ruiz et al. (1992b) for the Galician coast (Spain) and by Lubet (1976) for the Atlantic coast of France. Nevertheless, glycogen and lipid seasonal variation are not coincident with a gametogenic cycle. Abad et al. (1995) describe an increase in total lipid level throughout the gonad maturation period, in spring and early summer for adult *O. edulis* in the Galician coast. Oysters from Lima estuary show a decrease in lipid levels from the winter to the summer; whereas, at Aveiro, the highest lipid levels are in March and September through October.

In terms of glycogen, the seasonal variation at both sites is similar, with an increase from April until September. Walne (1970) describe a decline in the glycogen content during the breeding season.

The CI, observed throughout the experimental period for the European oyster, is considered low according to Walne (1974). Even in the summer months, the CI was below 70 in oysters from Lima estuary, those showing a higher CI (an average quality is indicated by an index  $\geq 80$ ). One possible explanation for the low performance of both populations can be the effect of TBT in the digestive gland. Axiak et al. (1995) exposed *O. edulis* to low levels of TBT (10 ng/l<sup>-1</sup>) and observed a significant digestive cell atrophy. In terms of shell morphology, we found no alterations in *O.*

*edulis*. No appreciable shell thickening was ever reported in the European oyster.

The only constraint to oyster culture at these locations was shell thickening of *C. gigas*. If a commercial oyster farming business is to be developed at these sites, a more detailed study about the effect of TBT compounds is needed because this poses serious problems for such endeavors.

The survey of Portuguese coastal environments demonstrates the occurrence of different degrees of butyltin contamination (Quevauviller et al. 1989, Cortez et al. 1993). The most contaminated sites are in enclosed bays or estuaries with high TBT inputs

from harbors and shipyards. The effect of high levels of contamination on molluscan species should encourage government action in Portugal to prohibit use of TBT-containing paints, as has been adopted in many other European countries.

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## FOUNDER EFFECT, GENETIC VARIABILITY, AND WEIGHT IN THE CULTIVATED PORTUGUESE OYSTER *CRASSOSTREA ANGULATA*

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**ABSTRACT** Existence of genetic variability is a prerequisite for successful implementation of breeding programs, and clarification of the relationships in such programs to quantitative traits is of great economic interest. We have studied the relationship between multilocus heterozygosity and/or allozyme genotypes and weight in the Portuguese oyster *Crassostrea angulata* (Lamarck). Two cohorts were obtained in a commercial hatchery by mass-spawning from wild oysters. Loss of genetic variability was shown in cultured oysters as compared with the wild population because of a founder effect caused by a low effective population size. Significant effects on growth rate were detected for the *Me-2*, *Xdh*, *Lap*, *Pgm*, and *Est* loci. However, these effects were not retained in the two cohorts, nor in the two ages of the same cohort, nor were differentiated effects detected in weight classes of the same age. At the same time, differences between genotypes were not associated with differences between heterozygous and homozygous genotypes. Positive correlations between multilocus heterozygosities and growth rate, as well as significant differences between mean body weights for different degrees of heterozygosity, were found only in the largest weight class. Moreover, significant results were obtained when the mean weight of different heterozygosity classes for total weight, body weight, and shell weight were compared only in the oysters selected for their larger size. This result points to the isozymes as markers for quantitative traits and confirms the existence of heterosis in *C. angulata*, indicating the possibility of establishing breeding programs based on the maintenance of inbred lines and crossing them to obtain hybrid vigor.

**KEY WORDS:** oysters, genetic variability, weight, *Crassostrea angulata*, isozymes

### INTRODUCTION

The commercially exploited *Crassostrea angulata*, also known as Portuguese oyster, is an oyster species naturally found on the coast of the southwest Iberian Peninsula, mainly in river mouths (Michinina and Rebordinos 1997). As natural populations became exhausted, the industry was forced to turn toward hatchery rearing of oysters. Nevertheless, some important aspects of shellfish hatchery practices must be considered, because they interact with the genetics of cultured species. In this sense, although progenitors from natural beds had been the source of seed for this culture, low effective population sizes caused the loss of rare alleles and a reduction in heterozygosity. Very often, oysters grown for hatchery seed are used as broodstock to produce the next generation, resulting in closed and small populations becoming propagated, which show the effects of inbreeding depression. In some cases, development of these populations is merely the result of general husbandry procedures. However, in other cases, rearing of populations from a reduced number of progenitors is intended to achieve some kind of genetic improvement in important traits (growth rate, healthy appearance, hard shells, etc.) without knowledge or evaluation of the validity of these programs that lack genetic controls.

The existence of genetic variability is a prerequisite for the successful application of breeding programs, and its estimation is easy for qualitative traits. In contrast, for quantitative traits, such an estimation is not trivial. However, quantitative traits are often more important for selective breeding. Therefore, it is important to determine the genetic variability of traits and clarify whether a relationship between traits can be established. In this way, the

genetic component of quantitative traits could be inferred from such markers as isozymes (Hedgecock et al. 1995).

Relationships between heterozygosities and/or allozyme genotypes and such quantitative traits as viability, growth rate, have been described for marine mollusks (Mitton and Grant 1984, Zouros and Pogson 1994). Nevertheless, there is no clear correlation between the degree of heterozygosity and characteristics related to fitness. In fact, results on the relationships with the growth and viability show that the studies reporting positive correlations only just outnumber those in which no correlations were found (Britten 1996). Some of these difficulties have been attributed to the use of unsuitable markers, because most of these studies were carried out using isozymes, but it is not completely established that allozymes (or at least some of them) are completely neutral markers.

Two main theories attempt to explain the positive correlations between the degree of heterozygosity and fitness. First is the overdominance hypothesis, which considers the enzyme variants as responsible for the correlation effect (Koehn et al. 1988, Sarver et al. 1992); and second is the associative overdominance hypothesis, which considers the scored loci as markers for those involved in the effect (Zouros and Mallet 1989).

Elucidating these two alternatives is important from the evolutionary point of view, but it also has clear relevance to breeding genetics. Our aims in this work are to compare the genetic variability within a wild population and some cultivated populations reared from the wild population, as well as to determine differential contribution to growth rate (determined as weight reached at a given time) in cultivated oysters (*Crassostrea angulata*). Genetic

data in natural populations of the Portuguese oyster are scarce (Michinina and Rebordinos 1997), and, as far as we know, there are no published reports relating quantitative traits to isozymic markers.

## MATERIALS AND METHODS

### Populations Sampled

Hatchery-propagated oyster stocks were maintained in the commercial hatchery AMALTHEA S.A. (Cádiz, Spain). A group of 20 adult oysters (selected for their large size and healthy appearance) were taken from a natural bed (wild population) located on the Atlantic coast of Cádiz on May 1993 and kept in a small flowthrough seawater system. Before spawning, some oysters were opened to check on gonadal maturation. Spawning was induced by raising the temperature from 20 to 28–30°C. Eggs were collected and placed in seawater at 25°C and then were counted and fertilized by adding several milliliters of dense sperm suspension; samples of the sperm-egg suspension were immediately examined microscopically, and, if necessary, more sperm were added to achieve a bound-sperm:egg ratio of about 10 (Dupuy 1974). Larval cultures were kept at a density of 10 larvae/mL for 10 days and then changed to two larvae/mL. After metamorphosis, oysters were transferred to tanks supplied with a flow of seawater.

Two cohorts were produced from the same progenitors. One was studied at 18 months old (18 m) and the other at both 8 and 12 months old (8 m and 12 m, respectively). During 4 months, oysters of the second cohort were subjected to overcrowded conditions to obtain harder shells, which are more valuable from the commercial point of view. The analysis at 12 months was performed on two different groups established by the hatchery, one of which was the group selected because of the large specimen size (12 mL), for additional growout, and the other contained the smallest samples (12 ms), which we chose to be discarded.

Once in the laboratory, oysters were numbered and weighed individually (total weight), then the shells were opened, the animal was removed, and weighed (body weight), and the shells were also weighed (shell weight). Before weighing, the samples were placed on filter paper to release most of the water contained within the shells.

A sample of 62 individuals from the natural bed was also collected, and upon arrival in the laboratory, the animals were removed from their shells and tissue samples (hepatopancreas) from wild and cultivated (after being weighed) oysters were frozen in liquid nitrogen and stored at -70°C until analysis.

### Electrophoresis and Genetic Interpretations

The allozyme genotype of each oyster was obtained for eight polymorphic genes: *Est* (esterase; E.C. 3.1.1.2.), *Lap* (leucine aminopeptidase; E.C. 3.4.11.1), *Mdh-1* and *Mdh-2* (malate dehydrogenase; E.C. 1.1.1.37), *Me-2* (malic enzyme; E.C. 1.1.1.40), *Pgi* (phosphoglucosomerase; E.C. 5.3.1.9), *Pgm* (phosphoglucosomutase; E.C. 5.4.2.2), and *Xdh* (xanthine dehydrogenase; E.C. 1.1.1.204). Electrophoretic techniques employed were those described in Michinina and Rebordinos (1997).

Activity zones were named by abbreviations followed by a number denoting from slower to faster migration. Loci were designated by italicized abbreviations with numerical suffixes corresponding to isozymes in order of ascending electrophoretic mobility. The most frequent allele for each locus was designated 100,

and alternative alleles were labeled according to their rates of migration relative to that of the most common allele. Null alleles (identified by absence of any enzymatic activity) were referred to by the number 00 (Michinina and Rebordinos 1997).

### Data Analysis

Allele frequencies were obtained by direct count of the presence or absence of bands from electrophoretic phenotypes, and numerical analyses were performed using the program Biosys-1 (Swofford and Selander 1981). Genotype frequencies were tested for Hardy-Weinberg equilibrium, and the degree of heterozygote deficiency relative to the Hardy-Weinberg expectations was recorded as the D index =  $\text{Het}_{\text{observed}} - \text{Het}_{\text{Expected}} / \text{Het}_{\text{Expected}}$ , where Het. is the number of heterozygotes.

An estimation of the effective size ( $N_e$ ) of the founder population was obtained from the reduction of expected heterozygosity with respect to the parental population, by the equation  $H_t = H_0(1 - 1/2N_e)^t$  (Crow and Kimura 1970), where  $H_t$  is the heterozygosity at time  $t$ ,  $t$  is the number of generations, and  $H_0$  is the initial heterozygosity. In the case described here,  $t = 1$   $H_t$  is the average heterozygosity in the cultivated population studied, and  $H_0$  is the average heterozygosity in the natural population from which the broodstock was collected.

Growth rate is one of the most commercially important traits, and it can be estimated as different weights reached by individuals of same age, grown in the same conditions. The relationship with each locus was tested in two different ways: first the mean weight of each genotype, and second the mean weight of homozygotes and heterozygotes were compared using one and two-way analysis of variance (ANOVA).

A basic assumption underlying the analysis of variance is the equality of variances, and this assumption was checked using Levene's test. When the test resulted in a significant value, we performed the Welch and Brown-Forsythe procedures, which are two alternate tests of the null hypothesis of equality of group means in which the group variances are not assumed to be equal. Most statistical tests were performed with the BMDP program package (Dixon et al. 1990).

## RESULTS

### Genetic Structure of Populations

Frequencies of allozyme alleles in each population are shown in Table 1, and heterozygosity estimates and measures of Hardy-Weinberg deviations are shown in Table 2. Some alleles present in the natural population were not present in reared populations. Thus, *Mdh1* and *Mdh2* showed two alleles in the natural population but became monomorphic in the oldest cultivated populations. Alleles *Lap*<sup>73</sup> and *Pgm*<sup>138</sup> from the wild population were absent in most of the cultivated ones. Nevertheless, a number of new alleles that were absent in the wild population did appear in cultivated ones. For example, *Lap*<sup>122</sup> was present in 8 m, 12 ms, and 12 mL; *Lap*<sup>00</sup> appeared in 8 m, and *Pgi*<sup>157</sup> appeared only in 8 m, probably because of a sampling error. Although contamination is very unlikely, because no spawning or larval settlement occurs in winter in natural populations of *Crassostrea angulata*, the hatchery did not install collector controls to check larval settlement, and, for this reason, external origin cannot be completely discounted for these new alleles. The average allele number (Table 1) ranged from  $3.14 \pm 0.40$  in the 8 m population to  $2.43 \pm 0.53$  in 18 m,

TABLE 1.

Allele frequencies in two cohorts of *C. angulata*, one analyzed both at 8 (8 m) and 12 months old (12 m) and the other at 18 months old, and in the source population (wild). At 12 months old, two weight classes of oysters were studied: the smallest (12 ms) and the largest (12 mL).

Locus Allele (n)	Wild 62	8 m 51	12 ms 69	12 mL 50	18 m 49
<i>Lap</i>					
73	0.123	0.010	0.000	0.000	0.000
91	0.711	0.173	0.255	0.210	0.030
100	0.167	0.769	0.667	0.770	0.970
122	0.000	0.029	0.078	0.020	0.000
00 <sup>a</sup>	0.000	0.019	0.000	0.000	0.000
<i>Mdh-1</i>					
85	0.034	0.029	0.014	0.000	0.000
100	0.966	0.971	0.986	1.000	1.000
<i>Mdh-2</i>					
100	0.992	0.990	1.000	1.000	1.000
118	0.008	0.010	0.000	0.000	0.000
<i>Me-2</i>					
90	0.108	0.029	0.359	0.190	0.020
100	0.650	0.875	0.535	0.590	0.950
110	0.242	0.096	0.106	0.220	0.030
<i>Pgi</i>					
77	0.086	0.183	0.021	0.030	0.260
100	0.914	0.779	0.979	0.970	0.740
157	0.000	0.038	0.000	0.000	0.000
<i>Pgm</i>					
94	0.057	0.000	0.042	0.040	0.030
96	0.131	0.019	0.028	0.020	0.140
100	0.230	0.615	0.542	0.720	0.460
114	0.189	0.240	0.197	0.070	0.230
132	0.369	0.125	0.141	0.150	0.140
138	0.025	0.000	0.049	0.000	0.000
<i>Xdh</i>					
99	0.438	0.298	0.303	0.260	0.220
100	0.479	0.404	0.500	0.400	0.500
101	0.083	0.298	0.197	0.340	0.280
<i>Est</i>					
94	nd	0.083	0.210	0.090	0.120
100		0.625	0.319	0.380	0.450
107		0.181	0.283	0.330	0.300
103		0.111	0.188	0.200	0.130
P <sub>95</sub>	71.43	71.43	57.14	57.14	57.14
n <sub>a</sub>	3.00	3.14	2.86	2.57	2.43
(SE)	(0.53)	(0.14)	(0.59)	(0.53)	(0.53)

<sup>a</sup> Null allele, identified by absence of any enzymatic activity; n: sample size; nd: not determined; P<sub>95</sub>: percentage of loci polymorphic at 95%; n<sub>a</sub>: average number of alleles per locus; SE: standard error.

although these differences were not statistically significant ( $t = 1.07$ ;  $df = 12$ ;  $p > .05$ ). The proportion of polymorphic loci (P<sub>95</sub>) was highest in the wild and 8 m populations (71.43%) and least in the other three cultivated populations ( $P = 57.14$ ). Nevertheless, the major differences between populations were observed when allelic frequencies between populations were considered, and the contingency  $\chi^2$  analysis at all loci was highly significant ( $p < .001$ ), except in the cases of the *Mdh1* and *Mdh2* loci, where this analysis was not significant.

Nine of the equilibrium tests carried out to determine deviations from Hardy-Weinberg proportions (D) were significant for

TABLE 2.

Expected and observed averages heterozygosities ( $h_e$  and  $h_o$ ) and deviations from Hardy-Weinberg proportions (D) in two cohorts of *C. angulata*, one analyzed both at 8 (8 m) and 12 months old (12 m) and the other at 18 months old, and in the source population (wild). At 12 months old two weight classes of oysters were studied: the smallest (12 ms) and the largest (12 mL).

Locus	Wild	8 m	12 ms	12 mL	18 m
<i>Lap</i>					
$h_e$	0.452	0.377	0.484	0.363	0.058
$h_o$	0.333	0.250	0.451	0.366	0.020
D	-0.263***	-0.081	-0.069	0.008	-0.656*
<i>Mdh-1</i>					
$h_e$	0.065	0.056	0.028	mn	mn
$h_o$	0.068	0.058	0.028		
D	0.035	0.030	0.014		
<i>Mdh-2</i>					
$h_e$	0.017	0.019	mn	mn	mn
$h_o$	0.017	0.019			
D	0.009	0.010			
<i>Me-2</i>					
$h_e$	0.183	0.224	0.573	0.567	0.096
$h_o$	0.507	0.212	0.479	0.680	0.100
D	0.639***	-0.033	-0.165***	0.198*	0.040
<i>Pgi</i>					
$h_e$	0.158	0.359	0.041	0.058	0.385
$h_o$	0.172	0.442	0.042	0.060	0.520
D	0.094	0.284	0.022	0.031	0.351
<i>Pgm</i>					
$h_e$	0.755	0.548	0.642	0.452	0.695
$h_o$	0.787	0.558	0.535	0.480	1.000
D	0.043	0.045	-0.167	0.061	0.438***
<i>Xdh</i>					
$h_e$	0.572	0.659	0.619	0.657	0.623
$h_o$	0.042	0.654	0.423	0.620	0.420
D	-0.927***	-0.008	-0.318**	-0.056***	-0.326***
<i>Est</i>					
$h_e$	nd	0.557	0.739	0.699	0.676
$h_o$		0.565	0.594	0.600	0.580
D		0.363	-0.196***	-0.141	-0.142***
Mean value					
D	-0.045	0.076	-0.091	0.004	-0.037
SD	0.154	0.149	0.115	0.093	0.328
$H_e$	0.361	0.323	0.341	0.300	0.265
SD	0.107	0.091	0.114	0.105	0.113
$H_o$	0.229	0.357	0.319	0.347	0.330
SD	0.102	0.091	0.089	0.103	0.128

$H_e$  and  $H_o$  are the averages of  $h_e$  and  $h_o$  over loci; <sup>a</sup> not significant after pooling; mn: monomorphic loci; SD: standard deviation.

\*  $p < .05$ ; \*\*  $p < .01$ ; \*\*\*  $p < .001$ .

cultivated oysters, mainly at 12 and 18 months. Considering that only one of the tests would be expected to be statistically significant by chance, there is a considerable deviation from equilibrium conditions. Significant  $\chi^2$  tests could be caused by low individual numbers in some phenotypic classes. Therefore, a further  $\chi^2$  test was calculated after pooling alleles in three classes: homozygotes for the most common allele, frequent-infrequent heterozygotes and a third class composed of infrequent homozygous and other heterozygous genotypes. Loci *Est*, *Me-2*, and *Pgm* fit the equilibrium conditions better when pooled classes are used, meaning that after pooling, three out of nine significant tests became nonsignifi-

icant. Hence, these cases could be explained by a high number of alleles present in low frequencies, although all scored alleles were present at high frequencies (Table 1), as compared to the low frequencies described in natural populations of mollusks in general (Sarver et al. 1992) and *C. angulata* in particular (Michinina and Rebordinos 1997).

On the other hand, only in two cases the lack of equilibrium was attributable to an excess of heterozygotes, the rest being attributable to an excess of homozygous genotypes. The natural population from which the progenitors were taken showed significant departures from Hardy-Weinberg proportions at three loci. One of the loci became nonsignificant after pooling of alleles (*Lap*) and another one yielded a homozygous excess (*Me-2*:  $D = 0.639$ ). As far as  $D$  values in every locus are concerned, they were negative in all cases in both cultivated and wild populations in the cases of *Lap* and *Xdh* loci. The *Lap* locus showed null homozygotes in the 8 m population, although they were not detected in the other populations or in the wild population, probably because of the low number of parental oysters used as progenitors.

Mean  $D$  values ( $\pm$  standard deviation) were positive in 8 m and 12 mL ( $D = 0.076 \pm 0.149$  and  $0.004 \pm 0.093$ , respectively); however, the 18 m population yielded a negative value ( $D = -0.037 \pm 0.328$ ), similar to that of the wild population ( $D = -0.045 \pm 0.154$ ), and 12 ms gave the highest negative  $D$  value ( $D = -0.091 \pm 0.115$ ). The average expected heterozygosity ( $H_e$ ) was higher in 12 ms ( $0.391 \pm 0.111$ ) than in wild population ( $0.361 \pm 0.107$ ), and also higher than in the other three populations (Table 2). However, when average observed heterozygosities ( $H_o$ ) were compared, the lowest value was given by the wild population ( $0.229 \pm 0.102$ ) followed by the small weight class of 12 months ( $0.319 \pm 0.089$ ); the populations of 8 months and large weight class of 12 months and 18 months showed slightly higher values (Table 2).

If we consider the over-all results on the occurrence of heterozygote excess at *Pgi* and *Pgm*, it can be seen that there is an absence in the cultivated populations of some alleles present in the natural population, marked differences in allele frequencies between the wild and the cultivated populations, and a lower value of the average heterozygosity ( $H_e$ ) in the cultivated populations as compared with wild ones. All these facts point to a founder effect on hatchery populations. The effective size of the founder population estimated by the reduction in average heterozygosity was two. This means a number of actual contributors to the progeny that is one order of magnitude lower than the total number of oysters set to spawn, and a similar situation has been described for other species (Hedgecock and Sly 1990).

Average genetic distances and genetic identities, calculated over the seven loci studied on the five populations (Table 3), gave a maximum divergence value of  $D = 0.991$  in the comparison of

TABLE 3.

Unbiased genetic identities above diagonal and unbiased genetic distances below diagonal (Nei 1978) between a wild population and four cultivated ones reared from it of *Crassostrea angulata*.

Population	Wild	8 m	12 ms	12 mL	18 m
Wild	—	0.890	0.919	0.892	0.849
8 m	0.116	—	0.967	0.981	0.991
12 ms	0.085	0.033	—	0.989	0.945
12 mL	0.115	0.020	0.011	—	0.955
18 m	0.163	0.009	0.057	0.046	—

TABLE 4.

Descriptive analysis of weight in grams in two cohorts of *C. angulata*, one analyzed both at 8 (8 m) and 12 months old (12 m) and the other at 18 months old. At 12 months old, two weight classes of oysters were studied: the smallest (12 ms) and the largest (12 mL).

Populations/Traits	N	M	SD	SE	CV
8 m					
Total weight	51	8.400	3.033	0.425	0.361
Body weight	51	2.108	0.934	0.131	0.443
Shell weight	51	6.293	2.178	0.305	0.346
12 ms					
Total weight	69	5.905	1.505	0.181	0.255
Body weight	69	0.812	0.265	0.032	0.326
Shell weight	69	5.093	1.295	0.156	0.254
12 mL					
Total weight	49	11.676	3.174	0.453	0.272
Body weight	49	1.467	0.527	0.075	0.359
Shell weight	50	10.236	2.737	0.387	0.267
18 m					
Total weight	49	49.558	7.769	1.110	0.157
Body weight	49	8.566	1.715	0.245	0.200
Shell weight	49	40.992	6.658	0.951	0.162

N: number of individuals; M: mean value; SD: standard deviation; SE: standard error; CV: coefficient of variation.

8 and 18 m, and a maximal identity value of  $I = 0.163$  between the wild population and 18 m.

#### Variability and Weight

Descriptive statistical data regarding the distribution of total weight, body weight, and shell weights of the samples are given in Table 4. Shapiro-Wilks tests using the mean and deviation values from the samples were used to assess whether the distribution of data was normal. Oyster data of 12 m were found not to be normal at any of the weight traits. In contrast, for the rest of the ages, the total weight and shell weight were found to be distributed normally, and, in addition, body weight at 8 months was also normal. Logarithmic transformation of data did not improve the adjustment to normality, and so this transformation was not used.

Twelve-months-old oysters came from the same cohort as those of 8 months, but were sampled 4 months later. These individuals (12 m) were subjected to overcrowded conditions to obtain harder shells, which became more resistant and, hence, healthier-looking in the long term. This is also the reason why 12-month-old oysters yielded a lower body weight than 8-month-old specimens. However, the largest class showed the highest mean value for both total and shell weight. The mean total weight at 8 m was 8.40 g, and 4 months later, the value was 11.68 g. Most of this increase was because of the increase in shell weight that resulted from the overcrowded conditions, because the mean body weight after this time period was lower. The mean body weight of 18-month-old oysters was four times that of the 8 m specimens. The coefficient of variation in the four groups studied was higher for the body weight variable, and it always decreased with age, with the value being 0.443 for body weight for 8 month and 0.200 for 18-month-old oysters.

The effect of the genotypes at individual loci on growth rate was assessed by one-way ANOVA on total weights of individuals



TABLE 5.

Analysis of variance for total weight within genotypes (Genotypes) and comparing total weight of homozygotes versus heterozygotes (Hom. vs. Het.) for eight allozymic loci in four cultivated populations of *C. angulata* with degrees of freedom (df), F-value (F), and probability of fitting to null hypothesis (p). \*Significant under Welch's and Brown-Forsythe's tests; nd: not determined; na: not applicable.

Locus	8 m			12 ms			12 mL			18 m		
	F	p	df	F	p	df	F	p	df	F	p	df
<i>Lap</i>												
Genotypes	0.71	.620	5	0.23	.918	4	2.17	.105 <sup>a</sup>	3	na		
Hom. vs. Het.	0.77	.471	1	0.31	.582	1	1.27	.266	1			
<i>Mdh-1</i>												
Genotypes	0.01	.927	1	0.17	.685	1	na			na		
Hom. vs. Het.	0.01	.927	1	0.20	.658	1						
<i>Me-2</i>												
Genotypes	2.92	.045*	3	0.13	.970	1	1.17	.337	4	3.08	.056	4
Hom. vs. Het.	2.43	.125	1	0.09	.772	1	0.62	.434	1	5.74	.021*	1
<i>Pgm</i>												
Genotypes	0.95	.392	2	1.26	.266	1	0.03	.853	1	0.01	.927	1
Hom. vs. Het.	0.02	.885	1	1.26	.266	1	0.03	.853	1	0.01	.911	1
Genotypes	2.13	.080 <sup>a</sup>	5	0.82	.619	1	0.63	.732	7	0.63	.674	7
Hom. vs. Het.	nd			nd			nd			nd		
<i>Xdh</i>												
Genotypes	0.65	.662	5	0.64	.669	5	4.79	.003**	4	0.30	.909	4
Hom. vs. Het.	0.00	.997	1	0.40	.530	1	3.26	.077	1	0.01	.910	1
<i>Est</i>												
Genotypes	0.82	.548	5	1.20	.311 <sup>a</sup>	9	1.13	.365	8	1.37	.238	8
Hom. vs. Het.	0.07	.797	1	2.12	.150	1	1.18	.283	1	1.29	.261	1

for the four studied populations (Table 5). Interactions were significant at loci *Me-2* at 8 m and *Xdh* at 12 mL. All variances were tested by a Levene's test, which gave nonsignificant results with the exception of locus *Pgm* at 8 m; in this case, the Welch test was also significant. At the same time, *Lap* at 12 ml gave rise to a Welch test with values of F = 18.61, resulting in p = .001 and Brown-Forsythe test F = 3.16 and p = .071. In addition, for *Est*, the Welch test at 12 ms gave values F = 6.52 and p = .001.

Further ANOVA analysis was conducted considering weights of heterozygotes versus homozygotes at each locus (Table 5). A significant value was detected at 18 m for locus *Me-2* and for locus *Xdh* in 12 mL p value was close to the significance level. In both cases, the mean body weight of heterozygous genotypes was higher than in homozygous genotypes.

To study the relationship between multilocus heterozygosity and growth rate, the correlation of individual heterozygosity (the number of loci for which one oyster was heterozygous) and indi-

vidual weight was computed (Table 6). None of the correlations was statistically significant, although two different trends can be deduced from Table 5. The two populations that were not selected (i.e., 8 m and 18 m) and the one selected for small specimen size (12 ms) showed values close to zero, indicating no correlation or a negative one. However, the population subjected to size selection (12 mL) yielded positive values and, for total weight and body weight, were close to being significant (r = 0.208 and r = 0.271, respectively), because the significant level at p < .05 started at r = 0.285.

Moreover, the existence of a relationship between multilocus heterozygosity and weight was examined by grouping individuals into classes according to the number of heterozygous loci in each individual and calculating the variance between weights of classes with different number of heterozygous loci. ANOVA values were not significant in any case, although in the 12 mL class, low p values were obtained and were close to significance in the case of body weight. When mean weights of each heterozygous class were compared by a Student's t-test, statistically significant (p < .05) results were found in 12 mL in the following cases:

- (1) The mean total weight of heterozygotes for two loci was 10.188 g and, for the class with three loci 12.960 g, being p = .018.
- (2) The mean body weight for the class with two loci heterozygotes was 1.155 g. For the class with three, the value was 1.642 g and for four 1.768 g. The p value between classes two and three was .009 and for classes two and four it was .004.
- (3) The mean shell weight for the group exhibiting two heterozygous loci was 9.214 g and for that exhibiting three such loci, it was 11.318 g. The p value was .036.

TABLE 6.

Correlation coefficients between individual heterozygosity and weight (total weight, body weight, and shell weight) in two cohorts of *C. angulata*, one analyzed both at 8 (8 m) and 12 months old (12 m), and another at 18 months old. At 12 months old, two weight classes of oysters were studied: the smallest (12 ms) and largest (12 mL).

Traits	8 m	12 ms	12 mL	18 m
Total weight	-0.001	-0.160	0.208	-0.116
Body weight	0.010	-0.189	0.271	-0.172
Shell weight	-0.006	-0.146	0.180	-0.098

## DISCUSSION

Growth rate is a very economically important trait, and it can be estimated as differences in weights reached by individuals from the same cohort grown under uniform conditions. Its relationship, if any, to gene markers would be very useful in any breeding program, but its estimation is not trivial because of the characteristics of the quantitative traits and also because methods and experimental designs used in research laboratories are likely to be different from procedures used in commercial hatcheries. This means that results cannot always be easily extrapolated. Hence, we decided to address this problem by checking, from a genetic point of view, oysters that had been classified as valuable for a hatchery and looking for relationships between allozymic genotypes and differential weights in individual oysters.

Loss of genetic diversity following hatchery culture of organisms has been widely reported and is caused by the effective population size of hatchery "mass spawning." Several factors contribute to this result, such as the sex ratio of progenitors, the mating behavior, physiology, and genetic organization (Beaumont 1994). Our results show a decrease in genetic variability of both the average allele number, and at the polymorphism level, and these are likely to be because of the small number of oysters (20) used as broodstock. Gosling (1982) estimated that at least 45 individuals (sex ratio of about 1:1) are needed to provide a 99% chance of retaining alleles at a frequency of 0.01 at a locus. Moreover, the estimated effective population size was estimated to be one order of magnitude lower than the number of progenitors, or, more likely, as low as two. Discrepancies between apparent and effective population size could be attributable to an insufficient number of progenitors used in the spawns that produced the cultivated populations and/or unequal spawning success among the chosen progenitors. The main practical meaning of this result is that hatchery managers should control the number of spawnings from the total number of oysters induced to spawn.

As far as the effects of loci on growth rates are concerned, we found only two (five, if we consider the significant values rendered by the Brown-Forsythe test) out of 28 cases producing significant effects on applying Welch's test. These were *Me-2* on 8 m ( $p < .05$ ) and *Xdh* ( $p < .01$ ) on 12 mL and *Lap*, *Pgm*, and *Est* ( $p < .05$ ) on 12 mL, 8 m and 12 ms, respectively. To accept that a real dependence of the growth rate on some isolated isozymic genes exists, we would expect a generalized effect on related species, populations of the same species, or at least individuals of the same cohort at different ages. However, none of these circumstances have been reported. First of all, we cannot compare our results to other results obtained for *C. angulata* species because of lack of relevant data. However, results published on *C. virginica* (Gmelin) species describe variable correlations between weight class and loci (Zouros and Pogson 1994, Foltz and Chatry 1986). Although methods described in the aforementioned papers were slightly different from ours, there should not be much difference, because all data are referred to specific enzymatic activities. Published data indicated that some biochemical characteristics in relation to quaternary structure and number of genes controlling each activity are conserved between taxa located phylogenetically far away. For this reason, it would be expected that extended relationships between specific genes and traits that are important in hatcheries would also have been conserved (Richardson et al. 1986).

At the same time, such effects were not retained when weights of heterozygotes and homozygotes were compared at those loci

affecting growth rate. Thus, this effect was only shown for *Me-2* at 18 m ( $p < .05$ ), and in 12 mL, the gene *Xdh*, reported as highly significant when comparing genotypes, was nearly significant when the weight of homozygous individuals was compared to the larger one of heterozygous individuals ( $p = .077$ ).

Moreover, if a consistent effect is caused by some specific loci, this effect should have been retained with age (at least) in individuals originating from the same cohort (8 m and 12 m), and/or differential effects should have been found between the lowest and highest weight classes of the same age (12 ms, 12 mL). However, these conditions did not occur, and no differences were observed between the two weight classes.

Another interesting characteristic of genes contributing to quantitative traits in marine bivalves is the deficit of heterozygotes, although there is no definitive explanation for this observation (Gaffney et al. 1990). The deficit of heterozygotes has been explained by the presence of null alleles, but also the relationship between heterozygote deficiencies and heterozygosity-fitness correlations has been attributed to selection against heterozygotes during the larval phase and overdominance at allozyme loci at the adult stage (Zouros and Pogson 1994). A different explanation considers the allozyme markers as neutral, but related to fitness, and this effect is seen after inbreeding and is also responsible for heterozygote deficiencies. Relationships between allozyme loci and overdominant or deleterious alleles could be established either by gametic disequilibrium caused by a small effective population size, or by partial inbreeding, resulting in a generalized homozygosity producing inbreeding depression (Zouros 1993, David et al. 1995).

It has been claimed that the use of real neutral markers could help to answer these questions. In fact, microsatellites can provide indicators of selection processes at linked loci attributable to linkage disequilibrium (Slakhtin 1995). By studying microsatellite in different larval stages, Bierne et al. (1998) found that these markers cosegregated with fitness-associated genes and significant multilocus heterozygosity-growth correlations were recorded at all stages, ruling out the hypothesis of differential selection between larval and juvenile or adult stages.

When relationships between heterozygosities and weight were analyzed, positive correlation and nearly statistical significances were detected only for 12 mL, showing an important difference between all of them and pointing to the expression of the selective effect acting upon selected individuals (more heterozygous). This result was corroborated by the analysis of variance carried out within different classes of heterozygosity in *C. angulata*, which rendered lower  $p$  values and even a significant value ( $p < .001$ ) under the Welch's test for body weight on this class. In addition, when a Student's  $t$ -test was performed, significant results were only obtained when comparing the weight of different heterozygosity classes for total weight, body weight and shell weight only in 12 mL. When the mean observed heterozygosities were compared between populations, a higher value was detected in the largest weight class ( $0.347 \pm 0.103$ ) as compared to the smallest one ( $0.319 \pm 0.089$ ), as expected. However, the value shown at 8 months was higher ( $0.357 \pm 0.091$ ); the decrease was probably the response to the selection caused by the stressful situation of oysters living in overcrowded conditions over 4 months. Finally, the difference between the average heterozygosity values of wild population ( $0.229 \pm 0.102$ ) and the considerably higher values found in all cultivated populations could be explained by the use of selected progenitors (by large size and healthy appearance) from the wild

population (quantitative traits were not determined in wild population, because the age of these oysters was unknown). Differences between populations are even more remarkable when expected average heterozygosities are also considered (Table 2). All these results would reinforce the role of isozymes as markers and, most importantly, as valid markers for quantitative traits.

Nevertheless, in the case of allozymes, it might also occur that both effects of linkage disequilibrium and overdominance would be operating within the genome, although it is very difficult to provide direct experimental evidence to estimate how much of the effect might be attributable to overdominance, or dominance at the scored loci, and how much could be attributable to other linked genetic conditions. Our results point to the associative hypothesis and the role of isozyme acting as markers rather than playing a

direct role. It is likely that this means that they can be used as markers for quantitative traits and, more importantly, confirm the existence of heterosis in *Crassostrea* spp. This phenomenon could be very valuable for breeding in aquaculture and also for establishing breeding programs in oysters based on the maintenance of inbred lines of oysters and crossing them to obtain hybrid  $F_1$  showing hybrid vigor.

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## GROWTH OF *PTERIA COLYMBUS* (RÖDING, 1798) IN SUSPENDED CULTURE IN GOLFO DE CARIACO, VENEZUELA

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**ABSTRACT** Over a 10-month period, we examined the growth of shell height and width, and of mass of the shell and tissues, of juveniles of *Pteria colymbus* (initially measuring 13.5 mm in shell height) placed in a suspended culture at 8-m depth at Turpialito in the Golfo de Cariaco, Venezuela. A high growth rate was observed for all body parameters. Shell parameters increased rapidly during the first 5 months and then at lower rates until the end of the study, and total tissue mass increased at a rapid and almost steady rate throughout the study. Mortality during the study was negligible. The maximum size predicted by the von Bertalanffy growth equation ( $L_{\infty} = 71$  mm) was near the maximal size observed in natural populations. *P. colymbus* seemed little affected by marked changes in environmental conditions during our study, because it showed continuous rapid growth rate. The rapid growth, low mortality, and availability of natural spat indicate that *P. colymbus* could be an excellent species for aquaculture in this region.

### INTRODUCTION

Although pearls are cultured in only a few species, all bivalve species secreting large amounts of iridescent nacreous material have the potential for use in pearl culture. The family Pteridae includes a number of such species, *Pinctada margaritifera*, *Pinctada maxima*, *Pinctada mazathantica*, and *Pteria sterna*, which have been exploited in tropical areas (Coeroli et al. 1984, Alagarswami et al. 1989, Gaytan-Mondragon et al. 1993, Monteforte and Garcia-Gasca 1994, Shirai 1994). In northeastern Venezuela, beginning with the Spanish colonization, considerable exploitation of the mother-of-pearl oyster *Pinctada imbricata* has been made on natural banks, particularly near Margarita Island ("the island of pearls") and Verginelli and Prieto (1991) report its growth in a natural population. Another Caribbean bivalve producing a thick nacreous layer is the winged pearl oyster *Pteria colymbus* (Röding, 1798), which presently is being considered as a potential species for aquaculture in Venezuela and Colombia (Borrero 1994, Marquez 1996, Velasco and Borrero 1996).

*P. colymbus* occurs along the coasts of North Carolina, Florida, and Texas, in Bermuda and the West Indies, and also as far south as Brazil (Abbot 1974, Díaz and Puyana 1994). In the Golfo de Cariaco in northeastern Venezuela, it is common in shallower waters attached to soft corals and suspended structures, such as the collectors and pearl nets used for bivalve culture. The present study examines the growth and survival of *P. colymbus* in suspended culture over a 10-month period.

### METHODS

The study was conducted at Turpialito in the Golfo de Cariaco, northeastern Venezuela (Fig. 1) and ran from 29 December 1993 to 29 October 1994. We began by placing 300 juveniles of *P. colymbus*, measuring 13.5 mm in mean shell height (95% CI = 2.09), in 6-mm mesh pearl nets suspended at 8-m depth from a longline. The juveniles originated from a natural settlement that had occurred on pearl nets in which the scallop, *Euvola ziczac*, was being cultured. At periodic intervals throughout the study (dates

given in Table 1), we collected all oysters to determine the proportion of living individuals and, at the same time, randomly selected 10 individuals for determinations of shell height (the maximum distance between the dorsal hinge and ventral margin), shell width (maximal distance through the animal from the outermost portion of the left and right valves), and the dry mass of the shell and tissues (drying at 60°C for ≈2 days). At each sampling date, the remaining oysters were returned to the sea in new pearl nets. As the oysters increased in size, we decreased their density in the experimental pearl nets (Table 1; scallops not needed to complete

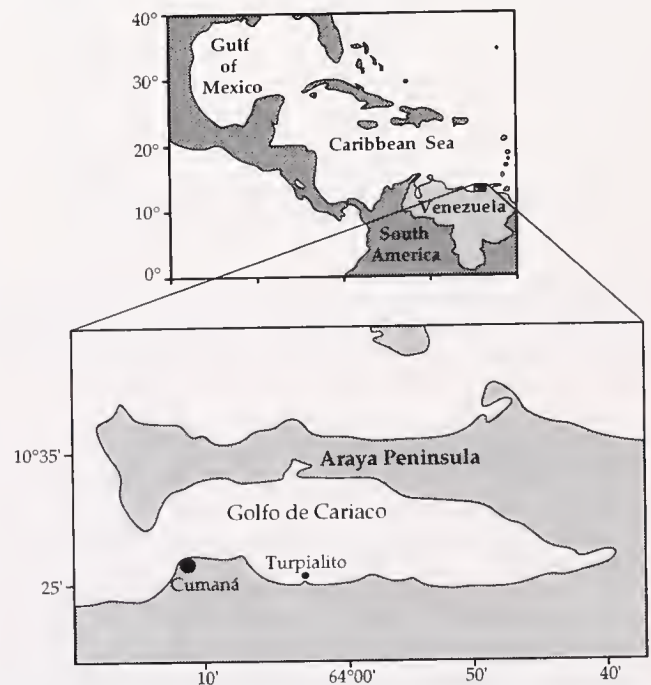


Figure 1. Location of study site at Turpialito in Golfo de Cariaco, northeastern Venezuela.

TABLE 1.

Mortality rates as documented on different sampling dates during our study of the growth of *Pteria colymbus* in suspended culture in Golfo de Cariaco.

Sampling Date	Set Out on Previous Date			Number Dead	Percentage Mortality/Day
	No. Nets	Density /Net	Total No. Oysters		
29 December 1993 (start of experiment)					
1 February 1994	3	100	300	3	0.010
28 February 1994	7	40	280	2	0.007
26 March 1994	6	40	240	0	0.000
26 April 1994	5	40	200	0	0.000
17 May 1994	4	40	160	0	0.000
23 June 1994	10	15	150	0	0.000
23 August 1994	9	15	135	0	0.000
29 October 1994	8	15	120	0	0.000

the desired density in the pearl nets were discarded), so that the oysters covered about 30–40% of the floor of the nets, as recommended for scallop culture by Ventilla (1982).

During the study, temperature was continuously recorded at 8-m depth in the experimental site using a SEALOG electronic thermograph (Vemco Ltd., Halifax, Nova Scotia, Canada), and other environmental factors were determined at weekly intervals during the first 7 months. (We were unable to take seston samples during the last 3 mo.) Water samples were collected using 5-L Niskin bottles and transferred to opaque containers for transport to the laboratory, where the seston was analyzed. Samples (500–1000 mL) were filtered through precombusted 0.7- $\mu$ m MFSF filters, which were then frozen at  $-20^{\circ}\text{C}$  for later analyses. The masses of the organic and inorganic sestonic fractions (suspended particles) were determined gravimetrically, and chlorophyll *a* concentrations were obtained using the spectrophotometric method (Strickland and Parsons 1972).

The general pattern of increase in shell height over time (in

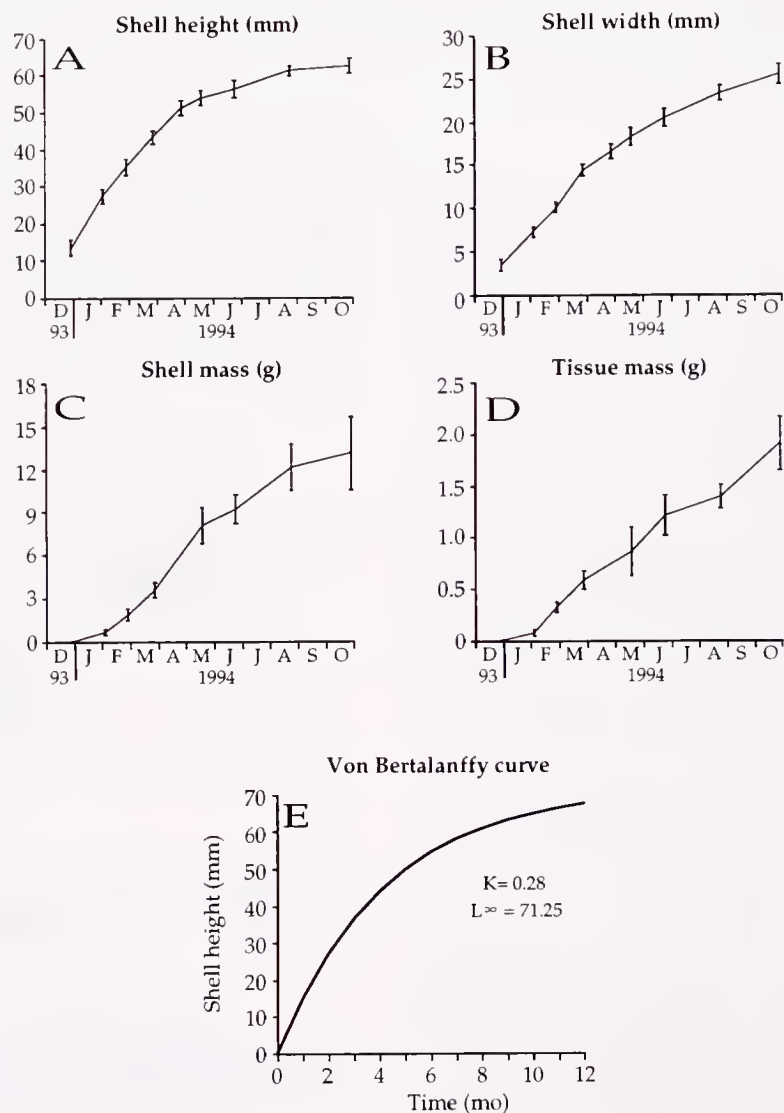


Figure 2. Changes in shell height (A) and width (B) and in the mass of shell (C) and tissues (D) between 29 December 1993 and 29 October 1994 and the calculated von Bertalanffy growth curve (E), for juveniles of *Pteria colymbus* maintained in suspension culture at 8-m depth at Turpialito in Golfo de Cariaco, Venezuela. Vertical bars represent standard deviations.

months) were described using the von Bertalanffy growth function and the K growth constant, and asymptotic size,  $L_{\infty}$ , was determined using the Gulland and Holt plot procedure (Sparre and Ursin 1989).

## RESULTS

Both shell and tissue growth were rapid, but they showed different patterns (Fig. 2). Growth in shell height and shell mass were greatest at the beginning of the study ( $9.1 \text{ mm mo}^{-1}$  and  $1.8 \text{ g mo}^{-1}$ , respectively, for the period 29 December 1993 to 17 May 1994), but decreased in the latter part of the study (by 81% for shell height and 48% for shell mass). The rate of increase in shell width was similar to the increases in shell height and mass, except that the decrease in the latter months was less marked. In contrast, the rate of increase in tissue mass was almost constant throughout the study, about  $0.18 \text{ g mo}^{-1}$ . The mortality rate was extremely low, there being only 3 of 300 oysters dead on the first sampling date, 2 of 280 on the second date, and none dead on all subsequent dates (Table 1). The general increase in shell height over time was well described by a von Bertalanffy growth curve, with a K growth rate of 0.28 and an asymptotic size ( $L_{\infty}$ ) of 71 mm (Fig. 2).

The thermograph record showed a slow temperature decrease from December 1993 ( $25^{\circ}\text{C}$ ) to early March 1994 ( $21.5^{\circ}\text{C}$ ) and then increased in April (to  $24.3^{\circ}\text{C}$ ) and early July (to  $25.0^{\circ}\text{C}$ ). The most marked temperature change during the study was in late August, when temperature increased from  $22.5$  to  $27.0^{\circ}\text{C}$ , and the level further increased to  $28.5^{\circ}\text{C}$  in late October (Fig. 3A). The high temperature levels during the last 3 months seemed to have no effect on the growth of *P. colymbus*, because the increase in tissue mass continued at the same rate throughout September and October, and the slowing in shell growth had begun in June; thus, several months before the abrupt temperature increase in August. Slowing in shell growth was likely part of the normal growth curve. High temperatures also did not affect survival, because no mortality occurred after February.

From December to July, we documented fluctuating, but generally high, levels of total seston, its organic content, and chlorophyll *a* (Fig. 3B, C, D). Chlorophyll *a* values likely dropped during the period of elevated temperatures in late August through October (when we did not collect seston samples), because studies during previous years demonstrated that primary productivity falls during the thermal stratification (caused by reduced upwelling) that develops in these months (Lodeiros and Himmelman 1994, Lodeiros 1996). Regular continuation of growth curves through September and October suggested that growth of *P. colymbus* did not decrease in response to decreased phytoplanktonic food availability. Furthermore, mortality was null during this period.

## DISCUSSION

Our study suggests that the growth and survival of *P. colymbus* is little affected by the variations in external environmental factors in the Golfo de Cariaco or by experimental manipulations. *P. colymbus* showed negligible mortality and rapid growth of all body parameters throughout the 10-month study period. The study period included the full range of environmental conditions typical of the gulf (Lodeiros 1996), from strong wind-driven upwelling and associated low temperatures and high primary productivity (January to March) to a well-developed thermal stratification and low productivity (September to October). The asymptotic size predicted by the von Bertalanffy curve (Fig. 2E, 71 mm) was similar

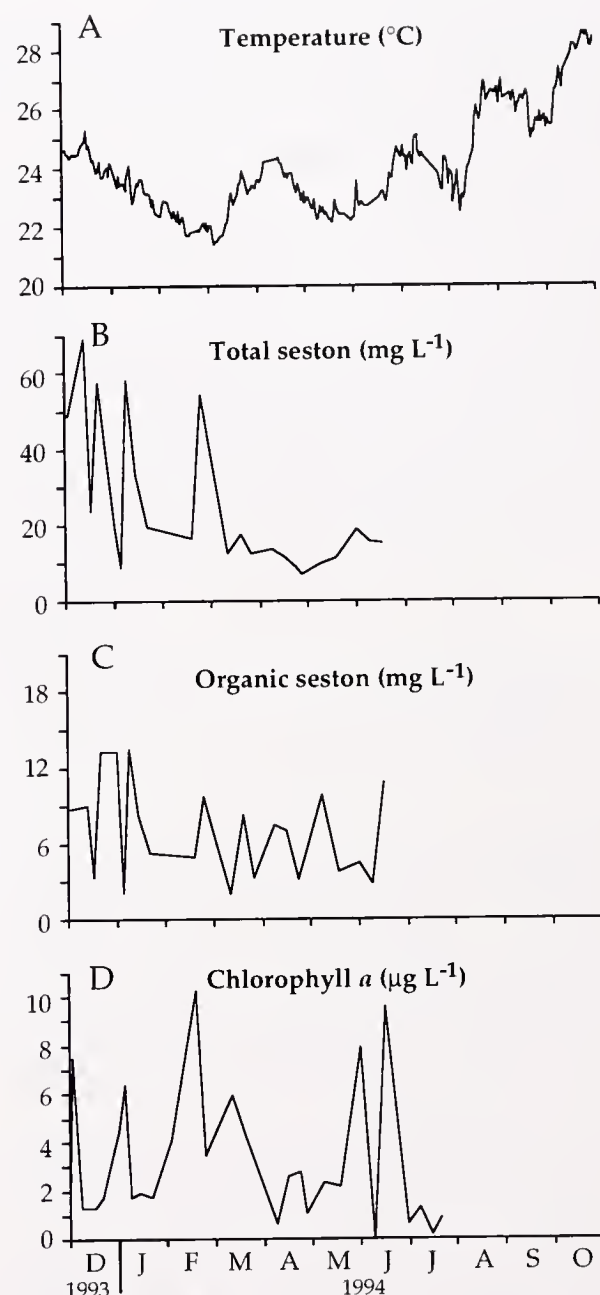


Figure 3. Variations in temperature (A), total seston (B), the organic fraction of the seston (C) and in chlorophyll *a* concentration (D) at the culture site at 8-m depth at Turpialito in the Golfo de Cariaco, Venezuela.

to the maximum size *P. colymbus* attains on a natural bank in the northern Golfo de Cariaco and on suspended structures ( $\approx 70$ – $75$  mm, pers. obs.). Furthermore, this suggests that the oysters were not stressed during our study. *P. colymbus* seems better adapted to culture conditions in the Golfo de Cariaco than other bivalves. For example, concurrent growth trials with the scallop *Lyropecten (Nodipecten) nodosus* at the same study site showed decreases in growth and increases in mortality associated with periods of high temperatures, red tide blooms, and abundant fouling (Lodeiros et al. 1998). A complete mortality of *L. nodosus* occurred during August, simultaneous with a sharp rise in temperature and a

bloom of the toxic diatom *Gymnodinium catenatum*. In earlier studies at Turpialito, the scallop *Euvola ziczac* was found to be highly susceptible to high temperatures and fouling (Lodeiros and Himmelman 1994, Lodeiros and Himmelman 1996, Lodeiros 1996). Fouling organisms colonized the shells of *P. colymbus* but seemed to have little effect on growth and survival.

Throughout our study, *P. colymbus* showed aggregating behavior; that is, individuals byssally attached to one another, as is characteristic of mussels. This suggests that *P. colymbus* is adapted to living at high densities and, furthermore, that mussel culture techniques may be suitable for this species. Spat of *P. colymbus* (5–15 mm shell length) are readily collected on spat collectors throughout the year (Marquez 1996). Velasco and Borrero (1996) studied *P. colymbus* in the Colombian Caribbean and observed rapid growth in suspended culture but high mortality (51% by the end of a 5-mo study) and low spat settlement. In our study, growth rates were higher than in the latter study, and mortality was negligible. The rapid growth, low mortality, and high availability of spat indicates *P. colymbus* could be an excellent species for aqua-

culture in the Golfo de Cariaco. Studies are required to optimize culture techniques (types of enclosures, stocking density, culture depth, and potential for using mussel culture techniques) and to determine whether the time of spat deployment affects growth rates and survival. Furthermore, studies are needed to develop techniques for inducing pearl formation. Our observations suggest that the tissues have a favorable texture and taste, but further studies are required to determine the marketability of the tissue for human or animal consumption.

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## GROWOUT OF BLACKLIP PEARL OYSTERS, *PINCTADA MARGARITIFERA* COLLECTED AS WILD SPAT IN THE SOLOMON ISLANDS

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**ABSTRACT** This study assessed growth and survival of juvenile blacklip pearl oysters (*Pinctada margaritifera*) in a number of intermediate culture systems: lantern nets, panel nets, perforated plastic trays, and attached to ropes enclosed by mesh. Juveniles with initial dorsoventral measurements of 8.3 to 51.5-mm increased in size by 20.4 to 24.8-mm in 3 months, and 30.7 to 36.5-mm in 5 months. Growth rates of juvenile *P. margaritifera* cultured in the open reef systems of the Solomon Islands compared favorably with those reported from the established pearl culture operations in French Polynesia and the Cook Islands. Initial experiments showed that survival of oysters in lantern nets in shallow reef areas was poor as a result of predation by fish and invertebrates. Siting of culture systems in deeper water decreased mortality by fish, although predation by invertebrates that recruited from plankton was still a potential problem. In general, there were no significant differences in growth or survival between juveniles held in lantern nets and panel nets; however, lantern nets were more difficult to clean and inspect for predators. Juvenile growth and survival did not differ significantly ( $p > .05$ ) between panel nets and trays after 5 months, although the rigid trays were easier to clean of fouling organisms. Juveniles placed loosely into trays tended to aggregate, and rates of growth and survival of oysters glued separately into trays were significantly greater ( $p < .05$ ) than those for oysters placed loosely into trays. There was no significant difference in growth between oysters glued into trays and those glued onto ropes and enclosed behind plastic mesh. Overall, this study shows that important criteria of the growout units needed for the intermediate culture of *P. margaritifera* in the Western Pacific include ease of cleaning and access for regular inspection and removal of predators.

**KEY WORDS:** Pearl oyster, spat, *Pinctada margaritifera*, intermediate culture, growth, survival, predation

### INTRODUCTION

Protocols developed by the Japanese for collection and culture of the pearl oyster, *Pinctada fucata martensii*, have been adapted in French Polynesia for blacklip pearl oysters, *P. margaritifera* (Coeroli and Mizuno 1985). Since adapting and developing their own spat collection and growout techniques and stimulating market demand for cultured "black" pearls, there has been rapid growth in round pearl production in Polynesia. For example, annual value from the export of loose "black" pearls from French Polynesia presently stands in excess of US\$145m (Fassler 1995, Remoissenet 1996, Doubilet 1997, N. Sims pers comm. 1998).

Oysters used for the production of black pearls are collected as spat from substrates (spat collectors) deployed in the surface waters of "closed" and semiclosed atoll lagoons in Polynesia (Sims 1992, Sims 1993a). In French Polynesia, spat collectors are generally deployed for 6 months (Coeroli et al. 1984, Cabral et al. 1985, Lintilhac 1987); however, this period is extended in some French Polynesian lagoons (Preston 1990) and, in Manihiki atoll in the Cook Islands, collectors are immersed for up to 2 years (J. Lyons pers comm. 1997). *P. margaritifera* are generally harvested from collectors when they are large enough [65–90 mm dorsoventral measurement (DVM) Nicholls 1931], to be hung from dropper ropes or "chaplets" (AQUACOP 1982, Preston 1990, J. Lyons pers comm. 1997). Oysters are drilled through the hinge of their shell and attached to the chaplets with wire or monofilament fishing line. Chaplets are then connected to submerged longlines, and this is the predominant method of holding adult *P. margaritifera* in Polynesia.

The success of the Polynesian pearl culture industry has not

gone unnoticed by other small island nations in the Pacific (Lucas et al. 1998), which historically have relied on a more modest income from the sale of *P. margaritifera* shell for its nacre or mother-of-pearl (MOP) (Richards et al. 1994, Gervis and Sims 1992). However, not all nations with stocks of *P. margaritifera*, have access to "closed" atoll lagoons. For example, in the central-western Pacific, most reefs fringe high islands or occur in shallow, sublittoral areas (Wells 1988) with few "closed" atolls. Between 1994 and 1997, Friedman et al. (1998) conducted trials to adapt spat collection and culture protocols used in Polynesia for collection and growout of *P. margaritifera* in the "open" reefs of the Solomon Islands. Their study found that commercial quantities of *P. margaritifera* spat could be collected from open reefs at certain sites and at certain times (Friedman and Bell 1996, in review, Friedman et al. 1998). The study also showed that collectors harvested after 6 months held large numbers of dead spat and that greater numbers of live spat could be amassed if collectors were harvested after 3 to 4 months (Friedman and Bell in review b).

Because *P. margaritifera* were removed from collectors at a small size (10 to 30-mm DVM), and invertebrate and fish predators are widespread in Solomon Islands (Friedman et al. 1998, Friedman 1998), it is essential to nurse juveniles until they attain a "size refuge" (Coeroli et al. 1984). This process has been termed "intermediate culture" (Ventilla 1982), because it covers the culture stage between spat collection and transfer of oysters to chaplets. Because there is less emphasis on rearing juvenile pearl oysters in the "closed" atolls of Polynesia, there is a paucity of information relating to this stage in the culture process. Therefore, the aim of this study is to compare growth and survival of *P. margaritifera* in a number of intermediate culture systems. The information generated by this study will not only assist in developing appropriate culture protocols for pearl oysters in open reef sys-

tems, but will be valuable for those developing nursery culture systems for hatchery-reared *P. margaritifera* spat (Southgate and Beer 1997).

#### MATERIALS AND METHODS

There were two phases of this study. In Phase One (1994 and 1995), spat were harvested from collectors deployed at 21 sites spanning over 500 km of the Solomon Islands (Fig. 1). Oysters were grown out at nine of these sites (Fig. 1) on submerged longlines (Fig. 2). In Phase Two (1996 and 1997), spat were collected at 36 sites within the Western Province of the Solomon Islands, and grown out at one site in Gizo lagoon (Fig. 1).

When spat collectors were harvested, live oysters were placed in a number of intermediate culture units; lantern nets, panel nets, trays, and glued onto ropes (Fig. 3). Lantern nets consisted of a maximum of eight platforms surrounded with 6- or 12-mm netting. The platforms were positioned on a frame before being stocked with juveniles and enclosed with mesh. The size of the mesh used to contain the juveniles depended on the size of the oysters in the experiment. Panel nets were constructed from a galvanized wire frame covered with 6- or 12-mm netting. They had five horizontal rows that could be accessed to insert juveniles from holes cut into one side of the netting (Fig. 3). Trays were made from stiff perforated plastic (8.5-mm mesh) and had removable lids that allowed for inspection and removal of predators (Fig. 3). In a fourth culture unit, oysters were glued to 4-mm rope enclosed behind stiff plastic mesh (18-mm mesh size) (see Fig. 3).

#### Phase One

##### Growth of Juveniles in Lantern Nets at 3–4-m Depth

*P. margaritifera* juveniles (936 individuals) were cultured at nine sites throughout the Solomon Islands, in lantern nets suspended at 3 to 4-m depth, on longlines in shallow water reef areas (8 to 25-m depth). Lantern nets were checked every 3 months to remeasure juveniles, remove predators, clean or change the meshes, and record survival.

##### Growth of Juveniles of Different Sizes in Lantern Nets at 6-m Depth

In April 1995, an experiment was set up to assess the effects of two husbandry regimes on the growth and survival of two sizes of juveniles (10 to 25-mm DVM and 26 to 55-mm DVM). Lantern nets were suspended from a longline set at 6 m in Gizo lagoon, outside the reef flat in front of ICLARM's Nusa Tupe Research Station (NTRS) (Fig. 1). The longline was deployed in deeper water (25–30 m), running parallel to the reef edge (~15 m out from the reef edge). For each husbandry regime, eight (4 × 2 juvenile sizes) replicate lantern nets, each holding eight oysters (four juveniles per platform) were deployed. All oysters were marked individually with glued tags ( $n = 128$ ). In the first husbandry regime, meshes alone were cleaned of predators, epibionts, and fouling. In the second regime, both oysters and meshes were cleaned. This process was carried out fortnightly for 6 months, after which the second cleaning regime was used for both sets of lantern nets. Growth (DVM) and survival of juveniles was recorded after 3, 6, and 12 months.

#### Phase Two

For this phase of the study, longlines for growout of juveniles were set at a depth of 9–12 m, in 35 to 45-m of water, just to the northeast of NTRS (Fig. 1). Longlines were sited >50 m from fringing reef, over sandy substrate. The area chosen for deployment of longlines was within a section of Gizo lagoon that was approximately 1 km<sup>2</sup>, had a mean depth of ~40 m, and had numerous passages and submerged reefs linking the lagoon to the open ocean. In Phase Two, the timing of husbandry checks was increased to 2–3 times a month; SCUBA divers brushed off algal fouling and manually removed predators from growout units.

##### Comparison of Growth and Survival of Juvenile Oysters in Lantern Nets and Panel Nets

In Phase One, lantern nets had two deficiencies for the growout of juveniles: (1) larvae of invertebrate predators and particulate matter settled onto the platforms within the nets, and these plat-

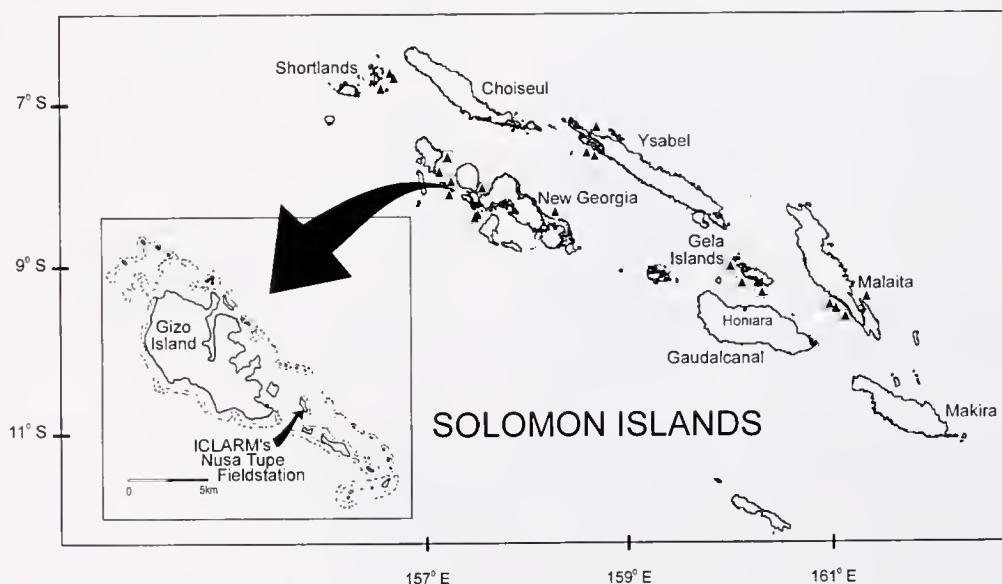


Figure 1. Twenty-one sites (triangles) where spat of *P. margaritifera* were collected and cultured (boxed triangles) in Phase One and, Gizo Lagoon (insert), where spat were held for growout trials in Phase Two of the study.

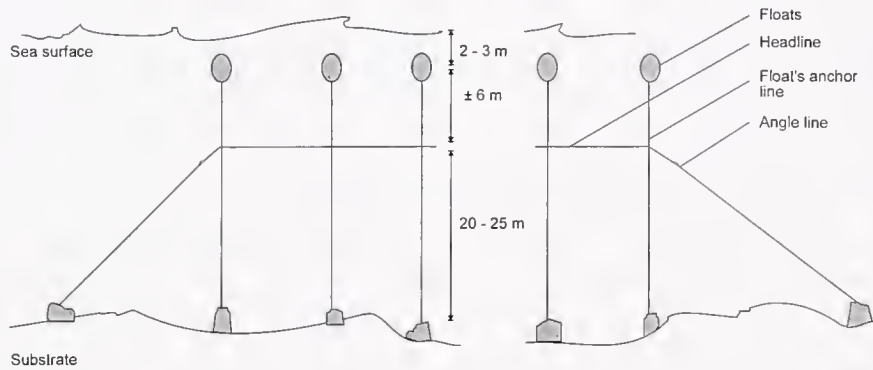


Figure 2. Longline system used to suspend growout units for juvenile *P. margaritifera*.

forms were difficult to access for cleaning; and (2) the flexible mesh on the sides of the nets made removal of algal fouling difficult.

Panel nets (Fig. 3) were trialed in an attempt to overcome some of these problems. Three experiments to compare growth and survival of juvenile *P. margaritifera* held in lantern nets and panel nets were conducted from 29 March to 29 September 1996 (Table 2). For the three experiments, juveniles of different mean sizes were used (DVM of 16- 24-, and 33-mm). To ensure that oysters were not lost through the meshes, juveniles of the smallest size class were enclosed behind 6-mm mesh; whereas, it was possible to use 12-mm mesh for the two experiments involving larger spat. Growth (DVM) and survival were recorded when units were removed from the water after 3 months.

#### Comparison of Growth and Survival of Juvenile Oysters in Panel Nets and Trays

Because panel nets also proved difficult to keep clean, an experiment was conducted using rigid perforated plastic trays that were easier to brush clean of algae. The first experiment compared growth and survival of juveniles of 24 mm in panel nets and trays and was run between 29 May 1996 and 29 August 1997 (Table 2). Growth (DVM) and survival were recorded when units were removed from the water after three months.

A second experiment was run from 29 January to 29 June 1997 (Table 2). In this experiment, we recorded growth (DVM), wet

weight, and survival of 11 mm juveniles when units were removed from the water after 5 months. Different colored threads were glued to 10 oysters per replicate at the start of the experiment, so that individual growth rates could be calculated.

#### Use of Cyano-Acrylate Glue in Intermediate Culture

Although management of plastic trays (Fig. 3) was faster and easier than panel nets, juveniles tended to form aggregations or "clumps" (Southgate and Beer 1997) in the trays. Juveniles "trapped" within these clumps had stunted development. Cyanoacrylate glue (Loctite 454 gel®) was assessed as a means of fixing juveniles to the sides and bottom of trays to prevent clumping and to determine whether spacing of oysters affected growth. Glue was also used to fix juveniles onto rope, which was then surrounded by stiff mesh of large size (Fig. 3). The mesh was too coarse to hold "loose" oysters but was stiff enough to be brushed clean of algal fouling.

Between 29 April and 29 September 1997, both growth (DVM and wet weight) and survival of juveniles were compared among four growout units: panel nets; trays with juveniles loosely added; trays with juveniles glued in place; and ropes with juveniles attached (glued) enclosed in large mesh. For each of the 10 replicates (25 juveniles per replicate), different colored threads were glued to 10 oysters at the start of the experiment so that individual growth rates could be calculated when growth units were removed from the water after 5 months.

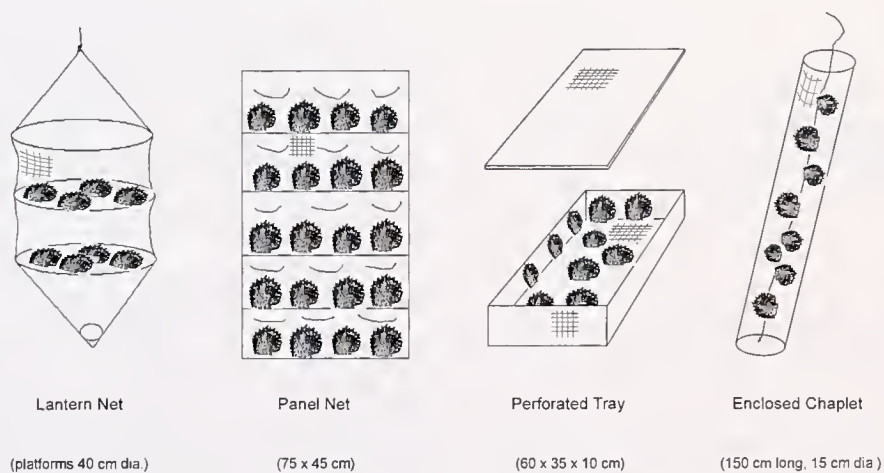


Figure 3. Units used for intermediate culture of *P. margaritifera* juveniles removed from spat collectors.

Growth (DVM and wet weight) and survival of juveniles attached to trays and ropes were also compared in a second experiment deployed between 2 May and 2 October 1997 (Table 2). Again, threads were used to mark juveniles individually.

#### General Growth Rates of Juveniles in Intermediate Culture

In 1996 and 1997, several different batches of oysters of different initial size, were reared in the intermediate culture systems described above. Growth (DVM) was measured after 3 and/or 5 months, and growth trajectories were plotted.

#### Analysis of Data

To examine differences in survival of juveniles reared in two types of culture units, *t*-tests were used in comparisons of live oyster number. In comparisons of survival among more than two types of culture units, a one-way analysis of variance (ANOVA) was used to analyze survival data.

To compare growth of juveniles marked individually, growth measurements from individual oysters were compared by *t*-test and, when appropriate, one-way ANOVA. To compare growth of juveniles in lantern nets, panel nets, and trays, the final size of juveniles were compared in each experiment using *t*-tests. In these tests, only subunits (e.g., platforms in lantern nets and rows in panel nets) and whole trays, which had 100% survival, were used.

Before *t*-tests or ANOVA, data were checked for homogeneity of variance using Levene's or Cochran's test, respectively, and transformed to  $\log_{10}(x+1)$  to meet this assumption, where necessary. Significant differences between means were identified using Tukey's HSD test.

## RESULTS

### Phase One

#### Growth of Juveniles in Lantern Nets at 3 to 4-m Depth

Spat removed from collectors immersed for 6 months had a mean DVM of 20.4 mm  $\pm$  0.4 SE ( $n = 936$ ), and a range of 3–61 mm. Annual growth increments of juveniles of 10–100 mm DVM, grown in lantern nets suspended from shallow water longlines, are shown as a Ford-Walford plot in Fig 4. The "growth performance

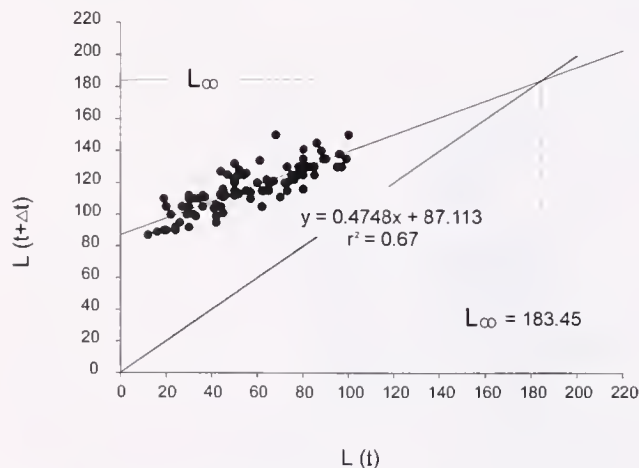


Figure 4. A Ford-Walford plot of annual growth of *P. margaritifera* juveniles ( $n = 101$ ) held in lantern nets at eight sites spanning 500 km of Solomon Islands.

indicator" or  $\phi'$  value, which can be calculated from the  $K$  and  $L_{\infty}$  derived from this plot ( $\phi' = \log K + 2 \log L_{\infty}$ , Munro and Pauly 1984) was 4.39. Survival of spat grown in lantern nets in shallow reef areas was poor and averaged 36.2%  $\pm$  8.4 SE, ( $n = 9$  sites).

#### Growth and Survival of Juveniles of Different Sizes in Lantern Nets at 6-m Depth

Both sizes of juveniles held in lantern nets at 6 m grew at rates similar to those held at 3 to 4 meters ( $y = 0.49x + 89.03$ ,  $r^2 = 0.29$ ,  $\phi' = 4.34$ ). Because of heavy mortality, it was impractical to analyze the differences in growth between husbandry regimes in this experiment. Only 49 (38.3%) of the 128 juveniles remained alive at the end of 6 months. Fish associated with a nearby reef caused the mortality, accessing the platforms by ripping the mesh of the lantern nets.

There were no significant differences in survival between the two husbandry treatments (Table 1). Mortality was, however, significantly greater than smaller-sized juveniles (Table 1). The decline in abundance of live juveniles over the course of this experiment was greatest in the first three months for the smaller size classes (Fig. 5).

### Phase Two

#### Comparison of Growth and Survival of Juvenile Oysters in Lantern Nets and Panel Nets

Growth of oysters was significantly greater in lantern nets for the smallest size class of juveniles, but no significant difference in growth was detected between the two growout units for the two larger size groups (Table 2, Fig. 6). Survival for juveniles held in lantern nets and panel nets for 3 months was 59.5 and 96.0%, respectively (Fig. 7). Survival of oysters was significantly greater in panel nets for the smallest size class of juveniles, but no significant difference in survival was detected between the two growout units for the two larger size groups (Table 2, Fig. 7). Survival for the two larger size groups of juveniles was good (mean 86.9%), corresponding to a period when settlement of predators (e.g., *Cyrtium* spp) was low.

TABLE 1.

Survival (%) at 3, 6, and 12 months of small (10 to 25-mm DVM) and large (26 to 55-mm DVM) *P. margaritifera* juveniles deployed in lantern nets and subjected to two husbandry regimes.

	Husbandry Regime	
	Clean Mesh and Oysters	Clean Mesh Only
3 Months	36.0 <sup>a</sup>	54.8 <sup>a</sup>
6 Months	34.4 <sup>a</sup>	42.3 <sup>a</sup>
Size of Oysters		
	Small (10–25 mm)	Large (26–55 mm)
3 Months	36.0 <sup>a</sup>	54.8 <sup>a</sup>
6 Months	25.0 <sup>a</sup>	51.6 <sup>b</sup>
1 Year	15.6 <sup>a</sup>	36.0 <sup>b</sup>

Means with the same superscript do not differ significantly by *t*-test ( $p < .05$ ).

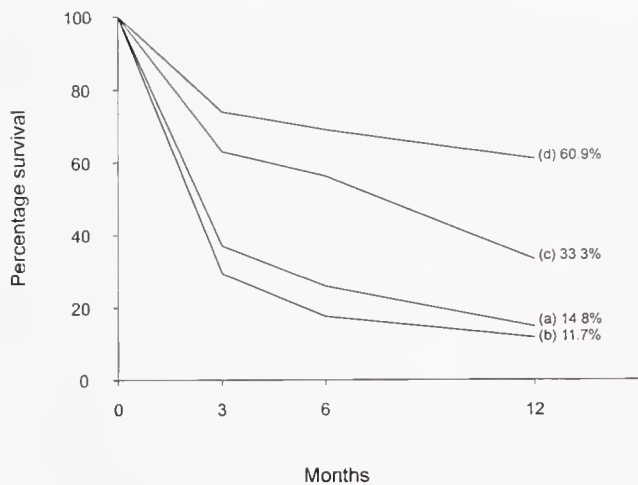


Figure 5. Percentage survival of *P. margaritifera* juveniles held in lantern nets after 3, 6, and 12 months. Oysters were divided into size classes: 10–19 mm (a), 20–29 mm (b), 30–39 mm (c), and 40 mm and above (d).

#### Comparison of Growth and Survival of Juvenile Oysters in Panel Nets and Trays

In the first experiment, juveniles in panel nets (46.97-mm  $\pm$  1.1 SE DVM) were significantly larger than juveniles held in trays (43.59-mm  $\pm$  0.7 SE DVM) after 3 months (Table 2). Survival in panel nets and trays averaged 94 and 96%, respectively, and did not differ significantly (Table 2).

In the second experiment, there was no significant difference in growth (DVM) of juveniles in panel nets (29.24-mm DVM,  $\pm$  0.56 SE) and trays (31.47-mm  $\pm$  1.3 SE DVM) after 5 months (Table 2). There was also no significant difference (df 16,  $t$ -value =  $-0.196$ ,  $p = .847$ ) in wet weight of juveniles between panel nets (11.68 g  $\pm$  0.47 SE) and trays (11.93 g  $\pm$  1.33 SE) after 5 months. Despite high mortality of oysters in two replicate trays because of predation by *Cymatium* spp. gastropods, there was no significant difference in survival between panel nets (70.0%  $\pm$  4.0 SE,  $n = 10$ ) and trays (60.5%  $\pm$  10.7 SE,  $n = 10$ ) (Table 2).

#### Use of Cyano-Acrylate Glue in Intermediate Culture

Growth (DVM) of glued oysters was significantly greater than that of oysters placed loosely into trays or in panel nets (Table 3, Fig. 8). There was no significant difference in growth increment between juveniles glued to ropes and juveniles glued to trays after 5 months (Table 3, Fig. 8). Survival was greatest for juveniles attached to rope enclosed behind mesh (88.4%  $\pm$  2.6 SE). Survival of oysters glued to trays (86.4%  $\pm$  2.4 SE) and in panel nets (82.4%  $\pm$  3.0 SE) was also high. Oysters not glued into trays had the lowest survival (74.8%  $\pm$  4.9 SE). Analysis of survival at 5 months was significantly different among the four culture units tested (df 3,36,  $F = 3.20$ ,  $p = .035$ ), however *post hoc* analysis (Tukey's HSD) only distinguished significant differences between oysters attached to rope and oysters "loose" in trays.

In the second experiment, average growth (DVM) of juveniles glued into trays (31.29 mm  $\pm$  1.71 SE) and onto rope (31.68 mm  $\pm$  0.64 SE) did not differ significantly (df 17,  $t$  value = 0.201,  $p = .843$ ) after 5 months. Difference in survival of juveniles glued onto trays (71.2%  $\pm$  6.10 SE) and glued onto rope (86.7%  $\pm$  3.26 SE) was significant (df 17,  $t$  value = 2.16,  $p < .05$ ); however,

because the two datasets were heterogeneous by Levene's test ( $p = .01$ ), this result should be viewed with caution.

#### Growth Rates of Juveniles in Intermediate Culture

The growth trajectory of 10 batches of oysters that entered intermediate culture at different sizes is shown in Figure 9.

## DISCUSSION

In the Solomon Islands, "intermediate" culture is required to nurse juvenile pearl oysters collected from the wild before they can be hung on chaplets. Juvenile *P. margaritifera* grew well in the culture units tested in this study; batches of juveniles with initial DVM of 8.3 to 51.5 mm increased in size by 20.4 to 24.8 mm in 3 months and 30.7 to 36.5 mm in 5 months. These growth rates compare favorably with growth of *P. margaritifera* reported from "closed" and semiclosed atolls in the Pacific. For example, in Takapoto atoll, French Polynesia, juveniles of 40 to 50-mm DVM grew 30 mm in 6 months (Coeroli et al. 1984, Lintilhac 1987); whereas, in the Cook Islands, Braley (1997) reported that hatchery produced *P. margaritifera* juveniles with a mean DVM of 10 mm grew approximately 16.4 mm DVM in 3 months. On the other hand, Sims (1993b) presented size-at-age data for 9-month-old *P. margaritifera* from Manihiki atoll as approximately 81 mm DVM, which indicate fast growth of juveniles in a "closed" atoll environment. However, the age of spat used in Sims's study may initially have been underestimated, because "median date" or "heaviest fall" was used to estimate the age of spat removed from collectors prior to the beginning of the growth study.

In the open reef systems of Dongonab Bay in Sudan, *P. margaritifera* juveniles collected as spat between 18 to 37-mm DVM grew by 13 to 24 mm and by 24 to 32 mm in 3 and 5 months of culture, respectively (Nasr 1984). However, in Sudan, there is little or no growth during winter (Nasr 1984). In Australia, hatchery-produced juveniles of *P. margaritifera* with a mean DVM of 13.9 mm, grew by 21.8 to 26.6 mm when held loosely in perforated plastic trays for 19 weeks (4.3 months) (Southgate and Beer 1997).

Our study demonstrates that growth is also dependant on the method used to hold juvenile oysters; that is, juveniles held under different intermediate culture conditions grew at different rates. A major difference between the systems used in this study was the ability to separate oysters. For example, oysters held in panel nets or glued into trays and on ropes were prevented from clumping; whereas, juveniles that were able to move around freely tended to form aggregations, such as those reported by Crossland (1957), Southgate and Beer (1997), and Sims and Sarver (1998). Oysters in the units where clumping occurred exhibited highly variable growth rates as a result of increased competition for food and space. In another study on *P. maxima*, Taylor et al. (1997b) reported that this behavior promoted an increase in the prevalence of growth deformities in juveniles.

Separation of juveniles in intermediate culture had the added advantage that units were easier to check for predators, because *Cymatium* spp. and crabs could not hide within clumps of oysters. The results of the trials where juveniles were stuck directly onto ropes highlighted the potential for holding juveniles behind meshes that were too large to contain oysters, but small enough to afford the growing juveniles some protection from fish. Although the use of cyano-acrylate glue gave some of the best growth rates in this study, adhesives are not a panacea for the problems en-

TABLE 2.

Results of experiments to assess growth and survival of *P. margaritifera* juveniles in various culture units in Phase 2 of the study (between March 1996 and October 1997).

Experiment	Replicates	Oysters per Replicate	Mean Starting Size (DVM)	Survival			Growth (DVM)		
				df	t-value	p	df	t-value	p
a) Lantern vs. panel nets									
29/3/96 to 29/6/96	12	20	16 mm	22	-3.77	0.001	250	3.81	<0.000
29/5/96 to 29/8/96	12	25	24 mm	22	-1.91	0.069	510	0.16	0.870
29/6/96 to 29/9/96	8	20	33 mm	14	-1.45	0.168	184	-0.66	0.507
b) Panel nets vs. trays									
29/5/96 to 29/8/96	12	25	24 mm	22	0.52	0.607	460	-2.79	0.006
29/1/97 to 29/6/97	10	20	11 mm	18	0.82	0.418	16	-1.66	0.116
c) Trays (glued) vs. ropes (glued)									
2/5/97 to 2/10/97	10	25	34 mm	17 <sup>a</sup>	2.16	0.045	133	0.64	0.521

<sup>a</sup>One rope was lost in this experiment.

countered in growout; cyano-acrylate is both expensive and difficult to apply.

Because the central south-western Pacific [e.g., Fiji, Vanuatu, Papua-New Guinea (PNG)] has few "closed," deep-water lagoons, pearl oyster growout under these conditions contrasts with growout in the atoll lagoons of the eastern Pacific. Whereas atoll lagoons are surrounded by low-lying carbonate islands, the reef systems in the Solomon Islands are subject to relatively large inputs of nutrients and particulate matter from high islands (Littler et al. 1991). The higher nutrient load in the lagoons of Solomon Islands may have been a factor in the good growth rates recorded in this study (Yukihira 1998). However, the negative side of this is the increased algal fouling when compared to the relatively nutrient-poor atoll lagoons of Polynesia. In the Solomon Islands, meshes of culture units required regular cleaning; thereby, increasing labor needs. Although meshes required cleaning, there was relatively little fouling by such "cementing" organisms as bivalves and polychetes ("hard" fouling). Algal fouling is easier to remove than "hard" fouling, and regular brushing for this purpose may have inhibited recruitment and survival of hard-fouling organisms and other byssally attached bivalves, which have been shown to be

a problem during growout of other pearl oyster species (Taylor et al. 1997a). In addition to being simpler to remove, algal fouling does not directly compete with juveniles for food resources and space. We also found that control of algal fouling was easier when culture units were made of stiff plastic meshes. This material was more practical and cost effective than flexible mesh, which could not be easily cleaned and had limited potential for reuse.

Coeroli et al. (1984) reported that 30% of 6 to 12-month-old *P. margaritifera* juveniles were lost in culture in French Polynesia and stated that fishes from the family Balistidae and Tetraodontidae were the chief predators of pearl oysters. In initial trials in the Solomon Islands, fish devastated juveniles in intermediate culture when longlines were deployed too close to reefs (Friedman et al. 1996). Although there was no direct comparison between growout culture in shallow and deeper water in this study, the lack of broken shells in culture units on longlines placed farther from reefs, and evidence presented in other studies (Sims and Sarver 1995), supports the inference that juvenile pearl oyster culture conducted at a distance from reefs and in deeper water reduces predation by fish. For example, in the Marshall Islands 5.5% of juveniles were lost to fish predation on longlines set in deeper

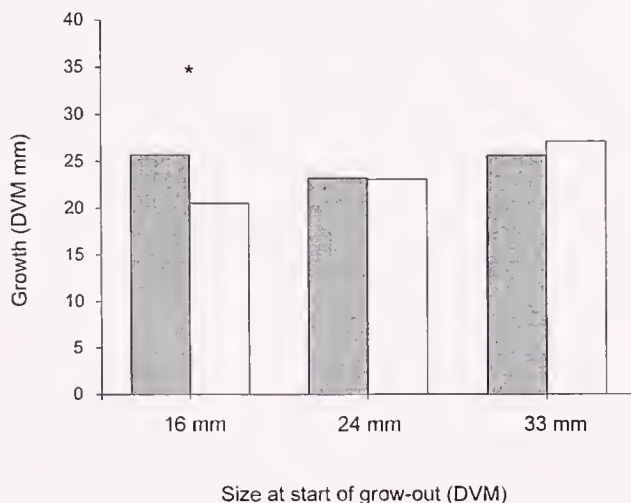


Figure 6. Growth (final DVM—mean DVM at start of experiment) in shell size (DVM mm) of *P. margaritifera* juveniles held for 3 months in lantern nets (shaded) and panel nets (open).

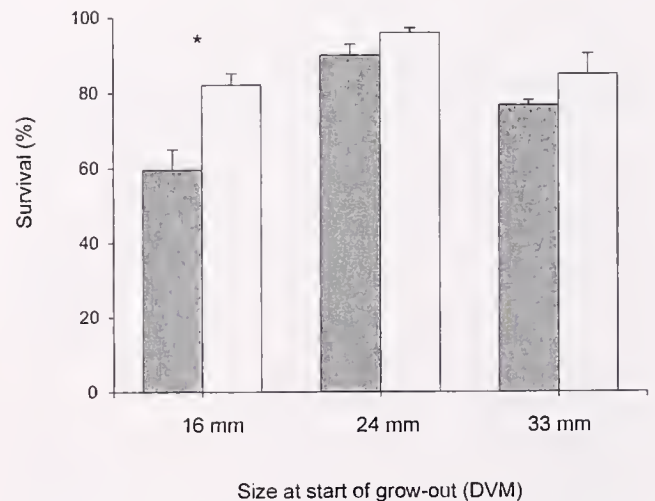


Figure 7. Percentage survival ( $\pm$ SE) of *P. margaritifera* juveniles held in lantern nets (shaded) and panel nets (open) for 3 months. Columns marked with an asterisk differed significantly in *t*-tests.

TABLE 3.

Results of one-way ANOVA for effects of culture unit on a) increase in mean shell height (DVM), b) increase in mean wet weight, and c) survival of juvenile *P. margaritifera*, grown for 5 months.

	df	MS	F	p
a) Growth (DVM, mm)				
Culture unit	3	231.771	29.299	.0000
Residual	36	7.911		
b) Change in wet weight (g)				
Culture unit	3	423.122	21.346	.0000
Residual	36	19.822		
c) Survival				
Culture unit	3	22.567	3.201	.0350
Residual	36	7.050		

The four culture units in this study were: panel nets; trays with juveniles loosely added; trays with juveniles glued in place; and ropes with juveniles attached (glued), enclosed in large mesh.

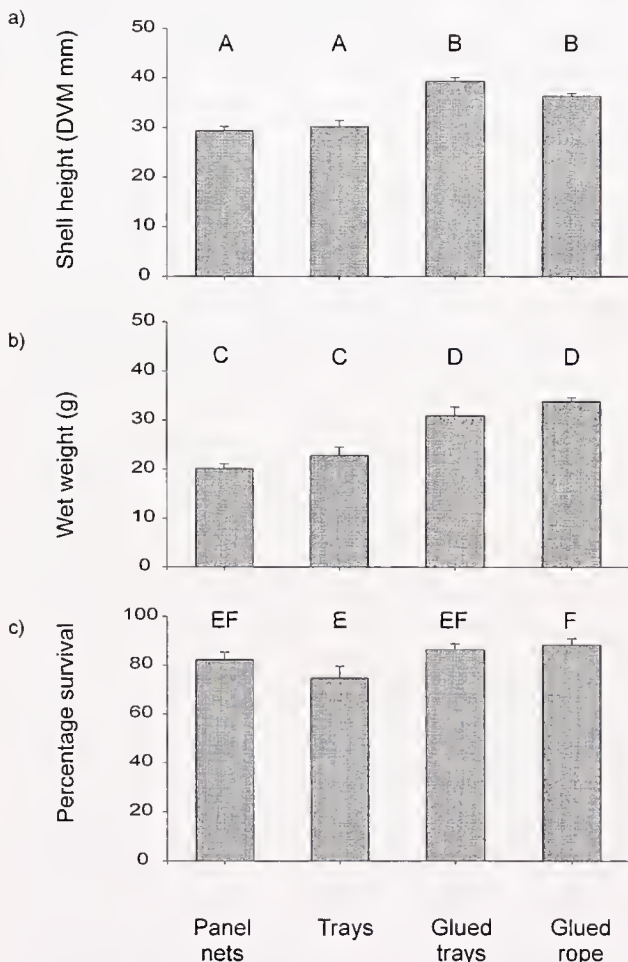


Figure 8. Changes in mean ( $\pm$ SE) a) mean shell height, b) wet weight, and c) survival of juvenile *P. margaritifera* held in growout units after 5 months. Columns with the same letter do not differ significantly ( $p < .05$ ) in *post hoc* tests (Tukey's HSD).

water, as compared to 25.5% losses on shallow water longlines (Sims and Sarver 1995). In French Polynesia, pearl farmers hold young oysters behind galvanized wire mesh to isolate oysters from attacks by balistid fish (Lintilhac 1987). Smaller fish also had an effect on growth. In this and other studies (Sims 1993a; Southgate and Beer 1997), small balistids and tetraodontids "grazed" on the non-nacreous shell margins of juvenile oysters in culture. In the Solomon Islands, this was only found to be significant at embayed inshore sites, and relocation of growout longlines to less turbid areas with greater water movement reduced or eliminated this problem.

Once longlines were located in areas free from predation by reef fish, survival of juveniles was influenced primarily by invertebrate predation. Invertebrate predators, such as *Cymatium* spp. (Govan 1995), crabs, and flatworms (Newman et al. 1993, Taylor et al. 1997b) recruited into growout units from the plankton (Dayton et al. 1989, Newman et al. 1993, Friedman and Bell 1996, Friedman 1998). The effect on the hydrodynamics of water flow has been presented as a major determinant of the success of culture unit design (Claereboudt et al. 1994). In the Solomon Islands, invertebrate predators were found in all growout units, but were more common in lantern nets and trays than in units that had smaller holding spaces (e.g., panel nets) or less scope for reducing water flow (e.g., glued ropes). This may have been caused by differences in water flow characteristics that could have influenced settlement of suspended particulate matter and the larvae of potential predators.

Predators that settle within nondivided culture units (e.g., trays) had access to all the juveniles within that unit. On two occasions

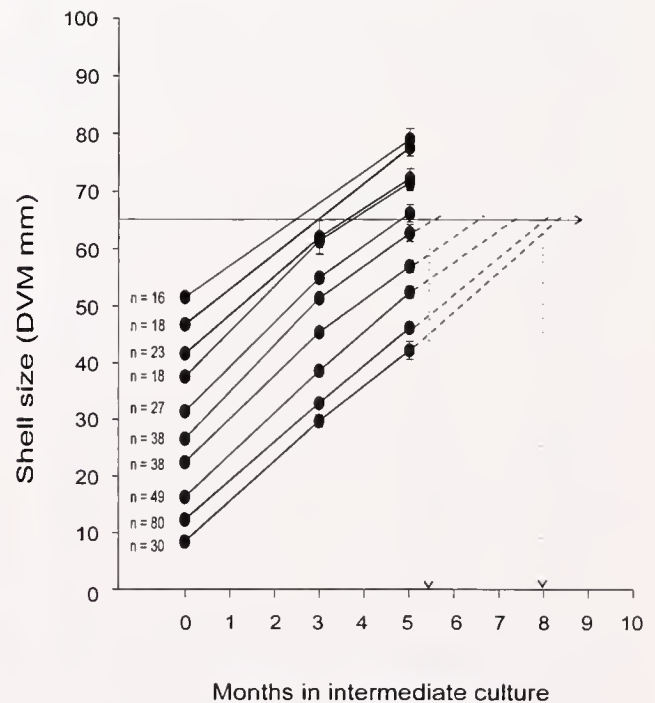


Figure 9. Growth trajectories for *P. margaritifera* juveniles of different sizes ( $\pm$ SE) grown in intermediate culture. Once oysters reach a size of approximately 65-mm DVM (marked on graph), they can be drilled for ear hanging. Trajectories that have been extended past the 5-month period (dashed lines), were added using real data from growth experiments involving larger juveniles.

in this study, 20 juveniles ( $\approx 11$  mm DVM) were killed by a *Cymatium* spp. within a single tray. To combat the problem of sequential juvenile predation in intermediate culture, one of two strategies can be adopted: (1) juveniles could be separated into small groups by mesh barriers; or (2) units could be designed to make them easier to inspect for predators. The first strategy relies on the predator outgrowing the mesh size of a single compartment in the growout unit and being prevented from entering other compartments of the culture unit. One such system has been successfully trialed in the Marshall Islands, where settlement of *Cymatium* spp. had previously devastated hatchery-produced *P. margaritifera* juveniles in nursery culture (Sarver et al. 1998, Sims and Sarver 1998). In the Solomon Islands, the second strategy was adopted for three reasons: (1) because in intermediate culture of spat taken from collectors, there is no need to deal with very small spat ( $< 6$ -mm DVM), which are difficult to handle and check for predators; (2) multispaced culture units are difficult to set up and keep free of fouling; and (3) because staff in the Solomon Islands could quickly recognize settlement periods of such predators as *Cymatium* spp. and respond accordingly. Although no reliable seasonal trends in settlement of the main predator groups have been recognized in the Solomon Islands, divers increased the intensity of inspections when large numbers of newly settled predators were found. In this way, predators were removed from growout units when they were small—before they caused significant juvenile mortality.

This study showed that there was a positive relationship be-

tween the size of juveniles placed into intermediate culture and their survival. Also, Coeroli et al. (1984) suggested that oysters over 50-mm DVM were "resistant to attacks from predators." In the Solomon Islands, oysters that had reached  $\approx 65$ -mm DVM could be removed from intermediate culture and grown on chaplets without a protective mesh covering.

In summary, this study has shown that within the open-reef systems typical of the western Pacific: (1) the rate of growth of juveniles compared favorably to that reported for the closed-atoll lagoons of Polynesia. In the Solomon Islands, juveniles of 10-mm DVM placed in intermediate culture generally attained a size suitable for transfer to chaplets ( $\approx 65$  mm DVM) in 8 months. Those entering intermediate culture at a size of 25- to 30-mm DVM were ready for moving to chaplets after 5–6 months; (2) the siting of longlines in deepwater decreased mortality attributed to fish associated with reef; (3) important characteristics of pearl oyster growout units include ease of cleaning and access for regular inspection and removal for invertebrate predators; and (4) separating oysters in intermediate culture resulted in more uniform growth.

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## A NEW EPIZOOTIC OF *HAPLOSPORIDIUM NELSONI* (MSX), A HAPLOSPORIDIAN OYSTER PARASITE, IN LONG ISLAND SOUND, CONNECTICUT

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**ABSTRACT** Epizootic prevalences of MSX, *Haplosporidium nelsoni*, were detected in eastern oysters (*Crassostrea virginica*) at several sites in Long Island Sound, Connecticut. Diagnosis was based on histological sections. Twenty-two sampling stations representing commercially important oyster grounds were tested. Three sampling stations were negative for MSX, the rest had prevalences from 7 to 89% (319 MSX positive specimens out of 653 processed). Two major seed areas (Housatonic River and Bridgeport Natural Bed) were uninfected, but all studied shallow (<15 feet) or deep (>15 feet) water growing areas were infected along the whole shoreline. Hatchery-raised seed, uninfected when imported to Connecticut, acquired infection in New Haven and Groton. Sporulation with acid-fast spores was detected in 1% of MSX-positive specimens. One specimen showed unusual pattern of sporulation with spores inside ciliated digestive duct cells and vesicular connective tissue in addition to digestive tubules. The percentage of potentially resistant specimens was low (<1%). A periodic range extension of an alternate host, usually restricted to the mid-Atlantic region of the U.S. coast, is suggested as the cause for the recent epizootics in Long Island Sound.

**KEY WORDS:** *Haplosporidium nelsoni*, epizootic, eastern oyster, *Crassostrea virginica*, Long Island Sound

### INTRODUCTION

MSX-disease, caused by a protozoan parasite *Haplosporidium nelsoni*, has caused heavy mortalities of the eastern oyster, *Crassostrea virginica* (Gmelin, 1791) in the Delaware Bay since 1957 (Haskin et al. 1966) and in the Chesapeake Bay since 1959 (Andrews and Wood 1967). MSX has been detected from Biscayne Bay, Florida, to Damariscotta River, Maine (Haskin and Andrews 1988), but has not been associated with mortalities in all areas. Systematic monitoring for prevalences of MSX in Long Island Sound had not been performed before the autumn of 1997. MSX was present in oysters (in 10 out of 1,337 oysters) collected 1966 to 1967 from New Haven Harbor, Connecticut, in Long Island Sound (Newman 1971). Hammonasset River, Clinton, Connecticut, had a prevalence of 32%, and Bridgeport had 18% in 1985 (Haskin and Andrews 1988). Oysters from the Hammonasset River were transplanted the year before to Cotuit, Massachusetts, where MSX-related mortality of 85% was estimated by the end of summer 1985 (Matthiessen et al. 1990). That same year, heavy MSX-related mortalities occurred on the south shore of Long Island Sound at the Flowers Hatchery, New York, where approximately 90% mortalities were reported (Haskin and Andrews 1988). MSX-related mortality data from Connecticut's shoreline at that time was not available, but production fell from 243,883 bushels in 1984 to 69,721 bushels in 1987 (DEP 1995). Haskin and Andrews (1988) wrote: "A nagging question over the past 20 years has been, What is preventing massive epizootics, like those in the Chesapeake and Delaware bays, from occurring in the eastern oyster areas extending from Great South Bay, Long Island, to the tip of Cape Cod?" We report a new epizootic of MSX in Long Island Sound, Connecticut.

### MATERIAL AND METHODS

Connecticut's oyster industry is based on grounds leased from the state. Over 46,000 acres of underwater farms are cultivated. Every oyster is transplanted on the average four times before it reaches market size (3–4 years), and, consequently, every oyster is exposed to possible parasitic infection in several different sites

during its lifetime. The oyster industry is based mostly on natural seed, but also hatchery-raised seed is deployed in some areas, especially in the eastern end of the state. Major seed areas are situated up such rivers as the Housatonic and Quinnipiac, and are also close to the shoreline in Bridgeport. These areas are prohibited from shellfishing for direct consumption. The oysters are transplanted to clean water before marketing. Growing areas are classified either approved (A), conditionally approved (CA), restricted (R), conditionally restricted (CR), or prohibited (P) according to National Shellfish Sanitation Program (1997). Sampling strategy for this study was to select different types of oyster grounds to represent the coastline: seed areas up the rivers and close to the shoreline, and shallow (<15 feet) and deep (>15 feet) water growing areas; 22 sites altogether. Several samples were taken from oyster growing areas of economic interest, such as Norwalk. Sampling started in the end of August 1997, and the last sample included in this paper was accessed in the end of May 1998. Sampling stations from west to east are listed in Figure 1 and Table 1. Thirty oysters were collected from each sampling station (28 from two, 27 from one sampling station). Tissues were fixed in Davidson's fixative (in 20‰ artificial seawater). Six- $\mu$ m thick paraffin sections were stained with hematoxylin-eosin. Advanced MSX cases with migration of plasmodia to the digestive tubules, possible prespores or spores were also stained with Ziehl and Harris' hematoxylin to detect acid-fast spores, according to Farley (1965). MSX infections were classified as initial, intermediate, advanced, terminal, or occult, according to Farley (1968).

### RESULTS

Six hundred fifty-three (653) oysters were sectioned for detecting MSX. Three hundred nineteen (319) were diagnosed with MSX disease. Prevalences of MSX at different sampling sites, listed in Table 1, varied from 0 to 89%. Major seed areas, Housatonic River and Bridgeport Natural Bed, were uninfected at this point, but another major seed area, Quinnipiac River, sampled in spring 1998, had a prevalence of 53%. Shallow as well as deep water growing areas were infected with MSX. Norwalk Bed 1131

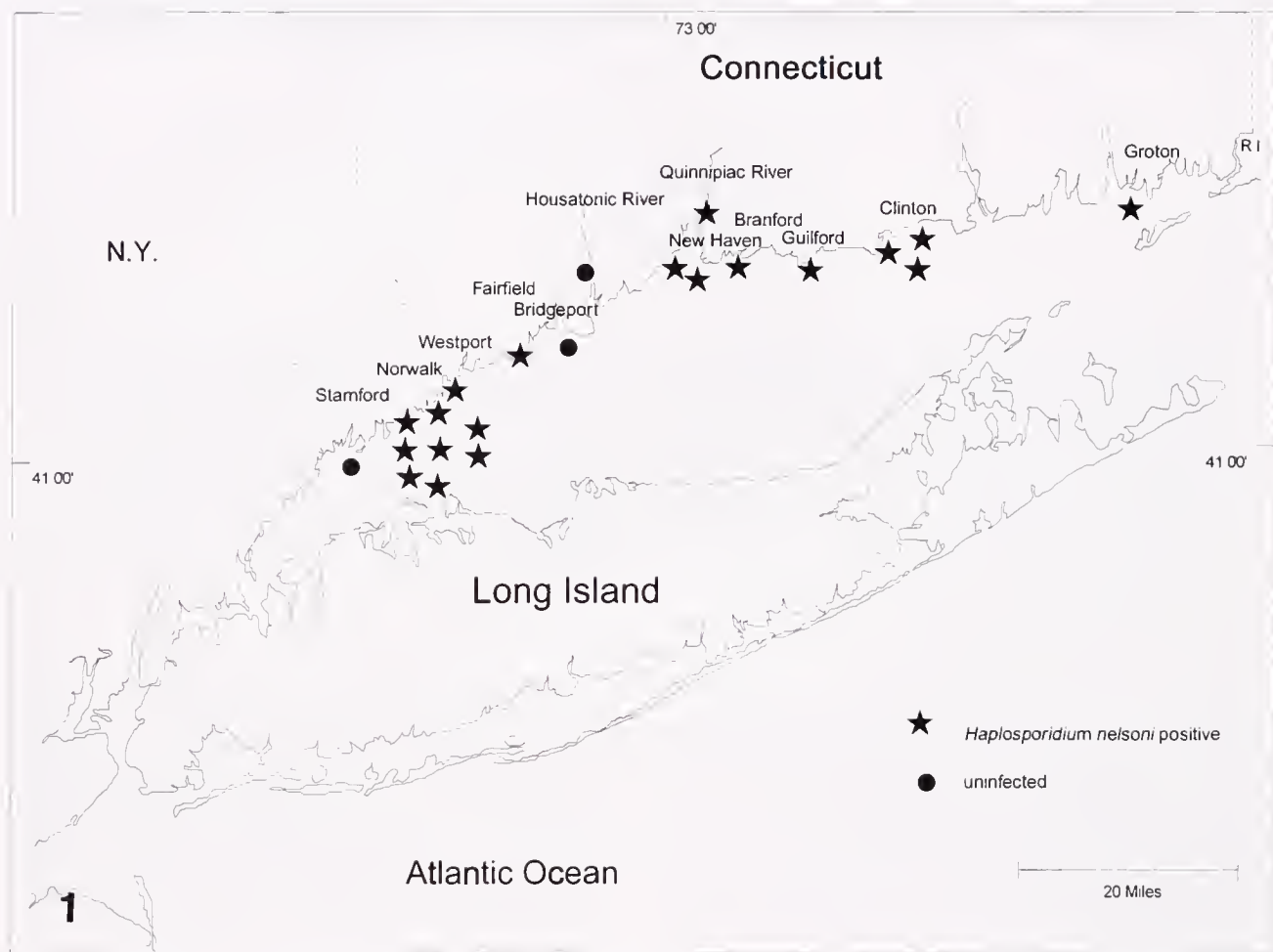


Figure 1. A map of sampling stations for detecting *Haplosporidium nelsoni* infection in Long Island Sound, Connecticut.

was sampled at the depth of 47 feet with prevalence of 70%; Westport 1088 at 43 feet with prevalence of 83%. Hatchery-raised seed acquired infection in Groton (27%) and two sites New Haven (43 and 29%). Seed, which was imported from Long Island, NY, has been uninfected when tested before import into Connecticut's waters. Infection stages were classified as follows.

**Initial Infection: Plasmodia Limited to Gill Epithelium**

First, plasmodia were detected between epithelial cells outside basement membranes. After that, plasmodia invaded hemolymph sinuses and interlamellar junctions. Hyaline hemocytes aggregated around parasitic infection areas. There was one case with initial infection originating from digestive epithelium (which might also be a secondary infection after sloughing of infected gill epithelial cells) and one case with infection starting from water tubes.

**Intermediate Infection: Isolated Plasmodia in the Digestive Diverticula in Addition to the Gill Infection**

Typical areas for plasmodia at this stage were the typhosole and vesicular connective tissue surrounding the stomach and intestine.

**Advanced Infection: Plasmodia Dispersed Throughout the Tissues**

Plasmodia have invaded the reproductive system and hemolymph vessels. There was usually a massive hyaline hemocyte response.

**Terminal Infection: Profuse Parasitemia in All Tissues**

This stage precedes death of the oyster. Tissue lysis may be present, and several specimens were sterilized by parasitic infiltration to the follicles.

**Occult: Tissues with Physiological Presentation of MSX Infection but No Plasmodia Detectable in Section**

Classical signs of MSX infection were massive hyaline hemocyte response and perivascular hyaline hemocyte infiltration in the mantle (cuffing).

**Resistant: Moribund and Phagocytosed Plasmodia or Plasmodia Retained Outside Basement Membranes in Gills**

Distribution of different stages—initial, intermediate, advanced, terminal, occult, or resistant, at different sampling stages are listed in Table 2.

Distribution of stages through the sampling period from August 1997 to May 1998 remained surprisingly unchanged. Active infection seemed to continue through the winter in the samples; for example, in Branford 179, which was collected in March, but had full-blown MSX disease with initial and intermediate infections. Four cases out of 319 MSX-positive specimens had the histological characteristics of resistance: one had gill infection with moribund plasmodia outside the basement membrane in the gills, one

TABLE 1.  
Prevalence of *Haplosporidium nelsoni* at different sampling stations in Long Island Sound, Connecticut.

Sampling Site	Location	Sampling Date	Shell Length (mm)	Prevalence (%)	Characterization of Site	Classification
Stamford 494	41°01.05'N: 73°31.96'W	01.02.98	61	0	Shallow-water growing area	RR
Norwalk 1131	41°02.42'N: 73°25.25'W	12.07.97	77	70	Deep-water growing area	A
Norwalk 173	41°03.38'N: 73°25.27'W	04.29.98	73	37	Shallow-water growing area	CA
Norwalk 162	41°03.36'N: 73°25.12'W	12.07.97	74	87	Shallow-water growing area	CA
Norwalk 176	41°03.65'N: 73°25.00'W	04.29.98	82	47	Shallow-water growing area	CA
Norwalk Manresa Island	41°04.42'N: 73°24.55'W	12.17.97	80	57	Shallow-water growing area	P
Norwalk town grounds	41°04.85'N: 73°23.55'W	09.16.97	91	89	Seed area	RR
Norwalk 40	41°04.57'N: 73°23.28'W	12.17.97	89	63	Shallow-water growing area	RR
Westport 1088	41°02.73'N: 73°22.21'W	12.07.97	78	83	Deep-water growing area	A
Westport 254	41°04.50'N: 73°22.60'W	08.27.97	85	11	Shallow-water growing area	CA
Fairfield Ash Greek	41°09.21'N: 73°14.00'W	12.16.97	78	7	Seed area	P
Bridgeport natural bed	41°08.50'N: 73°09.40'W	11.12.97	72	0	Seed area	RR
Housatonic River	41°11.00'N: 73°07.52'W	11.12.97	80	0	Seed area	P
New Haven lot 1B	41°15.47'N: 72°55.26'W	01.08.98	43	43	Hatchery seed on-bottom culture	RR
New Haven lot 1A	41°15.33'N: 72°55.56'W	01.08.98	46	29	Hatchery seed on-bottom culture	RR
Quinnipiac River	41°18.75'N: 72°53.12'W	05.26.98	94	53	Seed area	P
Branford 179	41°15.73'N: 72°45.63'W	03.02.98	87	83	Shallow-water growing area	A
Guilford East River	41°16.05'N: 72°39.62'W	09.28.97	91	73	Seed area	RR
Clinton Hammonasset River	41°15.97'N: 72°32.84'W	12.07.97	101	63	Seed area	RR
Clinton Cedar Island	41°15.97'N: 72°32.00'W	10.14.97	104	83	Seed and shallow-water growing area	RR
Clinton Hammock River	41°16.05'N: 72°31.30'W	12.07.97	97	70	Seed and shallow-water growing area	RR
Groton Pine Island Bay	41°18.99'N: 73°03.58'W	01.09.98	65	27	Hatchery seed off-bottom culture	CR

A = approved; CA = conditionally approved; R = restricted; CR = conditionally restricted; P = prohibited.

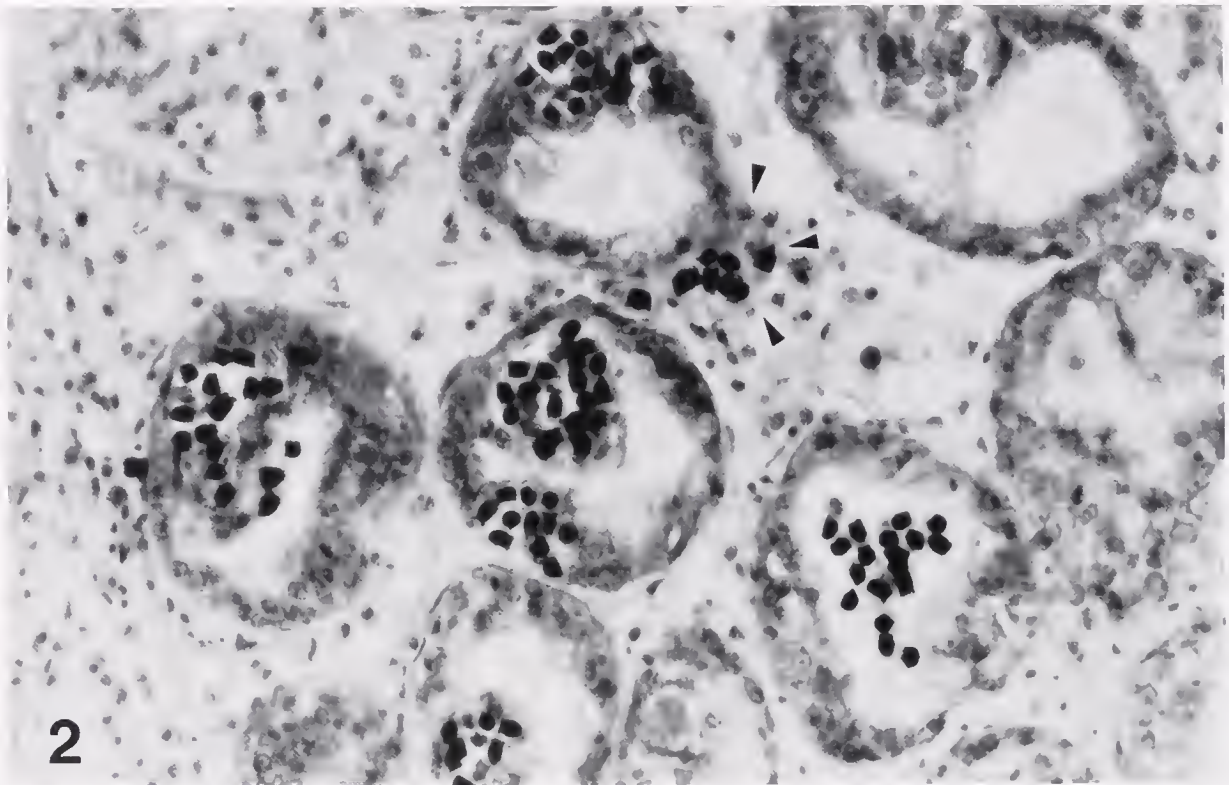
with moribund plasmodia outside the basement membrane and a few phagocytosed plasmodia inside interlamellar junction, and two with moribund and phagocytosed plasmodia inside a hemolymph vessel.

Sporulation rate was three out of 319 MSX-positive specimens (0.94%). Prespores and spores, which did not respond to acid-fast

stain, were observed in two additional animals. Sporulation showed an unusual pattern in one of the specimens. In addition to sporulation in digestive cells in digestive tubules (Fig. 2), there were several foci of spores in the ciliated digestive duct cells (Fig. 3). Nests of spores inside digestive cells broke outside digestive tubules creating disseminated accumulations of spores in vesicular

TABLE 2.  
Progression of *Haplosporidium nelsoni* at different sampling stations in Long Island Sound, Connecticut.

Sampling Site	Sample Size	MSX Positive	Initial	Intermediate	Advanced	Terminal	Occult	Resistant
Norwalk 1131	30	21	3	4	7	7	0	0
Norwalk 173	30	11	8	2	1	0	0	0
Norwalk 162	30	26	2	5	9	3	6	1
Norwalk 176	30	14	7	4	1	0	1	1
Norwalk Manresa Island	30	17	4	5	4	1	3	0
Norwalk town grounds	27	24	3	7	6	5	3	0
Norwalk 40	30	19	2	5	5	4	2	1
Westport 1088	30	25	2	7	7	0	9	0
Westport 254	28	3	3	0	0	0	0	0
Fairfield Ash Greek	30	2	0	2	0	0	0	0
New Haven lot 1B	30	13	0	3	4	6	0	0
New Haven lot 1A	28	8	3	3	2	0	2	0
Quinnipiac River	30	16	3	5	2	4	0	0
Branford 179	30	25	4	5	13	3	0	1
Guilford East River	30	22	3	3	9	2	5	0
Clinton Hammonasset River	30	19	1	3	1	0	14	0
Clinton Cedar Island	30	25	1	4	8	4	8	0
Clinton Hammock River	30	21	2	2	7	4	6	0
Groton Pine Island Bay	30	8	3	3	1	0	1	0



**Figure 2.** Sporulation of *Haplosporidium nelsoni* in the digestive diverticula of the eastern oyster. Note the presence of spores in the connective tissue (arrows) in addition to inside digestive cells and the lumen of digestive tubules. Paraffin section, Ziehl and Harris' hematoxylin for acid-fast spores. 400 $\times$ .

connective tissue (Fig. 2). Hemocytes were observed in the vesicular connective tissue with phagocytosed spores (Fig. 4). Usually, spores matured inside digestive cells and were released in the lumen of digestive tubules. Spores were also observed inside the lumens of digestive ducts and intestine. Plasmodia were observed in different stages of endomitosis (Fig. 5).

Gross macroscopic signs of MSX infection as described by Farley (1968); mantle recession, conchiolin deposits, and pale digestive diverticula, were not explicit in these samples. Pale digestive diverticula were recorded in 85 of 259 (33%) MSX positive specimens, but also in 44 of 334 (13%) uninfected specimens ( $\chi^2 = 28.6$ ; df 1;  $p < .005$ , occult MSX positives not included). Mantle recession was defined as an unprotected marginal area of the shell, which became heavily fouled. Mantle recessions were not often noticed, possibly because of the recent outset of the epizootics, so they had not developed. One shell with conchiolin deposits—an indication of resistance—was found from the Fairfield Ash Creek oyster bed. This bed, unlike the others, had a light level of infection and possibly represents a population with enzootic history of MSX.

There were co-infections with several other organisms. The probability for a co-infection with *Perkinsus marinus* (based on Ray/Mackin tissue assay, Ray 1954) in MSX-positive (176/259, 68%, occult not included) or MSX-negative specimens (240/334; 72%) showed no statistically significant difference ( $\chi^2 = 0.29$ ; df 1; NS). *Nematopsis ostrearium* was present in MSX-positive specimens (69/259, 27%), but also in uninfected specimens (81/334; 24%;  $\chi^2 = 0.34$ ; df 1; NS). The probability for co-infection with *Stegotricha*-like ciliates in the digestive diverticula was present in MSX-positive specimens (37/259, 14%), but also in uninfected

specimens (40/334; 12%;  $\chi^2 = 0.59$ ; df 1; NS). Ciliates on gills, mantles, or palps (several different species from the family *Ancistrocomidae*) were more likely to be found in uninfected (123/334, 37%) than in MSX-infected specimens (53/259; 20%;  $\chi^2 = 13.25$ ; df 1;  $p < .005$ ). This result was polarized by a high prevalence of a species of a small ciliate in one of the uninfected sites (Stamford, 37%) and the off-bottom site in Groton (53%). Several fouling organisms affected the shells—boring sponges (*Cliona* sp. several different species), and mud worms (*Polydora websteri*) were commonly found. *Cliona* sp. were found on MSX-positive specimens (83/259; 32%) and uninfected specimens (120/334; 36%;  $\chi^2 = 0.64$ ; df 1; NS). *Polydora websteri* was found in uninfected (155/334; 46%) and MSX-infected specimens (92/259; 36%;  $\chi^2 = 4.14$ ; df 1,  $p < .025$ ). This result was influenced by a 100% prevalence in an uninfected Housatonic River seed sample.

## DISCUSSION

The beginning of epizootics in the Chesapeake and Delaware Bays demonstrated that infection started in the bay proper and spread to seed areas or rivers later (Andrews and Wood 1967, Ford and Haskin 1982). A similar pattern may appear in the Long Island Sound epizootics. At the time of sampling, some major seed areas were uninfected, but some, sampled later, were infected (Table 1), and the uninfected seedbeds may acquire infection later. The limiting low salinity parameter does not necessarily exist in Long Island Sound. Major oyster grounds, both seedbeds and growing areas, are situated within optimum salinity levels for MSX, from 15 to 25‰ (Andrews 1979). In Delaware Bay, disease-free stocks

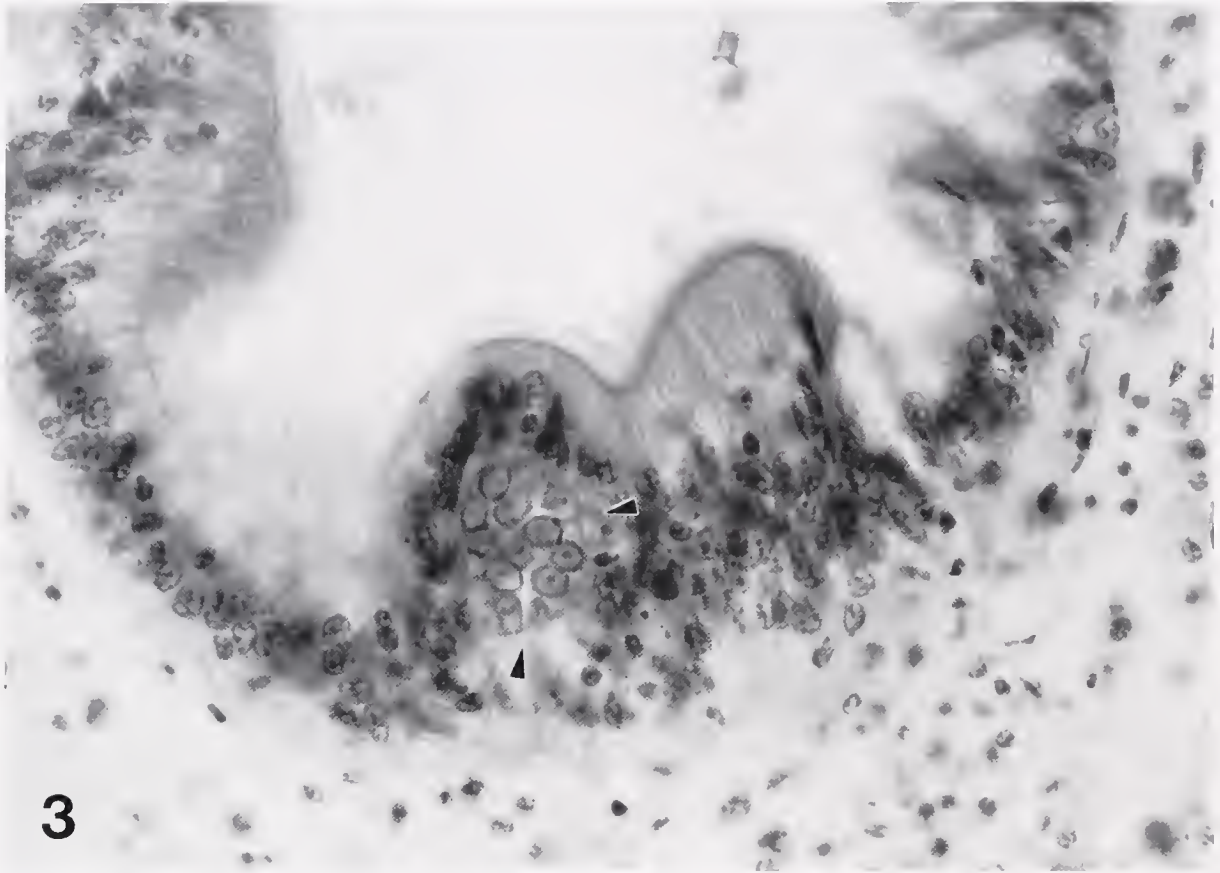


Figure 3. Unusual sporulation of *Haplosporidium nelsoni* inside digestive duct cells (arrows). Note ciliated digestive duct cells. Paraffin section, H & E, 600x.

are obtainable from low-salinity rivers (Andrews 1979). According to Farley (1975), drought and accompanying higher salinity facilitated spreading of the disease in the estuaries in the Chesapeake Bay. Management strategies for MSX and Dermo diseases in the Chesapeake Bay are based on using low-salinity sanctuaries in rivers for the limited oyster industry (Krantz and Jordan 1996).

Water temperature cannot be considered to be critical to the ability of MSX to reach epizootic prevalences, because epizootics have been reported in the northeast in Massachusetts (Matthiessen et al. 1990), but not in MSX-positive areas south of the Chesapeake Bay, such as North and South Carolina, Georgia, or Florida (Haskin and Andrews 1988).



Figure 4. Phagocytosis of *Haplosporidium nelsoni* spores by hemocytes (arrow). Paraffin section, Ziehl and Harris' hematoxylin, 1,000x.

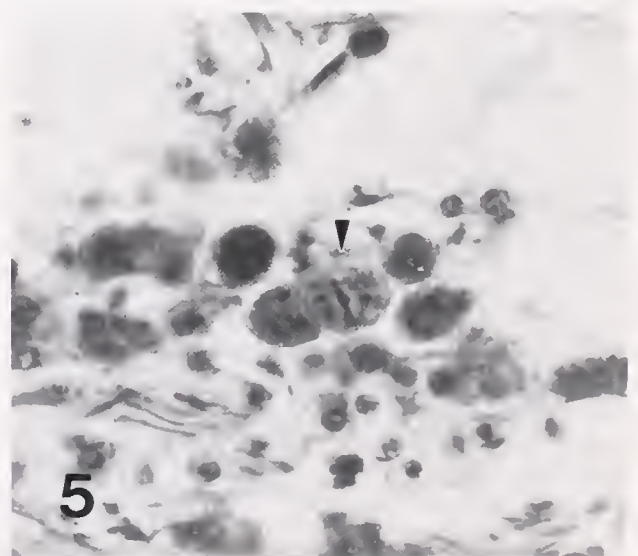


Figure 5. Endomitosis of *Haplosporidium nelsoni* plasmodia (arrow). Paraffin section, H & E, 500x.

Classification of different MSX stages was adopted from Farley (1968), because it has clinical implications and gives opportunity for predicting mortality rates and expected life spans in the field, which is crucial for management purposes. Distribution of stages with serious infections decreasing at the end of a year (Ford and Haskin 1982, Farley 1975) was not apparent in this set of samples. On the contrary, infections seemed to rage through the oyster populations over the entire winter. Appearance of plasmodia underwent transformation from endomitotic "Kernstab" stages (Fig. 5) (Farley 1967) into passive, smaller plasmodia in some samples (the Clinton Hammonasset, and Hammock Rivers), but continued multiplication in some samples, such as Branford 179. The number of specimens with histological characteristics with potentially resistant animals was low, 4 of 653 animals (<1%). This might be attributable to the recent outset of Long Island Sound epizootics. Farley (1968) reported an increase of oysters with characteristics for resistance after 1.5 years or more of exposure to the disease. Haskin and Ford (1979) reported increasing survival with each year class in naturally resistant oysters in the beginning of the epizootics in the Delaware Bay. During the first year of exposure in 1957, oyster set had 84% mortality, 1958 set had 48% mortality, and 1959 set had 29% mortality.

Sporulation rate (three cases of sporulation in 319 cases of MSX-positive specimens) in this article is higher than usually reported. Andrews (1979) reported fewer than one case of sporulation per 2,000 cases of MSX. Unusual sporulation in the digestive duct cells and in connective tissue (Figs. 2 and 3) was probably caused by *Haplosporidium nelsoni* and not *H. costale* (SSO, Seaside Organism), because the spore size was  $6 \times 8 \mu\text{m}$  and not  $3 \times 5 \mu\text{m}$ , as reported for *H. costale* (Wood and Andrews 1962). Plasmodia in the specimen had size and characteristic capped nu-

clei of *H. nelsoni*. Furthermore, *H. costale* sporulates in May to June (Andrews 1979), and the specimen in this article was collected in September. *H. costale* has not been reported in field samples collected from Long Island Sound, Connecticut. However, SSO-like haplosporidians were reported in oysters transplanted from New Haven, Connecticut to Tomales Bay, California in 1967 (Katkansky and Warner 1970).

The Bureau of Aquaculture will monitor the prevalence of MSX annually. Long Island Sound experienced a MSX-epizootic in 1985 (Haskin and Andrews 1988), which was reflected in a collapse in production figures. After that, Connecticut's production rose from 69,721 bushels in 1987 to 893,964 bushels in 1992 (DEP 1995). At the same time (1987), state and private oyster companies started a massive culch program in Connecticut. This prevented habitat loss, which has drastically changed the ecosystem in other MSX epizootic areas. No other MSX enzootic areas, Chesapeake or Delaware Bay, have reported total recovery of oyster stocks.

We propose the following hypothesis for the transmission of MSX. Cyclic patterns have been observed in disease activity in the Delaware Bay, with peaks every 6 to 8 years (Ford and Haskin 1982). Similar fluctuations have been observed in the Chesapeake Bay (Farley 1975). Cyclic patterns in stock densities with several year intervals are characteristic for several estuarine species. The alternate host may be such a species. According to our hypothesis, MSX is transmitted via an alternate host, which is restricted to the mid-Atlantic states. The alternate host's northern limit of distribution is Long Island Sound, and periodically it establishes itself there and then withdraws south to the Chesapeake and Delaware Bays. If the present epizootic in Long Island Sound will pass by, as it did after 1985, this hypothesis is defensible.

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## DISTRIBUTION OF THE TURBELLARIAN *URASTOMA CYPRINAE* ON THE GILLS OF THE EASTERN OYSTER *CRASSOSTREA VIRGINICA*

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**ABSTRACT** *Urastoma cyprinae* has been reported from the gills of various bivalve species. In Atlantic Canada, it has been identified in the eastern oyster *Crassostrea virginica*. Recent studies have demonstrated that *U. cyprinae* can cause serious alterations to gill tissue in mussels. It has been suggested that, in oysters, the worm feeds on mucus secreted by the gills. Our work has revealed that *U. cyprinae* is attracted to oysters and that the source of attraction is mucus. The current study focuses on the distribution of *U. cyprinae* on the gills of oysters in relation to the presence and the state of mucus from different regions of the gills. Findings demonstrate that the worms occur throughout the gill surface, but that they are most heavily concentrated along the basal food tract, the major pathway for the transport of food particles to the labial palps. If mucus plays an important role in the parasite's survival, then *U. cyprinae*'s preference for this location may be a way of ensuring intimate contact between itself and the mucus slurry characterizing this site.

**KEY WORDS:** *Urastoma cyprinae*, turbellaria, eastern oyster, *Crassostrea virginica*, gills, oyster mucus

### INTRODUCTION

*Urastoma cyprinae* Graff occurs as a free-living organism in mud and algae (Marcus 1951, Westblad 1955) and on the gills of various molluscan bivalves (Fleming et al. 1981, Goggin and Cannon 1989, Noury-Sraïri et al. 1990, Murina and Solonchenko 1991, Teias dos Santos and Coimbra 1995, Trotti et al. 1998). In Atlantic Canada, the "gill-worm" has been reported in the eastern oyster *Crassostrea virginica* Gmelin (Burt and Drinnan 1968, Fleming et al. 1981, Boghen et al. 1993).

Robledo et al. (1994) have shown that *U. cyprinae* can induce pathological changes in the mussel *Mytilus galloprovincialis* Lamarck, resulting in disorganization of gill filaments and infiltration of hemocytes. Brun et al. (in press) have demonstrated that *U. cyprinae* is strongly attracted to the mucus that coats the gills of oysters, and Fleming (1986) has suggested that the worm may actively feed on this substance. Such findings bring into question to what extent *U. cyprinae* is a facultative commensal, as has previously been suggested (Burt and Drinnan 1968).

The role of mucus in particle processing of bivalves has been studied extensively (Ward et al. 1993, Ward et al. 1994, Ward 1996, Beninger and St-Jean 1997). The attraction of *U. cyprinae* to mucus may depend upon such factors as its viscosity, composition, and transport velocity, which vary significantly between different areas of the gills (Ward et al. 1993, Ward 1996). Considering the affinity of *U. cyprinae* to mucus (Brun et al. in press) and the possibility that the worm feeds on this substance (Fleming 1986), a comparative study of its attraction to different regions of the gills where mucus is present in a form that is most readily accessible becomes an interesting consideration. In this paper, we examine the distribution of *U. cyprinae* on the gills of oysters in relation to some of the characteristics of the mucus on the different parts of the gills, as mentioned above.

### MATERIALS AND METHODS

Twenty adult oysters infected with *U. cyprinae* were collected from Shippagan Bay (New Brunswick, Canada) on October 8, 1997. The animals were transported to the Université de Moncton on ice and were kept at 6°C in a walk-in cold room for 2 days before the start of the study.

Of the 20 molluscs collected, five were randomly chosen, and their right valves were removed (Fig. 1). The animals were individually dipped into liquid nitrogen (-196°C) for 30 s, which allowed immediate sacrifice of both oysters and worms. At the same time, it immobilized *U. cyprinae* in their respective positions on the gills, thereby facilitating worm counts at the specific location where they occurred.

The oyster lamellae were divided into three zones: ventral, medial, and dorsal, from which *U. cyprinae* were identified and counted (Fig. 2). These zones generally correspond to the gill regions of bivalves occasionally referred to by other authors (Ward et al. 1993, Ward et al. 1994, Ward 1996, Beninger and St-Jean 1997). The surface area occupied by each of the three zones for each lamella was determined by measuring its length and width (Fig. 2). Based on these measures, boundaries were delineated so that the dorsal zone, representing the area beginning at the basal food tract and extending toward the ventral axis of the oyster, would occupy 10% of the surface of the lamella. The medial zone, commencing at the lower limit of the dorsal zone toward the ventral axis, represented 80% of the surface of the lamella, and the ventral zone, stretching from the lower limit of the medial zone to the marginal indentation of the marginal food groove, occupied the remaining 10% (Fig. 2). Both the ascending and the descending sides of the basal and ventral tracts were examined and treated as separate zones for each of the lamellae. The combined surface areas, therefore, occupied by all eight ventral, eight medial, and eight dorsal zones were 10, 80, and 10%, respectively, for each of

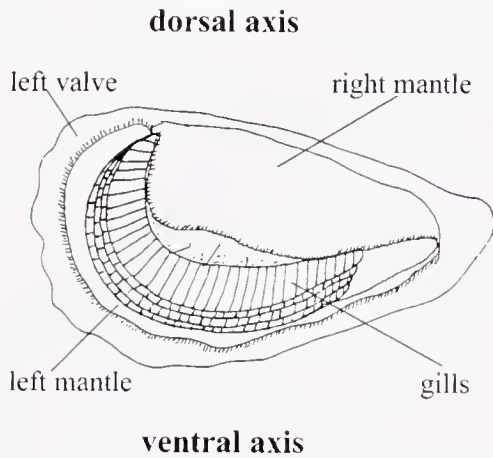


Figure 1. Schematic diagram of the oyster, after removal of the right valve, displaying the dorsal and ventral axes, left mantle, and raised right mantle exposing the gills.

the five oysters. In our study, the mantle area was not used for determining the counting zones or for the calculation of worm numbers.

Each oyster was partially thawed and examined in a semi-frozen state using an Olympus SZ30 stereomicroscope. This condition ensured minimum dislodging of the worms from their point of attachment on the gills during examination. Worm counts were conducted on successive demibranchs from each of the zones as defined above, using a hand-held counter, starting with the demibranch immediately adjacent to the right mantle (Fig. 1).

A log-linear model (Agresti 1990) was employed using Systat® 8.0 for Windows® (SPSS 1998) to analyze the worm counts as a function of three factors: oysters, zones, and lamellae, and their interactions. This analysis allows the user to identify significant factors and estimate their importance with an adequate control of Type 1 error. The alternative would be for the user to employ numerous chi-square tests on separate contingency

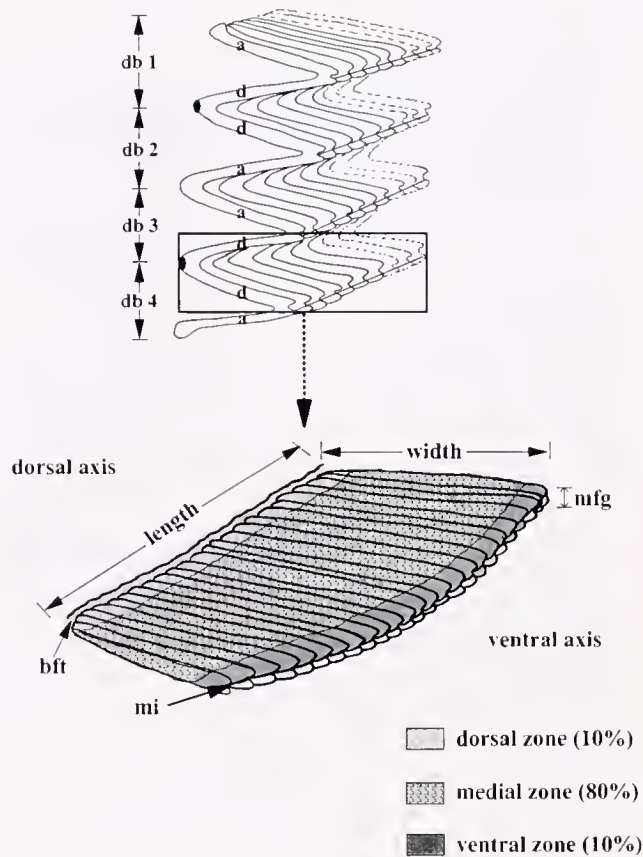


Figure 2. Schematic diagram of the four gill demibranchs of an oyster displaying the ascending and descending lamellae. Enlargement of area in rectangle to show details of one of the two lamellae forming an oyster demibranch. Each lamella is divided into three zones from which *Urastoma cyprinae* were counted: ventral, medial and dorsal. The shaded areas, with corresponding percentages, represent the surface area of the lamella occupied by each zone (10, 80, and 10%, respectively). (a) ascending lamella (bft) basal food tract, (d) descending lamella, (db1) demibranch 1, (db2) demibranch 2, (db3) demibranch 3, (db4) demibranch 4, (mfg) marginal food groove, (mi) marginal indentation.

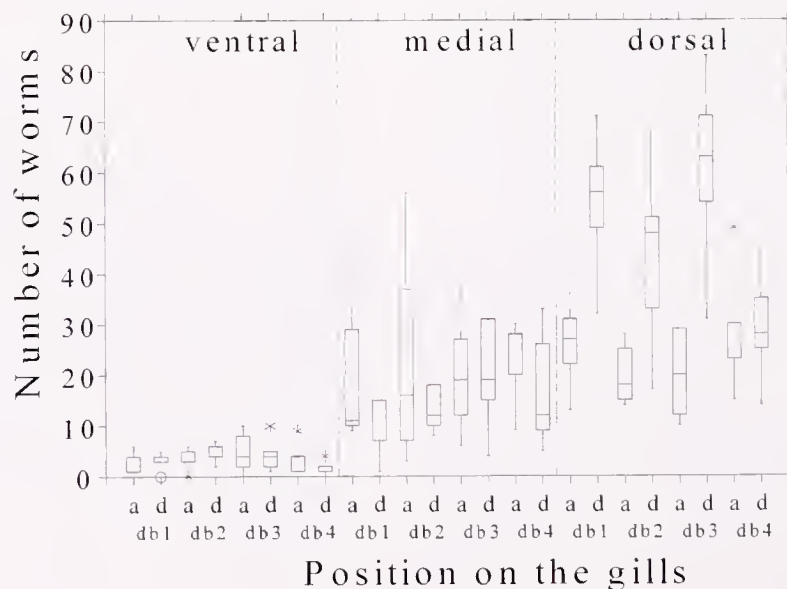


Figure 3. Box and whiskers plot representing the number of worms observed in oysters for each of the counting zones (ventral, medial, dorsal) of the ascending and descending lamellae for the four demibranchs. Due to the small number of oysters sampled, it is possible to identify single counts in most cases from the box and whiskers plot.

tables, but with less control on the over-all probability of error. For factors found to be significant in the log-linear model, the results of chi-square tests are given as a simple measure of significance.

In the log-linear model, the Pearson chi-square and the likelihood-ratio test are used to identify the most significant factors and Raftery's BIC (Bayesian Information Criterion) is employed to select the most appropriate model. The model is reported in a multiplicative form as follows:  $F_{ijk} = F_0 m_i m_j m_k m_{ij} m_{ik} m_{jk}$ , where *i* represents the oysters, *j* the zones, and *k* the lamellae.  $F_0$  is the reference frequency, and *m* represents the effects. An estimated *m* value close to 1 suggests that the effect of the individual factor or the interaction between two factors is small. The standard scores [ $\lambda$  (lambda)/se(lambda)] for the parameters of the additive expression of the model ( $\ln F_{ijk} = \theta + \lambda_i + \lambda_j + \lambda_k + \lambda_{ij} + \lambda_{ik} + \lambda_{jk}$ ) were calculated and used with the Bonferroni correction test to determine their significance. However, for the sake of conciseness, they are only reported as required for analysis. For each position, the counts for all oysters were added. These total counts were arranged in a simple two-way table (zones × lamellae) and analyzed using the chi-square test (Zar 1984). To make comparison with other studies easier, mean counts per oyster are reported.

RESULTS

Grouped box and whisker plots (Fig. 3) represent the observed worm counts graphically. The level of infection/parasitism for the five individual oysters are 513, 514, 507, 279, and 466, respectively. The results of the log-linear model indicate that the test of fit is significant for both the Pearson chi-square ( $\chi^2 = 189.3735$ , *df* = 56, *p* < .000005) and the likelihood-ratio test ( $\chi^2 = 199.4746$ , *df* = 56, *p* < .000005). A triple interaction factor  $m_{ijk}$  was removed, because it was nonsignificant. The model retained, therefore, is  $F_{ijk} = F_0 m_i m_j m_k m_{ij} m_{ik} m_{jk}$  with a Raftery's BIC value of -233.4889.

Table 1 shows that the three factors and their respective interactions are significant. The variable having the greatest impact on worm numbers is the zones ( $\chi^2 = 1208.88$ ). When considering the multiplicative effects for the latter, the results in Table 2 indicate that the worms are more abundant in the dorsal zones of the gills (2.688) as compared to the medial (1.345) and ventral (0.276) zones, respectively. The standard scores for the additive expression of the model show that each value within the zones is highly significant: ventral (-20.054); medial (6.881); and dorsal (25.246). In most instances, the multiplicative effects of the other two factors as well as all the interactions are close to 1, except for

a few values (Table 2) that represent particularly low or high worm counts.

Table 3 shows the mean number of worms observed and expected for each position. The results of the chi-square test comparing the total (5 × mean number) number of worms observed with the expected number of worms observed at each position indicate that *U. cyprinae* are more numerous along the dorsal zones as compared to the medial and ventral zones. This is reflected by the total number of worms counted in each of the three

TABLE 2. Multiplicative effects for each of the three factors and their interactions.

TABLE 2.							
Multiplicative effects for each of the three factors and their interactions.							
a.	$m_i$						
	1	2	3	4	5		
	1.257	1.355	1.049	0.571	0.981		
b.	$m_j$						
	Ventral		Medial		Dorsal		
	0.276		1.345		2.688		
c.	$m_k$						
	1a	1d	2a	2d	3a	3d	4a 4d
	0.974	0.888	0.912	1.200	1.018	1.346	1.060 0.727
d.	$m_{ij}$						
		1	2	3	4	5	
	Ventral	1.009	1.264	0.800	0.987	0.992	
	Medial	1.122	1.134	1.099	0.747	0.958	
	Dorsal	0.883	0.698	1.137	1.357	1.053	
e.	$m_{ik}$						
		1	2	3	4	5	
	1a	0.804	0.981	0.644	1.334	1.478	
	1d	0.851	1.209	1.127	0.736	1.171	
	2a	1.330	1.499	0.731	1.006	0.682	
	2d	0.825	0.609	1.229	1.510	1.073	
	3a	0.990	1.062	1.165	1.016	0.810	
	3d	0.963	1.151	0.867	0.688	1.150	
	4a	1.183	0.981	0.938	0.894	1.029	
	4d	1.181	0.771	1.631	1.073	0.627	
f.	$m_{jk}$						
		1	2	3			
	1a	0.950	1.266	0.831			
	1d	0.939	0.567	1.878			
	2a	1.037	1.382	0.675			
	2d	1.304	0.687	1.116			
	3a	1.369	1.175	0.621			
	3d	0.888	0.821	1.371			
	4a	1.029	1.163	0.835			
	4d	0.640	1.307	1.196			

TABLE 1. Results of the log-linear model analysis.

Term Tested	Model Without the Term			Removal of Term from Model		
	Ln (MLE)	Chi-sq	df	Chi-sq	df	P-value
Oysters	-388.580	275.72	60	76.25	4	0.0000
Zones	-855.156	1208.88	58	1009.40	2	0.0000
Lamellae	-363.095	224.75	63	25.28	7	0.0007
Zones-oysters	-377.011	252.59	64	53.11	8	0.0000
Lamellae-oysters	-419.180	336.92	84	137.45	28	0.0000
Zones-lamellae	-441.028	380.62	70	181.15	14	0.0000

*m* represents the effects, *i* the oysters, *j* the zones, and *k* the lamellae. The calculated reference frequency ( $F_0$ ) is valued at 11.516. Values significantly different from 1 using the Bonferroni correction test are indicated in boldface. a. Multiplicative effects for each of the five oysters. b. Multiplicative effects for the three counting zones. c. Multiplicative effects for the four ascending (a) and four descending (d) lamellae. d. Multiplicative effects for the interactions between the oysters and the zones. e. Multiplicative effects for the interactions between the oysters and the lamellae. f. Multiplicative effects for the interactions between the zones and the lamellae.

TABLE 3.

Observed and expected mean number of worms for each of the three zones (ventral, medial, dorsal), and the differences in percentages between them for the ascending (a) and descending (d) lamellae for the four demibranchs (db). Negative values indicate an observed worm count lower than expected; positive values demonstrate a higher one.

Lamellae	Zones								
	Ventral			Medial			Dorsal		
	Observed mean $\pm$ SE <sup>a</sup>	Expected mean	difference (%)	Observed mean $\pm$ SE <sup>a</sup>	Expected mean	difference (%)	Observed mean $\pm$ SE <sup>a</sup>	Expected mean	difference (%)
db 1 a	3.2 $\pm$ 1.0	5.6	-43	20.4 $\pm$ 6.7	45.6	-55	25.8 $\pm$ 3.9	5.6	+361
db 1 d	3.0 $\pm$ 0.8	5.6	-46	9.0 $\pm$ 2.7	45.6	-80	53.8 $\pm$ 6.5	5.6	+861
db 2 a	5.0 $\pm$ 0.9	5.6	-11	13.2 $\pm$ 2.1	45.6	-71	43.4 $\pm$ 8.6	5.6	+257
db 2 d	3.8 $\pm$ 1.1	5.6	-32	23.8 $\pm$ 10.0	45.6	-48	20.0 $\pm$ 2.8	5.6	+675
db 3 a	4.8 $\pm$ 1.9	5.6	-14	20.6 $\pm$ 5.8	45.6	-55	20.0 $\pm$ 4.0	5.6	+257
db 3 d	4.4 $\pm$ 1.6	5.6	-21	20.0 $\pm$ 5.1	45.6	-56	60.4 $\pm$ 8.8	5.6	+979
db 4 a	1.6 $\pm$ 0.7	5.6	-71	17.0 $\pm$ 5.3	45.6	-63	29.4 $\pm$ 5.2	5.6	+425
db 4 d	3.8 $\pm$ 1.5	5.6	-32	21.4 $\pm$ 3.7	45.6	-53	28.0 $\pm$ 5.8	5.6	+400
	<b>3.7 <math>\pm</math> 0.4</b>			<b>18.2 <math>\pm</math> 2.0</b>			<b>35.1 <math>\pm</math> 3.0</b>		

<sup>a</sup>Standard error on the mean.

zones: dorsal (1404), medial (727), ventral (148). Differences in percentages between the observed and expected worm counts further underline the preponderance of *U. cyprinae* in the dorsal versus the medial and ventral zones (Table 3).

#### DISCUSSION

Previous studies have shown that *U. cyprinae* are highly attracted to mucus on the gills of oysters (Brun et al. in press). Mucus is secreted by pallial organs and plays a predominant role in activities associated with bivalve-suspension feeding (Ward et al. 1994, Ward 1996).

The use of mapping for distinguishing between particular regions of pallial surfaces has previously been applied in studies on mucocyte identification in bivalves (Beninger et al. 1993, Beninger and St-Jean 1997). The current work employed the principle of gill mapping to determine whether there existed a relationship between infestation levels of *U. cyprinae* and specific areas on the gills. This approach contrasts with previous investigations on oysters (Fleming et al. 1981, Fleming 1986) and such other species as mussels (Robledo et al. 1994, Teias dos Santos and Coimbra 1995, Trotti et al. 1998), which reported the occurrence of the parasite in the host without specific reference to a niche.

Statistical analyses of three variables (oysters, zones, lamellae) and the interactions between them, demonstrated that it was the zones that exerted the greatest influence on the distribution of *U. cyprinae*. It was also noted that the highest numbers of worms were consistently found along the dorsal regions as compared to the medial and ventral zones. Having already established that *U. cyprinae* display a strong attraction for mucus (Brun et al. in press), the existing situation raises the question as to why there is such a marked preference for the dorsal zones. Several possible explanations could be proposed.

Mucus contained in the basal food tract consists primarily of mixed mucopolysaccharides (acid and neutral); whereas, the marginal food groove is mostly composed of acid-dominant mucopolysaccharides (Beninger and St-Jean 1997). As a result, trapped particles in the dorsal regions are transported in a low-viscous mucus slurry (Ward 1996), at a velocity of more than twice that occurring in the ventral grooves (Ward et al. 1993). This differs from the material in the marginal food grooves, which is contained

in a viscous mucus cord (Ward et al. 1994). The likelihood that the worms prefer to be in contact and possibly feed on the more "fluid" mucus in the dorsal zones is an attractive prospect, both from the point of accessibility and ease of uptake.

*Urastoma cyprinae* possesses an oral-genital pore located in the posterior end (Cannon 1986), and a tegument that takes the form of a ciliated epithelium (Schmidt and Roberts 1989, Noury-Sraïri et al. 1990). Although *U. cyprinae* obviously feeds with its oral-genital pore, the tegument may also play an important role in food acquisition and the transfer of material across the body wall (Noury-Sraïri et al. 1990). The dorsal regions may, therefore, represent choice sites where extensive contact between the body of *U. cyprinae* and the fast-moving, "fluid" mucus slurry prevails.

In the oyster, the basal food tract is the predominant route for transportation of food in preparation for final sorting and ingestion (Ward et al. 1998). In contrast, mucus-bound particles contained in the marginal grooves may have a lower nutritional value (Ward et al. 1998) and are eventually rejected as pseudofeces (Newell and Langdon 1996, Ward 1996). If we assume that the worms do not feed solely on the mucus, but also rely on particles bound in the mucus, then *U. cyprinae*'s preference for the dorsal regions, where high-quality nutritional materials are present, can be expected. The dorsal regions may also provide added protection for the worms, because the latter would be less vulnerable to water currents when the oyster's valves are open. Finally, given the fact that *U. cyprinae* are negatively phototactic (Burt and Bance 1981, Pike and Wink 1986), the dorsal regions may also provide the worms with a darker environment as compared to the ventral regions. Morphological and behavioral studies are presently under way to understand the parasite's adaptations and possible host-parasite interactions better in the dorsal regions of the oyster's gills.

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## FECUNDITY OF THE VELVET SPIDERCRAWB *STENOCIONOPS OVATA* (BELL, 1835) (BRACHYURA: MAJIDAE) IN THE GULF OF CALIFORNIA, MÉXICO

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**ABSTRACT** The fecundity of the velvet spidercrab *Stenocionops ovata* from the Gulf of California was determined based on an incidental capture realized in the Gulf of California. Evidence suggested that adult females of *S. ovata* can produce more than one batch of eggs in a breeding cycle. The number of eggs per brood by *S. ovata* range from 35,187 eggs to 170,057 eggs with a mean of about 94,200 eggs. This species showed lower fecundity than other Majidae of deep water (e.g., *Maiopsis panamensis*) from the Gulf of California. Partial fecundity was positively correlated with both length of carapace without rostral horns ( $r = 0.655$ ) and branchial width of carapace ( $r = 0.617$ ), without a clear tendency of one measure to be a more useful morphometric measure in the determination of the relation with fecundity in this species.

**KEY WORDS:** Fecundity, spidercrab, Majidae, *Stenocionops*, Gulf of California

### INTRODUCTION

*Stenocionops ovata* (Bell 1835) has been captured from 15 to 275 m of depth over sandy-lime bottoms or over coral, shell, seaweed, or sponge substrates (Hendrickx 1995). According to Hendrickx (1989) this species is represented by abundant populations in the central Gulf of California, between 60 and 100 m depth, where they are accessible to commercial drag nets. The great number of *S. ovata* obtained by incidental capture by one trawler at Isla Danzante supports this idea. Therefore, great populations exist in the gulf that may be commercially exploited. This species represents a potential resource not currently being exploited, which merits further study (Hendrickx 1995).

Studies on fecundity in a population with economic potential are of special interest because of its relation with the renewal intensity of the population. Moreover, it is a basic to our knowledge of the reproductive strategy and evolution of the population from what it is an essential component in the population dynamic (García-Montes et al. 1987).

Fecundity is an import parameter in crustaceans, among others, in determining the reproductive potential of a species and/or the stock size of a population (Medina and Fransozo 1997). Among brachyuran crabs, there is considerable variation in fecundity (Medina and Fransozo 1997). Body size of female brachyurans is the principal determinant of fecundity per brood and reproductive output (brood mass) (Hines 1988). The fecundity of crabs in deep water of the Gulf of California has not been determined, except for *Maiopsis panamensis* (Faxon 1893, Villalejo-Fuerte et al. 1998).

In this work, the estimation of fecundity was carried out from the ovigerous mass. The intention of this paper is to present the first data about reproductive effort and fecundity of *S. ovata* in the Gulf of California and their relation to the carapace length and width. We also present a comparison with the results of fecundity obtained for another Majidae of deep water (*M. panamensis*) from the Gulf of California.

### MATERIALS AND METHODS

Three hundred ninety-one specimens of velvet spidercrabs *S. ovata* were incidentally captured in the northern extreme at Isla Danzantes, Gulf of California, Mexico (25°48'54"N and 111°15'45"W) in October 1994. Captures were made with gill nets

of monofilament with 6-in mesh size at 180-m depth. Velvet spidercrabs obtained were identified with the Keys of Garth (1958).

For each ovigerous female, length of carapace (mm) without rostral horns (CL), branchial width of carapace (CW), total weight, and the weight of the brood were registered. The pleopod structure with the egg mass was removed immediately after capture and was preserved in a 10% buffered solution of formaldehyde prepared with sea water. The eggs were removed from the pleopods by means of the method described by Choy (1985). They were stored in 7% formalin until they were processed.

For the fecundity estimation, only 25 ovigerous females were considered, according to the criterion of Medina and Fransozo (1997). The fecundity estimation, considered in this paper as the number of eggs per brood, was done by means of the gravimetric method described by Bagenal (1978).

The number of eggs per brood was determined by direct counting of samples of 0.1 g. The relative fecundity was determined as the number of eggs per gram of total weight. The reproductive effort had been estimated with the ratio of brood weight per body weight, because it has often been used as an index of current reproductive effort (Fukui and Wada 1986). To determine the relation of partial fecundity with length of carapace without rostral horns (CL) and branchial width of carapace (CW), dates were fitted to four models. Gonads were removed and fixed in a neutral formalin solution 10%. Gonad sections were taken and dehydrated in alcohol, embedded in paraffin, and sectioned at 7  $\mu$ m. Sections were placed on slides and stained with hematoxylin-eosin (Humason 1979).

### RESULTS

From the 391 velvet spidercrabs obtained, 304 (77.7%) were males, and 87 were females (23.3%). The sex ratio for the total sample was 3.5 M:1 F and differed significantly ( $p \leq .05$ ) from the expected ratio of 1:1. All the 87 females obtained were ovigerous. Ovigerous females reach 71.5–102.5 mm CL (90.1 mm mean, 7.5 SD) and 65.6–88.5 mm CW (76.6 mm mean, 5.84 SD). The ratio of brood weight to body weight ranged from 0.0375 to 0.1112 (0.0835 mean, 0.0193 SD) and was not correlated with CW or CL ( $p > .05$ ). Relative fecundity ranged from 236 eggs/g (90 mm CL, 82 mm CW) to 972 eggs/g (86 mm CL, 75 mm CW) with a mean of 587 eggs/g.

Partial fecundity ranged from 35,187 eggs (90 mm CL, 82 mm

TABLE 1.  
Results of the fits between partial fecundity with carapace length and carapace width.

Model	With Carapace Length	With Carapace Width
Geometric	$Y = 5530.98X^{0.0069X}$ $r^2 = 0.5361$	$Y = 6057.77X^{0.0081X}$ $r^2 = 0.4599$
Exponential	$Y = 2993.42e^{0.0377X}$ $r^2 = 0.5341$	$Y = 3242.57e^{0.0434X}$ $r^2 = 0.4587$
Linear	$Y = -178093.82 + 3018.66X$ $r^2 = 0.4904$	$Y = -197944.2 + 3804.99X$ $r^2 = 0.4364$
Logarithmic	$Y = -1062616.4 + 257173.25\ln X$ $r^2 = 0.4736$	$Y = -1142520 + 285099.48\ln X$ $r^2 = 0.4285$

CW) to 170,057 eggs (102 mm CL, 88.5 mm CW) with a mean of 94,200 eggs. Partial fecundity was significantly correlated ( $p < .05$ ) with both length of carapace without rostral horns (CL) and branchial width of carapace (CW), demonstrating that fecundity increases with the increase in both length of carapace without rostral horns and branchial width of carapace.

The equations of regression obtained for the four models are summarized in Table 1. The best fit between fecundity and CL or CW was with the geometric model ( $r^2 = 0.5361$  and  $r^2 = 0.04599$ , respectively) (Figs. 1, 2). Histologically, the ovaries presented mature and previtelogenic oocytes.

#### DISCUSSION

Although incidental captures of velvet spidercrabs were realized only in October 1994, ovigerous females were present. According to Garth (1958), in the Gulf of California, the breeding of *S. ovata* occurs in April, but the fact that ovigerous females had been encountered in October is evidence that *S. ovata* have a larger breeding cycle. However, an annual study of the reproductive biology of the species is necessary to give conclusive statements.

In addition, there is histologic evidence to suggest that *S. ovata* females can produce more than one batch of eggs in a breeding cycle. The occurrence of ripening ovaries simultaneous with brooded eggs has been interpreted as an indication of the potential to produce a second brood during the same breeding season (Pillay and Nair 1971). This condition has been reported for the panamic spidercrab *M. panamensis* (Villalejo-Fuerte et al. 1998), as well as for other brachyuran species (Ryan 1967, Pillay and Nair 1971, Perez 1990) and several anomuran species (Arneyaw-Akumfi 1975, Varadarajan and Subramoniam 1982).

Reproductive effort wasn't correlated with CL or CW, but the fecundity was correlated with both CL and CW. Although the correlation between fecundity and CL ( $r = 0.655$ ) was slightly higher than that obtained between fecundity and CW ( $r = 0.617$ ), we do not consider that this difference is sufficient to believe that the CL is the more useful morphometric measure in the determination of the relation with fecundity in this species, such as occurs for *M. panamensis*, in which the CW is the more useful morphometric measure in the determination of the relation to fecundity (Villalejo-Fuerte et al. 1998). The lower fecundity observed in *S. ovata* (mean of 94,200 eggs) as compared to the sympatric spidercrab *M. panamensis* (mean of 676,039 eggs), may probably be attributed to size, considering that this species is smaller than *M. panamensis*.

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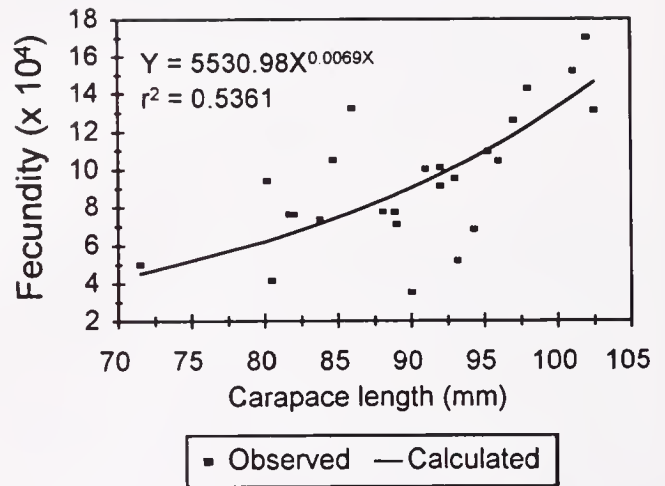


Figure 1. Relationship between partial fecundity and carapace length in ovigerous female *Stenocionops ovata* from the Gulf of California.

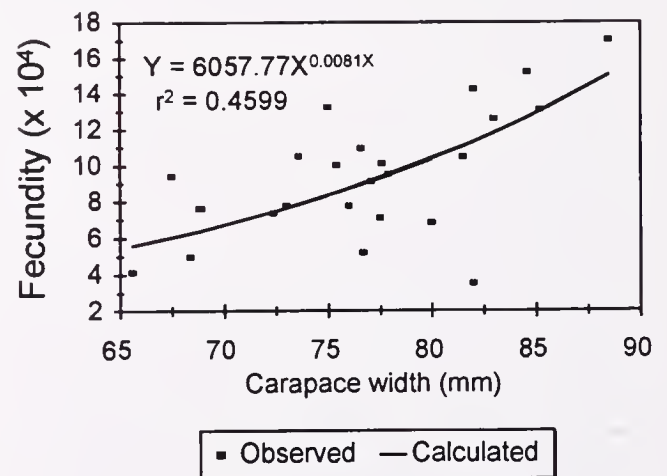


Figure 2. Relationship between partial fecundity and carapace width in ovigerous female *Stenocionops ovata* from the Gulf of California.

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## DEVELOPMENT OF A RECRUITMENT INDEX FOR FORECASTING SEASONAL LANDINGS OF THE KUWAIT SHRIMP FISHERIES

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**ABSTRACT** The seasonal landings and pre-season survey data for the Kuwait shrimp fishery were analyzed. Mean catch rates in the pre-season surveys in the southern area in July and August might serve as a recruitment index for the fishery. A combined-species model and a single-species model for *Penaeus semisulcatus* explained 96 and 89% of the variance, respectively. These parametric models together with nonparametric methods were then employed to forecast the 1997 to 1998 season's shrimp landings before the season opened. The nonparametric models produced better forecasts than the parametric models. Finally, a general discussion about parametric and nonparametric methods for fishery forecasting was presented.

**KEY WORDS:** Recruitment index, shrimp, parametric method, nonparametric method, Kuwait

### INTRODUCTION

Recruitment to a fishery is known to vary greatly between years, sometimes by orders of magnitude, in unpredictable ways. In a single-cohort fishery, such as the Kuwait shrimp fishery, variation in recruitment leads directly to changes in seasonal landings. A reliable recruitment index is not only useful for investigating stock–recruitment relationships, but also for forecasting seasonal landings. Establishing a stock–recruitment relationship is a top priority of fishery managers, because this relationship provides guidelines for the maximum limits of fishing mortality for sustainable production. Forecasts of annual landings before the season opening are of considerable importance to commercial fishermen as well. This information is crucial for decisions concerning the best fishing areas and what their investment strategy should be with regard to fishing equipment. Such a forecast also provides valuable information to the fishery management authority that controls the number of licensed boats and results in opportunities for improved biological management, through planning to take advantage of strong recruitment events and to protect stocks when poor recruitment is expected (Walters 1989).

Morgan and Garcia (1982) developed a recruitment index for the Kuwait shrimp fishery to investigate the possible stock–recruitment relationship. Their recruitment index was calculated by dividing the observed catch during a biological year by the estimated yield-per-recruit for that year. In this calculation of yield-per-recruit, there is an implicit assumption that the fishing effort coefficient is constant. However, a recent study (Ye and Mohammed 1999) found that catchability of shrimp has a high interannual variability in the Kuwait shrimp fishery. This variability may mask the real variation of recruitment and lead to an error in the stock–recruitment relationship (Garcia 1983). To solve this kind of problem, a recruitment index independent of fishing effort is required.

To date, a pre-season forecast for the shrimp fishery in Kuwait has not been attempted. In general, there are two types of fisheries forecasting. One involves the traditional parametric method, including methods of ordination and canonical analysis and univariate and multivariate linear, curvilinear, and logistic regressions. The other is the nonparametric method (Rice 1993). Traditional statistical methods have many disadvantages (James and McCulloch 1990), and nonparametric methods, in which the predicted variable is described by a probability density function, have gained more favor (Evans and Rice 1988, Rice 1993).

This study attempts to establish a recruitment index for the

Kuwait shrimp fishery that best represents the variation in recruitment. Both parametric and nonparametric methods were employed to forecast seasonal landings. Finally, a general discussion about the difference between the parametric and nonparametric is made.

### MATERIALS AND METHODS

#### *Data Used*

To develop an index independent of fishing effort for the shrimp fishery, survey data are the first choice. Before the Gulf War, the Mariculture and Fisheries Department, Kuwait Institute for Scientific Research (KISR) carried out year-around surveys for 6 years from May 1985 to April 1990. Unfortunately, the computer database was destroyed during the Iraqi occupation. Although a great effort was made by KISR to rescue the original survey record files, some were permanently lost. Because a recruitment index mainly concerns the period before season opening, survey data for the period of April to August from 1985 and 1987 to 1989 were used together with the postwar pre-season surveys.

After the Gulf War, a pre-season survey was initiated in 1993. Because of financial constraints, the pre-season surveys were conducted for different numbers of months in different years, 4 months for some years and only 2 months for the others. Its survey design was also not consistent. Fixed stations were sampled in 1993 and 1994, but a random sample scheme was introduced afterwards.

Either of the two survey periods, 4 to 6 years depending upon the month for which the data were used, is too short for a rigorous time-series analysis. Therefore, the two surveys were pooled together as a single time series after standardization. The prewar survey used the RV *Bahith*, a stern trawler (679 tons gross tonnage) of KISR with one flat trawl of 30-meter foot rope between otter boards and a cod end of 50-mm stretched mesh. The postwar pre-season survey employed *Mutaheda 5*, a Gulf of Mexico-type, double-rigged shrimp trawler leased from the United Fisheries of Kuwait. This boat towed two flat trawls of the same cod-end mesh size and foot rope length as RV *Bahith*. Because both boats and nets used were similar, a factor of 2 was used to standardize roughly the density index of the prewar survey.

Monthly surveys from April to August sampled 10 to 17 stations from Kuwait Bay to Umm Al-Maradem Island in the southern Kuwait waters (Fig. 1). One tow was made at each station lasting for 15 to 60 minutes, varying with catch level. All data were standardized to catch per boat-hour for analytical purposes.

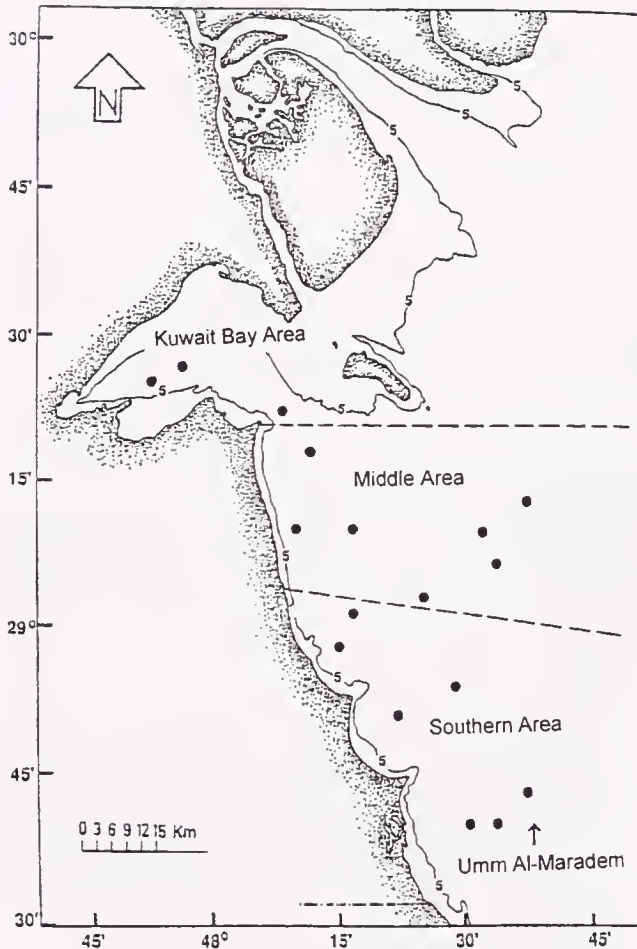


Figure 1. The Kuwait waters covered by the shrimp surveys and its stratification. Dots indicate the survey stations in 1993.

The Kuwait shrimp fishery captures mainly three species: *Penaeus semisulcatus*, *Metapenaeus affinis*, and *Parapenaeopsis styliifera*. *P. semisulcatus* dominates in the central and southern areas, but all three species occur in the northern waters (Ye et al. 1996). Catch rates of either combined species or single species varied greatly from station to station. To improve the shrimp density index and to account for geographical differences in distribution, the entire survey area was stratified into three areas: Kuwait Bay, middle area, and southern area (Fig. 1) (Sparre and Venema 1992, Xu et al. 1995).

The Kuwait shrimp fishery consists of two sectors: industrial trawlers and dhow trawlers. The industrial sector consists of three fishing companies, United Fisheries of Kuwait, Bubiyan Fisheries Company, and National Fishing Company, with a total of 35 fishing vessels. Its annual landings constituted about 75% of the total shrimp landings in Kuwait from 1985 to 1990 and dropped to 50% after the war (Ye et al. 1996). Each company provided KISR with monthly shrimp catch and effort data for each vessel.

The artisanal fleet had more than 70 dhow boats after the war (Ye et al. 1996). This fleet sells shrimp at three local fish markets. The Kuwait Central Statistics Office (CSO) collects shrimp landings only from whole sales. Some fishermen, however, sell shrimp directly to retailers; thus, the CSO statistics are conservative estimates. KISR established a dockside interview system to collect dhow boats' landings since 1977 when KISR, in conjunction with

the United Nations Food and Agriculture Organization, started the Shrimp Fisheries Management Project (van Zalinge 1981, Mohammed et al. 1998). A 1-in-5 systematic sample of daily interviews with a random start date each season has been in effect; that is, every fifth day throughout each season, interviews were carried out with skippers of all dhow boats landed that day, and consisted of the following queries: total shrimp catch, days spent fishing, trawl tows per day, hours per tow, and fishing ground (for a detailed description of this interview sampling see van Zalinge 1981). The estimated precision, expressed by the estimated coefficient of variation; that is, standard deviation of the mean divided by mean, of the seasonal estimate of shrimp landings was 5.84% in 1980 to 1981 (van Zalinge 1981). This sampling program has been in use since 1980.

#### Parametric Method

Traditional parametric methods have concentrated on the development of parametric relationships (linear or curvilinear equations) between the observed catch or recruitment and explanatory variables, such as a recruitment index or environmental factors. This method requires a perceived understanding of the physical or biological characteristics of the system that may affect the shrimp species. Its predictive ability has generally been readily variable with blackbox techniques, such as regression and time series analysis, often producing good forecasts of catch or recruitment (Noakes et al. 1989, Stocker and Hilborn 1981).

The Kuwait shrimp fishery is a single cohort fishery, with seasonal total landings based mainly on recruitment from the same season (Morgan 1989). It seems straightforward that the density index derived from a pre-season survey is directly related to the yield of the coming season. However, thorough analysis is necessary to determine indices from which area and month have the best-fit relationship with seasonal total landings.

In this study, a set of monthly mean catch rates from the sampling stations within each area during the study period was treated as a time series. Thus, 15 sets (3 areas  $\times$  5 months from April to August) were chosen as independent variables and seasonal catch as the dependent variable. A backward, stepwise multiple regression analysis was used to identify the best variables. The probability-to-remove value was set at 0.10 and the probability-to-add value at 0.05.

#### Nonparametric Method

Several probabilistic approaches have been applied in fishery forecasts over the last few years as alternatives to parametric models (Evans and Rice 1988, Fried and Hilborn 1988, Noakes 1989, Rice 1993, Chen and Shelton 1996). Nonparametric methods allow the data to "speak for themselves" instead of merely having to conform to some arbitrary mathematical equation. If the assumptions associated with the parametric methods are not satisfied or if the wrong parametric model has been selected, then these probabilistic or nonparametric approaches may produce more accurate forecasts than the competing parametric methods (Noakes 1989). Evans and Rice (1988) developed a modified "kernel estimator" for similar purposes, which showed that the nonparametric method was superior to the alternatives tested.

The problem, however, is this: given a set of past observations of recruitment indices and seasonal landings, and the current recruitment index, estimate the current corresponding landings probability distribution function (PDF). Thus, the landings PDF is a

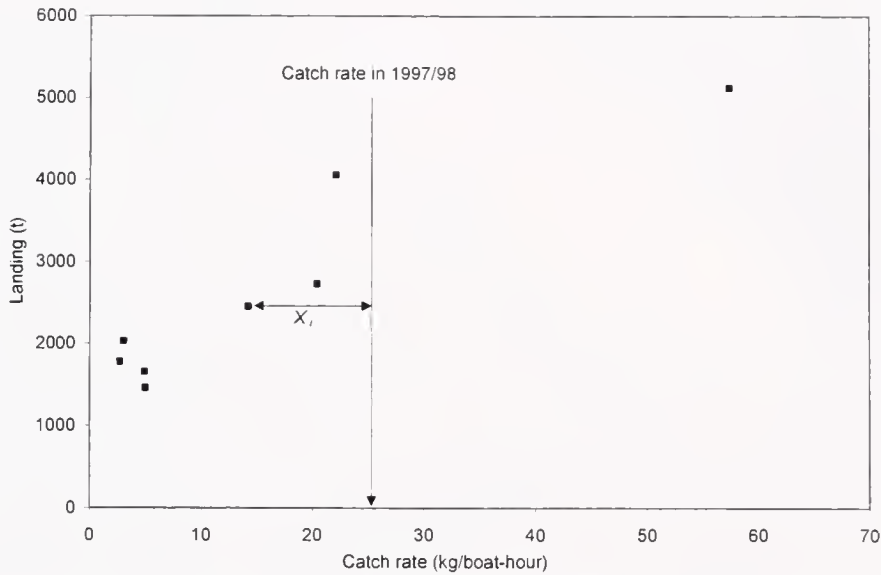


Figure 2. Illustration of nonparametric algorithms for assigning probabilities of occurrence of observed recruitment indices during a projection (scatter plot of species combined landings vs. mean survey catch rate in July).

function of past observations and this year's recruitment index, and will, in general, be different if next year's recruitment index is different. The basic rule is to pay more attention to past observations whose recruitment indices are close to this year's recruitment index.

There are several algorithms for estimating landings PDF, but Evans and Rice (1988) found that the Cauchy algorithm gave the best results; it also seemed to present the least risk of going seriously wrong. Therefore, the Cauchy algorithm is also used here.

$$W_i = \frac{1}{1 + \left(\frac{X_i}{D}\right)^2} \quad (1)$$

where  $W_i$  is a weighting factor for  $i$ th observation,  $X_i$  is difference between value of  $i$ th past recruitment index and this year's index value (Fig. 2).  $D$  is the "tuning" parameter, in the same unit as  $X_i$ . When  $X_i$  is small relative to  $D$  (sites are similar),  $W_i$  is large; when  $X_i$  is large relative to  $D$  (sites differ greatly),  $W_i$  is small.

Among the reasons for using nonparametric density estimation methods instead of parametric methods are lack of knowledge of the true distribution of errors and lack of smoothness in the unknown true functional relations. Therefore, to measure goodness-of-fit, the deviations of observations from the PDF median may be better than deviations from the PDF mean (Rice 1993).

The optimum value of  $D$  is chosen through crossvalidation (Efron and Gong 1983, Hall and Marron 1990). Begin with a trial  $D$  much larger than the range of values of the index observations, and estimate the crossvalidated sum of squared values. Delete one observation in the historical data set. Use Eq. 1 to estimate the PDF for that observation using the value of the deleted recruitment index and all other pairs of observations in the historical dataset. Calculate the squared difference of the seasonal landings at the deleted site from the median of the estimated PDF of the landings. Repeat for each observation in the historical dataset, and sum the squared differences. This crossvalidated sum of squares measures goodness-of-fit, assuming the null hypothesis of no relationship (Rice 1993).

## RESULTS AND DISCUSSION

### Combined Species Model

The Kuwait shrimp fishery is a multispecies fishery. In the commercial sector, fishers and managers of fishing companies and the management authority, a combined-species index or forecast for the coming season's success might be more important than single-species forecasts. As an initial step, mean catch rates (kg per boat-hour) of combined species from the three areas from April to August were used as independent candidate variables, and seasonal total landings were used as a dependent variable.

A stepwise regression built the following model of two independent variables.

$$C = a_0 + a_1X_1 + a_2X_2 \quad (2)$$

where  $C$  = yearly catch in kg;  $X_1$  = mean catch rate in July from the southern area in kg per boat hour;  $X_2$  = mean catch rate in August from the southern area in kg per boat hour; and  $a_0$ ,  $a_1$ , and  $a_2$  are constants (Table 1). This model fits the data very well ( $R^2 = 0.96$ ,  $p = .0004$ ,  $n = 8$ ) (Fig. 3).  $t$ -statistics show that the intercept and two coefficients of the model are statistically significant (Table 1). A residual plot showed that there is no serious autocorrelation of the data.

TABLE 1.  
Statistics of the forecast models.

Model	Constant	Estimate	p Statistic
Combined species	$a_0$	1255.5	0.001
	$a_1$	52.2	0.001
	$a_2$	24.2	0.028
	$R^2 = 0.96$	$F = 54.33$	$n = 8$
<i>P. semisulcatus</i>	$b_0$	325.1	0.140
	$b_1$	14.9	0.013
	$b_2$	15.3	0.074
	$R^2 = 0.89$	$F = 21.04$	$n = 8$

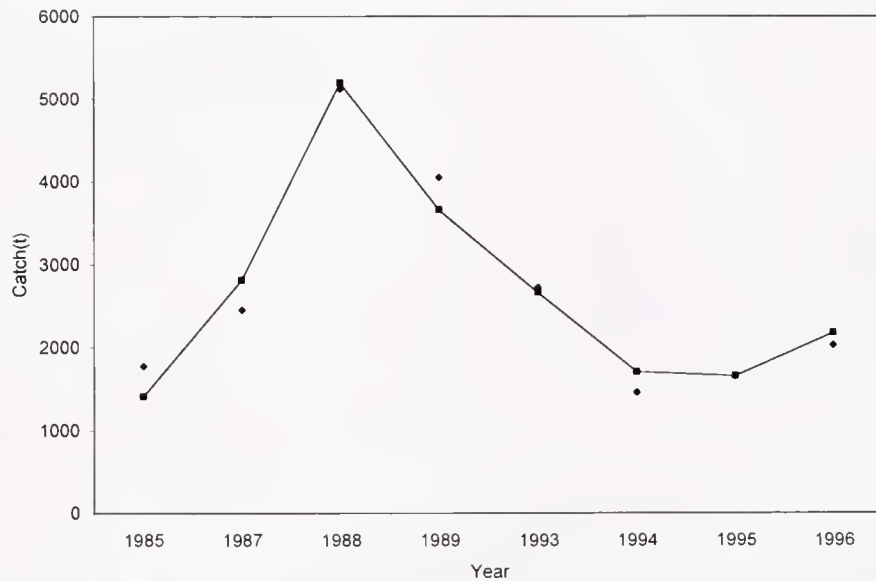


Figure 3. Comparison between the observed and predicted seasonal landings of species combined shrimp in Kuwait waters.

Traditionally, Kuwait Bay has been recognized as an important nursery area (Bishop and Khan 1991, Ye et al. 1996). Surprisingly, no significant correlation between the mean catch rate in Kuwait Bay and seasonal yield was found. The survey catch rate was highly variable, as expected in fisheries. Because of lack of statistical consideration in the survey design, only two stations were sampled in Kuwait Bay each month in both the pre- and postwar surveys before 1997. This makes the index from Kuwait Bay more unreliable. To increase the precision of the catch rate estimates, the number of stations was increased to five in Kuwait Bay and seven in the middle and southern areas in the 1997 survey. The average catch rates with standard errors and percentage of *P. semisulcatus* are listed in Table 2.

The most important conclusion from Table 2 is that the percentage of *P. semisulcatus* in the catch of Kuwait Bay was very low, 10.3% in weight in July and 5.7% in August 1997, respectively. In contrast, the seasonal landings were dominated by *P. semisulcatus*, from 50% in bad seasons to 98% in good seasons (Siddeek et al. 1994). Although the shrimp densities of combined species in Kuwait Bay were much higher than in the other areas, the low percentage of *P. semisulcatus* in Kuwait Bay, combined with its small area, underplays the significance of its contribution to the seasonal total landings of shrimp. It may be more reasonable to say that Kuwait Bay is a more important and significant nursery ground for *M. affinis* and *P. stylifera* than for *P. semisulcatus*. Some major nursery grounds of *P. semisulcatus* may be located in the southern area rather than in Kuwait Bay (Bishop et al. 1994). Further investigation is needed in the future.

Because *P. semisulcatus* dominates the shrimp catch and is more abundant in the central and southern area, a high correlation between the index from the southern area and total yield may be expected. The coefficient for the mean catch rate in July from the southern area ( $X_1$ ) doubled that for the August mean catch rate ( $X_2$ ) (Table 1). This hints that the stock in the southern area in July has a more important effect on seasonal landings than that in August, if the estimation is not biased. The decrease of importance of the southern index from July to August may be caused by the much larger standard errors associated with the mean catch rates in August (Table 2).

#### Single-Species Model

To develop a recruitment index for each single species, both the survey data and total landings were separated by species based on species composition data from commercial fishing and research survey. A comprehensive examination of the data of the three main species indicates that a single species model is possible only for *P. semisulcatus*. This may be because both the pre- and postwar surveys, because of regional conflicts, did not cover the northern waters where *M. affinis* and *P. stylifera* are dominant. Because fishing effort was also affected by regional conflicts, landings in Kuwait probably do not reflect their population magnitudes. Thus, no significant correlation between the survey density estimates and seasonal landings could be established.

The same stepwise regression technique as for the combined-species model was used. A multivariate linear model was found.

$$C = b_0 + b_1X_1 + b_2X_2 \quad (3)$$

TABLE 2.  
Average catch rates and their standard errors of the 1997 preseason shrimp survey

	n	Species Combined (Mean $\pm$ SE)		<i>P. semisulcatus</i> (Mean $\pm$ SE)		% of <i>P. semisulcatus</i>	
		July	August	July	August	July	August
Kuwait Bay	5	243.44 $\pm$ 84.81	457.58 $\pm$ 97.22	25.02 $\pm$ 7.89	26.21 $\pm$ 9.79	10.28	5.73
Middle area	7	10.34 $\pm$ 4.81	111.60 $\pm$ 68.60	9.22 $\pm$ 3.92	12.17 $\pm$ 6.09	89.15	10.90
Southern area	7	25.91 $\pm$ 12.06	48.51 $\pm$ 41.57	25.64 $\pm$ 11.89	48.49 $\pm$ 41.57	98.96	99.97

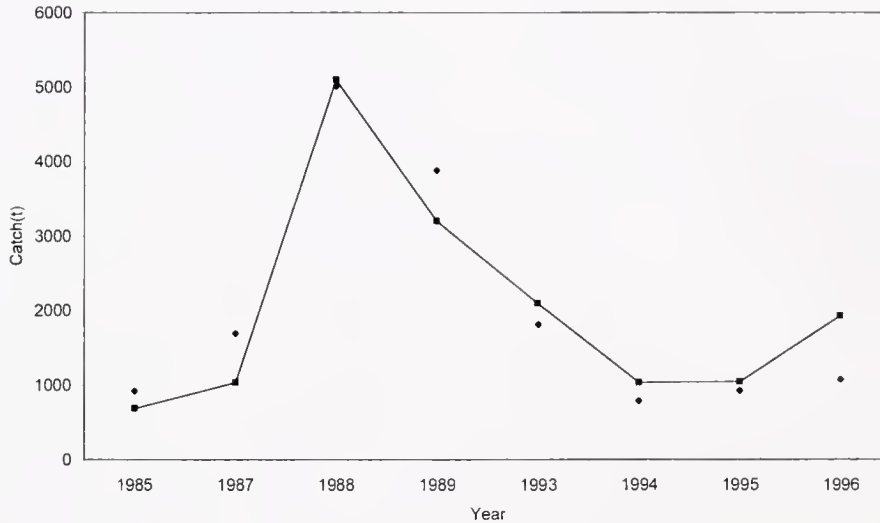


Figure 4. Comparison between the observed and predicted seasonal landings of *Penaeus semisulcatus* in Kuwait waters.

where  $C$  = total landings of *P. semisulcatus*;  $X_1$  = mean catch rate of *P. semisulcatus* in the southern area in July;  $X_2$  = mean catch rate of *P. semisulcatus* in the southern area in August; and,  $b_0$ ,  $b_1$ , and  $b_2$  are constants (Table 1). The model fits the data well with  $R^2 = 0.89$  (Fig. 4).  $t$ -statistics show that the hypothesis of zero coefficient for  $X_1$  could be rejected at  $p = .013$ , and that for  $X_2$  at  $p = .074$ . Although the latter is bigger than the traditional criterion of  $p = .05$ , we decided to keep it, because the time series is only 8 years ( $t$ -statistics are not very reliable for short time series data). By keeping  $X_2$ , we will significantly reduce the standard deviation of the model, resulting in greater accuracy of the model forecasts.

The last datum point in Fig. 4 fits the model more poorly than the others. This may be caused by imprecise species composition data from the industry sector in the 1996 to 1997 season. Species composition was estimated roughly by wholesale grade-size data, and the fit is expected to improve if more precise species composition data are available.

#### Recruitment Indices and Forecasts from Parametric Methods

The above regression analyses show that the mean survey catch rates in the southern area in July and August can explain a very high percentage of variation in seasonal landings (Table 1) and can serve as an index of recruitment to the fishery in the coming season. The index could be calculated from July mean catch rates alone or July and August mean catch rates, depending upon the requirements of practical aspects. If only 1 month is to be used, then the July mean catch rate is preferred, because the coefficient of variability in August is much higher (Table 2). The increase of the coefficient of variability may be caused by the patched schooling behavior of shrimp before recruitment (Matthews et al. 1994). In general, the time series is short, and the predictive ability of the models is expected to improve as more data become available.

Test forecasts for the 1997 to 1998 season were made on the basis of the above-developed models before the opening of the shrimp season on 1 September. The indices from the southern area in July and August were used (Table 2). The point estimate of the species combined total catch was 3781.6 tons with 95% confidence boundaries of 2802.1 to 4761.0 tons (Table 3). The *P. semisulcatus*

catch was predicted at 3684.5 tons with 95% confidence limits from 1751.5 to 5617.6 tons (Table 3).

The observed catch of all species was 2551.5 tons in the 1997 to 1998 season, falling outside the 95% confidence interval, 2802.1 to 4761.0 tons, forecast by the parametric method (Table 3). The landed catch of *P. semisulcatus* in this season was 2117.7 tons, much lower than the forecast of 3684.5 tons, although it was within the 95% boundaries of 1752 to 5618 tons (Table 3).

#### Forecast from the Nonparametric

The regression analysis above suggests that the best recruitment indices are the mean catch rates from the southern area in July and August. Although multivariate extrapolations of kernel estimators have been developed (Loftsgaarden and Quesenberry 1965), the multivariate methods are computationally demanding. As a first step, an univariate one was used here. Between these two indices, the July mean catch rates were chosen, because of their greater contribution (Table 1) and a lower standard error (Table 2).

The estimated PDF is shown in Figure 5. The prediction for the 1997 to 1998 season, in fact, was a full PDF, not a single expected value. Therefore, the plot was probability (y-axis) versus catch (x-axis) for a given recruitment index, not the usual regression-based plot of expected value of catch across the range of the recruitment index. The full uncertainty of each prediction was an intrinsic part of the results. The median, the catch corresponding to the 0.5 cumulative proportion in Figure 5, of the 1997 to 1998 season's total landings was estimated 3229.9 tons (Table 3), 14.5%

TABLE 3.

Comparison between observed shrimp landings and forecasts in the 1997 to 1998 season

	Species combined	<i>P. semisulcatus</i>
Parametric forecast (t)	3781.6 (2,802–4,761) <sup>a</sup>	3684.5 (1,752–5,618)
Nonparametric forecast (t)	3229.9 (1,600–4,000)	2031.9 (900–4,000)
Observed landings (t)	2551.5	2117.7

<sup>a</sup> Numbers in bracket are 95% boundaries.

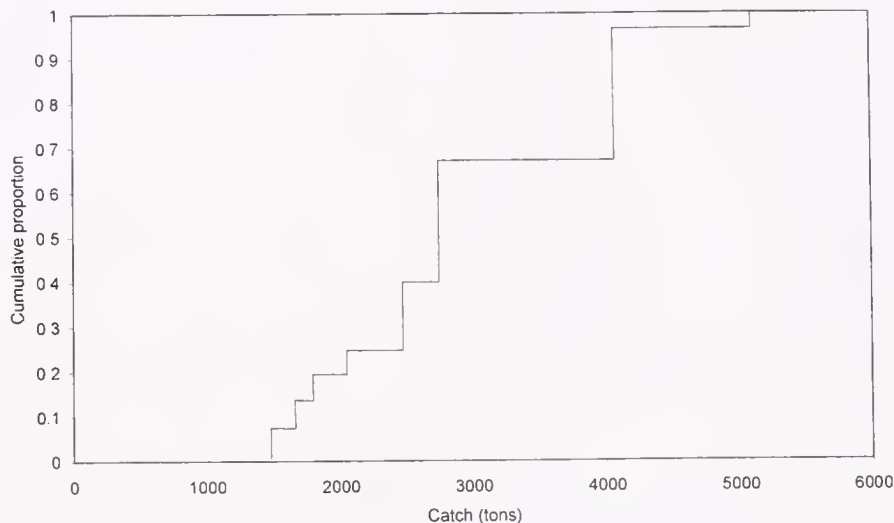


Figure 5. The cumulative probability of species combined shrimp landings for the July 1997 recruitment index in the southern area.

lower than the prediction from the regression model. The ogive suggests that the 1997 to 1998 season has a very low probability (<3%) of landings over 4,000 tons, and a probability of 5% of landings less than 1,600 tons. The nonparametric method suggests that both the lower and upper 95% boundaries are lower than the estimates given by the regression model.

The estimated median of the *P. semisulcatus* catch in 1997 to 1998 was 2,031.88 tons (Table 3). The probability of landings over 4,000 tons was less than 3%, and there was a probability of about 5% that the total shrimp catch would be below 900 tons (Fig. 6).

The actual species combined catch in 1997 to 1998 was 2,551.5 tons, 21% lower than the forecast 3,229.9 tons, but still within the 95% confidence interval of 1,600 to 4,000 tons forecast by the nonparametric method. The single-species catch of *P. semisulcatus* was 2,117.7 tons, very close to the nonparametric forecast of 2,031.9 tons (Table 3).

#### General Discussion

All the predictions have wide confidence intervals. This is attributed to the great variation of the survey data, which is usual in

fisheries. The parametric forecasts are quite poor. Actual species combined landings in 1997 to 1998 fell outside the 95% confidence boundaries, and the parametric forecast for *P. semisulcatus* was 74% higher than what was landed, although its 95% confidence interval covered the real catch value (Table 3). In contrast, the nonparametric methods made better forecasts than traditional parametric methods. Both landed catches of combined species and single species of *P. semisulcatus* were within the 95% confidence intervals of forecasts. Impressively, the forecast for *P. semisulcatus* was only 4% lower than the landed in the 1997 to 1998 season (Table 3).

The parametric methods made poor forecasts, although they fitted the historical data very well (Figs. 3 and 4). The best estimator that provides the best fit to the historical data may not produce the best forecasts (Noakes 1989). One or two outliers (see the point at the upper right corner of Fig. 2) will affect parameter estimates of regression strongly, and hence, all predictions using this model. In the nonparametric methods, the outliers will be present as an extended limb of the PDF, but the influence of the outliers diminishes quickly outside the area of the recruitment index

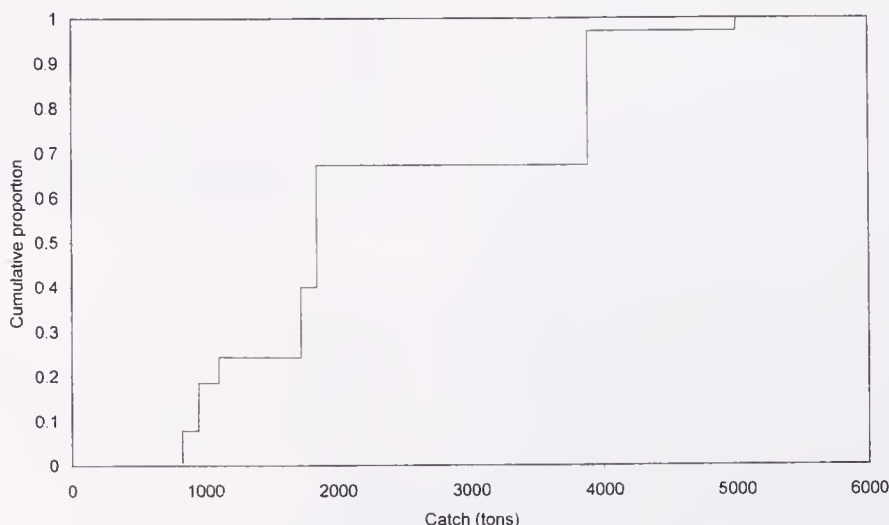


Figure 6. The cumulative probability of *Penaeus semisulcatus* landings for the July 1997 recruitment index in the southern area.



axis where the exceptional observations lie. Predictions from non-parametric methods may be biased by distribution characteristics of the observations, but the predictions do not necessarily preserve those characteristics. This may partly explain why the predictions of the parametric methods are higher than those of nonparametric methods (Table 3).

The predicted PDF facilitates interpretation in the context of the biology of the population under study and differs from model-based analysis methods. The full PDF is rich with information and can be used in many ways. When testing *a priori* hypothesis, the slope of the PDF provides important information about the range of estimates consistent with a prediction. Relatively high probabilities of 20% of the combined species catch below 2,000 tons can easily be seen in Fig. 5. The direct estimation of the probability that a seasonal catch falls above or below some value given a special set of conditions may be useful in risk assessment.

In this study, because of particular difficulties, only a short time series of observations are available. This will deteriorate the effects of outliers on predictions. Although a few outliers do not introduce serious bias to all predictions from density estimation methods, bias is still a concern. Large numbers of observations are needed before kernel estimators provide completely unbiased estimates of PDFs (Bowman 1985, Silverman 1986). The PDF's forecasts by the kernel methods are likely to contain some bias, but

they are likely to be wrong by less than predictions from routine model fitting to a few dozen noisy datapoints (Evans and Rice 1988, Rice 1993).

The parametric models used two variables, the mean catch rates of the southern area in July and August; the nonparametric approach, however, employed only one. The nonparametric forecast results from one index, the mean southern catch rate in July, are quite promising. The cause-effect relationship between the seasonal landings and recruitment is neither simple nor knowable. There are many potential effects of such environmental variables as water temperature, salinity, Shatt Al-Arab discharge, or area of nursery habitat. In principle, the Cauchy method can be extended to two or more independent variables, but details have not been worked out (Rice 1993). Therefore, difficulties may exist for selecting one or two best sets of independent variables for nonparametric methods from a great number of variables. Parametric methods may well serve as an approach to identify independent variables primarily for nonparametric methods.

#### ACKNOWLEDGMENTS

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## SKewed SEX RATIO IN AN ESTUARINE LOBSTER (*HOMARUS AMERICANUS*) POPULATION

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**ABSTRACT** A total of 19,485 lobsters were caught at eight sites in the estuarine and coastal waters of New Hampshire from 1989 to 1992, and their size and sex were determined. The sex ratio of lobsters caught farthest from the coast, in Great Bay, was heavily skewed in favor of males. Sex ratios in other estuarine and river sites were also skewed toward males, and there was a tendency for the number of males per female to decline as one moved down the estuary toward the coast, where the sex ratio was nearly 1:1. The single offshore site was dominated by females, with about 0.6 males for each female. There were also seasonal trends in the sex ratios in the upper estuarine sites, where the number of males per female tended to decline from summer through autumn. In general, differences in the sex ratios between sites were those of primarily adult lobsters larger than 80 mm carapace length (CL). At all sites, the sex ratio of lobsters smaller than this size was close to 1:1, whereas in the upper estuary the mean sex ratio of lobsters greater than 80 mm CL was more than 14:1. These data, in conjunction with seasonal variations of sex ratios, suggest that differential movements of adult male and female lobsters is the primary cause of skewed sex ratios in the Great Bay Estuary.

**KEY WORDS:** Estuary, lobster, *Homarus americanus*, sex ratio

### INTRODUCTION

The American lobster, *Homarus americanus* (Milne-Edwards), is broadly distributed in the western north Atlantic from Labrador to North Carolina (Squires 1990). Within this range, the species supports important commercial and recreational fisheries, particularly in New England and the Canadian Maritime provinces. Because of their commercial importance, lobsters have received a considerable amount of attention from the scientific community (see reviews by Cobb and Phillips 1980a, Cobbs and Phillips 1980b, Factor 1995). Not surprisingly, most of these studies have focused on coastal and off-shore populations where lobsters are most abundant.

Although lobsters are considered to be stenohaline, and generally limited to marine ( $\geq 25$  ppt salinity) habitats (Dall 1970), there are smaller exploited populations found in estuarine habitats (Thomas 1968, Thomas and White 1969, Munro and Therriault 1983, Reynolds and Casterlin 1985, Vetrovs 1990). The physiological ecology and population structure of these lobsters is poorly understood. In recent years, we have been studying one such population located in the Great Bay Estuary of New Hampshire, USA (Jury et al. 1994a, Jury 1994b, Jury et al. 1995, Crossin et al. 1998, Watson et al. 1999). This system, located in the southeastern portion of the state, is characterized by extensive mudflats separated by deep (10–20 m) channels, strong tidal mixing and flushing, and marked seasonal changes in temperature and salinity. Monthly mean temperatures can vary from 0–18°C at the coast, and from 0–25°C in the upper estuary (Loder et al. 1983). The system receives freshwater from seven rivers that drain an area of approximately 2400 km<sup>2</sup>. Salinities in the upper estuary may drop to 10–15 ppt in the spring, as freshets associated with snow and ice melt, and heavy rains enter the system. At the coastal terminus, average salinities are much more stable, typically ranging from 30–33 ppt (Loder et al. 1983).

Among the data we have gathered is information on sex ratio by location, season, and size class. The sex ratio of many geographically separate American lobster populations has been reported. Although most coastal lobster populations that have been examined approximate the expected 1:1 ratio (Cooper 1970, Stew-

art 1972, Krouse 1973, Cooper et al. 1975, Pecci et al. 1978), there are several instances where skewed ratios have been observed. These include reports of populations with more males than females (Briggs and Mushacke 1979, Munro and Therriault 1983, Karnofsky et al. 1989), as well as reports of populations with more females than males (Skud and Perkins 1969, Estrella and McKiernan 1989). Explanations for these skewed sex ratios have included differential catchability (Krouse and Thomas 1975, Fogarty and Borden 1980, Miller 1990, Tremblay and Eagles 1997), segregation of the sexes by depth (Skud and Perkins 1969, Briggs and Mushacke 1979), differences in migratory behavior (Munro and Therriault 1983), physiological and behavioral differences between the sexes (Jury et al. 1994a, Jury et al. 1994b), and fisheries regulations that protect some females (Estrella and McKiernan 1989). In this paper, we report consistent spatial differences in lobster sex ratio within a New England estuary, and differences in sex ratio between size classes of lobsters found in upper estuarine areas.

### MATERIALS AND METHODS

The Great Bay estuarine system lies in the southeastern corner of New Hampshire, USA. It receives freshwater from seven rivers, which mixes with saltwater entering from the western Gulf of Maine. Lobsters were sampled at eight sites in the estuarine and coastal waters from 1989 to 1992 (Fig. 1). These spanned a distance (by water) of approximately 37 km, ranging from Great Bay proper, which is about 26 km inland, to the Isles of Shoals, which lie 11 km offshore. The eight sites fall into three broader spatial categories, which we have arbitrarily designated as "estuarine" (Great Bay, Little Bay, Bellamy River), "riverine" (upper, mid-, and lower Piscataqua River), and "coastal" (Coast, Isles of Shoals). Along this line of sites, physical and chemical characteristics vary from those of a typical New England estuary (greatly fluctuating temperature and salinity, strong tidal mixing, soft substrate) to those of a typical New England coast (relatively stable temperature and salinity, less tidal current, and harder substrates of cobble and rock).

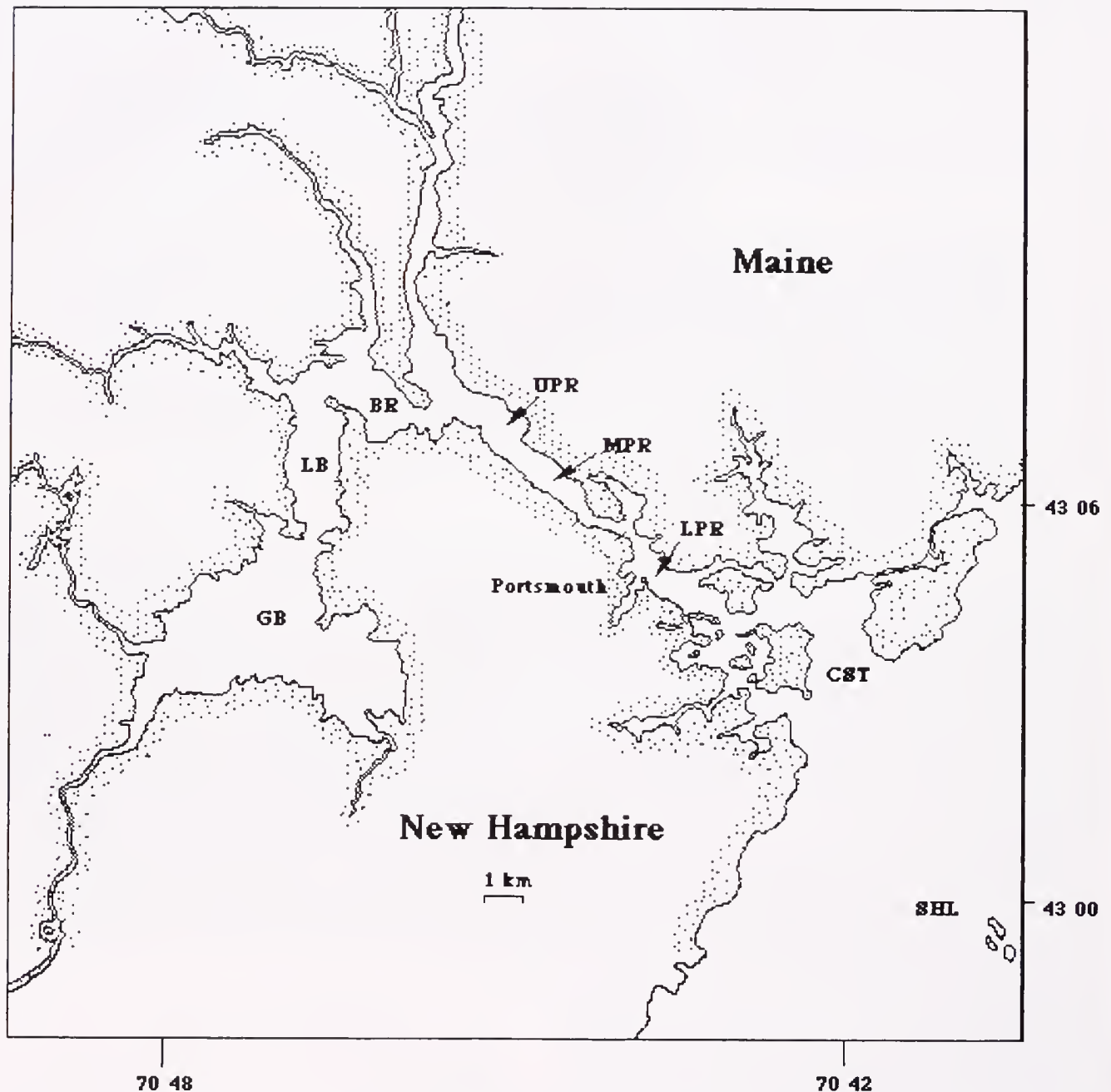


Figure 1. Location of the study sites within the estuarine and coastal waters of New Hampshire. GB (Great Bay), LB (Little Bay), BR (Bellamy River), UPR (Upper Piscataqua River), MPR (Middle Piscataqua River), LPR (Lower Piscataqua River), CST (Coast), SHL (Shoals).

All lobsters were caught in traps baited with herring and tended two to three times per week. Most were caught in our own traps as part of a larger study on estuarine lobsters, but many were caught by commercial lobstermen with whom we fished, and a small number were caught by the New Hampshire Department of Fish and Game. All traps from which we collected data were made of vinyl-coated wire, equipped with one or two escape vents (1 7/8" H x 6" W), and had either a single (research traps) or double parlor (commercial traps). Although winter sampling was limited because of upper estuarine ice cover and general lack of commercial fishing activity, we were able to sample all sites adequately during the spring (April–June), summer (July–September), and autumn (October–December) in most years. All lobsters had their carapace

length (CL) and abdomen width measured to the nearest millimeter, all were molt-staged using external shell criteria and/or pleopods (Aiken 1973, Aiken 1980), and all were sexed by examining the first pair of pleopods (Templeman 1944). Most were also tagged, before release, with numbered modified sphyron tags (Scarratt 1970), because in another part of the study, we were examining movement and growth (Watson et al. in press).

At each study site, except the Shoals, both temperature and salinity (YSI Meter Model 33) were measured at the surface each time our traps were hauled. In 1991, data were collected from surface and bottom waters. There was always <2°C and 2 ppt difference between surface and bottom values because of extensive vertical mixing (Loder et al. 1983).

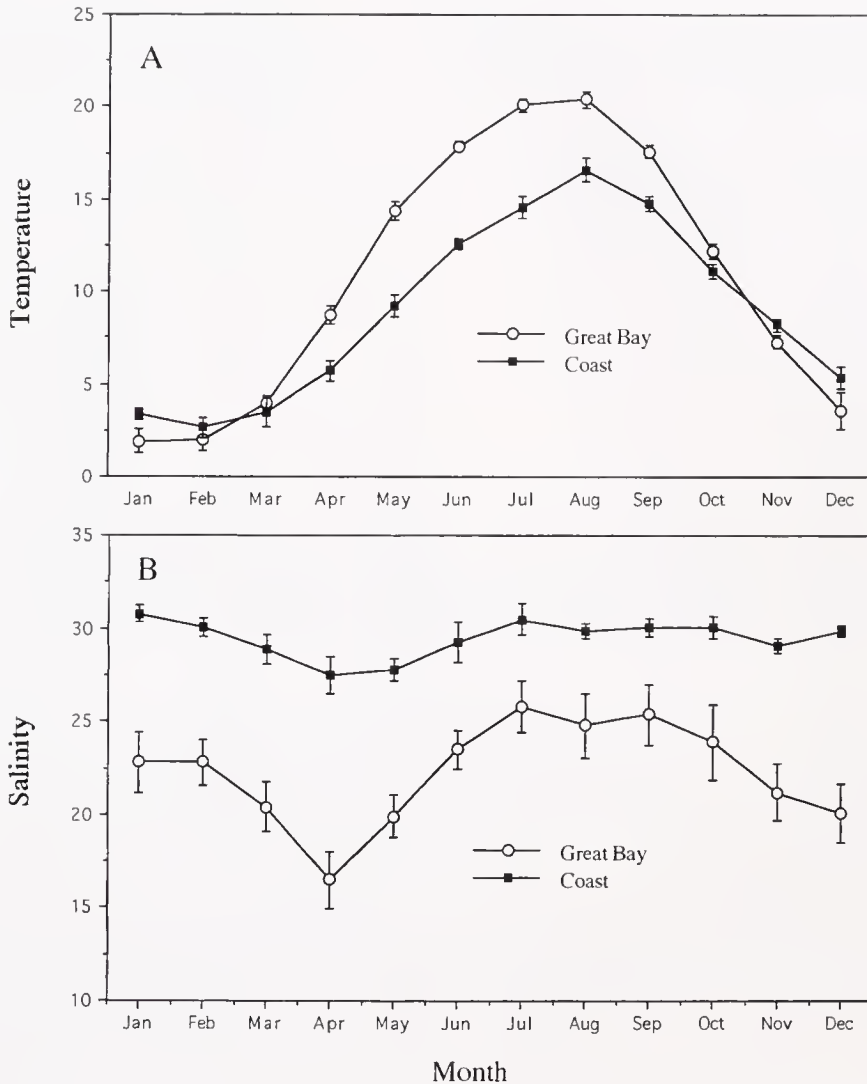


Figure 2. Mean ( $\pm$ SE) monthly temperatures ( $^{\circ}$ C) and salinities (ppt) at the Great Bay and Coastal sites from 1989 to 1992.

The null hypothesis of equal numbers of males and females was, in all cases, tested using chi square analysis. Comparisons of sex ratio between sites, and between seasons within sites, were done using one-way analysis of variance (ANOVA) followed by Tukey's posterior test. Mann-Whitney U tests were used to compare the sizes of males and females at each site within each year. The alpha level for all statistics was 0.05.

## RESULTS

Mean monthly temperature and salinity, from 1989 to 1992, at two of our sites is depicted in Fig. 2. In the upper estuary (Great Bay site, GB), mean monthly temperatures were 3–7 $^{\circ}$ C warmer than the Coastal (CST) site from April through October (Fig. 2a). Mean salinity in Great Bay was highest in late summer (approx. 27 ppt), and lowest (approx. 16 ppt) in the spring when freshwater input was more abundant because of heavy rains and snowmelt. At the Coastal site, the salinity was relatively constant throughout the year (Fig. 2b). As expected, both temperatures and salinities at the sites that occur between Great Bay and the Coast are intermediate

to those depicted. Although complete temperature and salinity records are not available from the Isles of Shoals, the small amount of data available indicates this site is very similar to the Coastal site, located approximately 11 km away.

Observed mean sex ratios, from 1989 to 1992, at each of the sampling sites are given in Table 1. Although there was some interannual variation at each site, in each of the estuarine sites (Great Bay, Little Bay, Bellamy River), there were significantly more males than females in every year ( $p < .05$ ). In the riverine sites (upper-, mid-, lower Piscataqua River) and at the Coast, the numbers of males and females were more nearly equal. However, even at these sites, there were significantly more males than females in some years (Upper Piscataqua River 1991; mid-Piscataqua River 1989, 1990; lower Piscataqua 1991; Coast 1990). As with the other sites, there are some interannual variation in sex ratio at the Isles of Shoals, but in each year for which data were available, there were significantly more females than males ( $p < .05$ ).

When the data from all years and seasons were combined, sex ratio departed significantly from the expected 1:1 ratio in seven of

TABLE 1.  
Mean number of males per female at each sampling site from 1989 to 1992 and in all years combined.

Site		Year				All Years
		1989	1990	1991	1992	
Great Bay	Mean	6.92*	5.32*	5.88*	3.03*	5.29*
	SE	2.65	0.86	3.28	0.53	0.82
	<i>n</i>	83	136	358	130	707
Little Bay	Mean	1.81*	1.86*	2.04*	1.68*	1.85*
	SE	0.15	0.12	0.39	0.14	0.07
	<i>n</i>	297	876	1,165	516	2,854
Bellamy R.	Mean	1.32*	1.32*	2.30*	1.56*	1.63*
	SE	0.16	0.01	0.37	0.37	0.63
	<i>n</i>	614	1,318	1,236	902	4,070
Upper Pisc. R.	Mean	1.27	1.29	1.39*	—	1.31*
	SE	0.21	0.19	0.20	—	0.04
	<i>n</i>	486	316	724	0	1,526
Middle Pisc. R.	Mean	1.42*	1.28*	0.95	0.93	1.14*
	SE	0.10	0.05	0.16	0.15	0.12
	<i>n</i>	1,197	1,210	489	342	3,238
Lower Pisc. R.	Mean	0.89	1.02	1.44*	1.11	1.12
	SE	0.16	0.06	0.08	0.02	0.12
	<i>n</i>	278	480	244	284	1,286
Coast	Mean	1.02	1.33*	1.22	1.00	1.18*
	SE	—	0.24	0.14	0.12	0.08
	<i>n</i>	253	1,248	463	777	2,488
Shoals	Mean	0.64*	—	0.52*	0.56*	0.57*
	SE	—	—	0.07	0.06	0.03
	<i>n</i>	222	0	1,893	1,201	3,316

Mean and standard error within years is based on three seasons. Mean and standard error for all years is from all years and all seasons combined. (*n*) = number examined; \* denotes a significant departure from a 1:1 sex ratio (chi square,  $p < .05$ ).

the eight sampling sites (Table 1). There were significantly more males than females in each of the five upper sites and at the coast ( $p < .05$ ), and significantly more females than males at the Isles of Shoals ( $p < .05$ ). In the remaining site (lower Piscataqua River) there were approximately equal numbers of males and females.

Using the aggregate data from all years and seasons, we found that the mean number of males per female in Great Bay (5.29) was significantly higher ( $p < .001$ ) than every other site. Although there was a tendency for the number of males per female to decline as one moved toward the coast (Table 1), there was no significant difference in the mean sex ratio among these other sites ( $p > .05$ ). This same tendency was also observed in each of the three upper estuarine sites. Although there was some interannual variation in each of these sites, in each year except 1991, sex ratio declined as one moved down the estuary from Great Bay to Little Bay to the Bellamy River. Unlike the three upper estuarine sites, there was no obvious clinical trend in sex ratio found in the riverine and coastal sites. The Shoals site, however, had the lowest mean sex ratio of all sites in each of the years for which we had data (Table 1).

In addition to the observed spatial differences in sex ratio, there were also some seasonal trends observed (Fig. 3). At the Great Bay site, there was a considerable amount of interannual variation within seasons. Although the mean number of males per female tended to be highest in the spring (6.37), and then to decline through the summer (5.45) and autumn (4.38), there were no significant differences ( $p > .05$ ) between seasons. A similar, but less pronounced pattern was observed in Little Bay, but again, there were no significant differences between seasons ( $p > .05$ ). Interestingly, at the Bellamy River site, the seasonal trend was reversed.

Although there was no significant difference between seasons, sex ratio tended to increase from spring (1.42), to summer (1.50), to autumn (1.84). Seasonal trends were much less pronounced at the other five sites. Significant seasonal differences in sex ratio were found only at the upper Piscataqua River site, where the mean number of males per female was significantly higher ( $p < .001$ ) in the spring (1.71) than in either summer (1.10) or autumn (1.13), between which there was no significant difference ( $p > .05$ ).

The mean size (CL) of male and female lobsters at each site and year is given in Table 2. In general, the mean size of males were significantly larger than that of females in the three upper estuarine locations. In the Piscataqua River sites, males and females were more similar in mean size. The only significant differences were found in the middle Piscataqua River, in 2 of the 4 years, and in the lower Piscataqua River, in 1 of the 4 years. In each of these instances, males were larger than females. At the coastal site, the mean size of males was larger than that of females in 1989; whereas the reverse was true in 1990. No difference in mean size was found in the remaining 2 years. Finally, at the Isles of Shoals site, where we had only 3 years of data, females were significantly larger than males in two of these (1989, 1992), but not in the other (1991).

Sex ratio varied with size class at certain sites (Table 3). The site where the change in ratio with size class was most pronounced was in Great Bay, which is the site farthest up the estuary. In the other estuarine sites (Little Bay and Bellamy River), males also tended to dominate the larger size classes, but not to the same extent as in Great Bay. In the riverine sites, there tended to be more males than females in many size classes, but the sex ratio was most

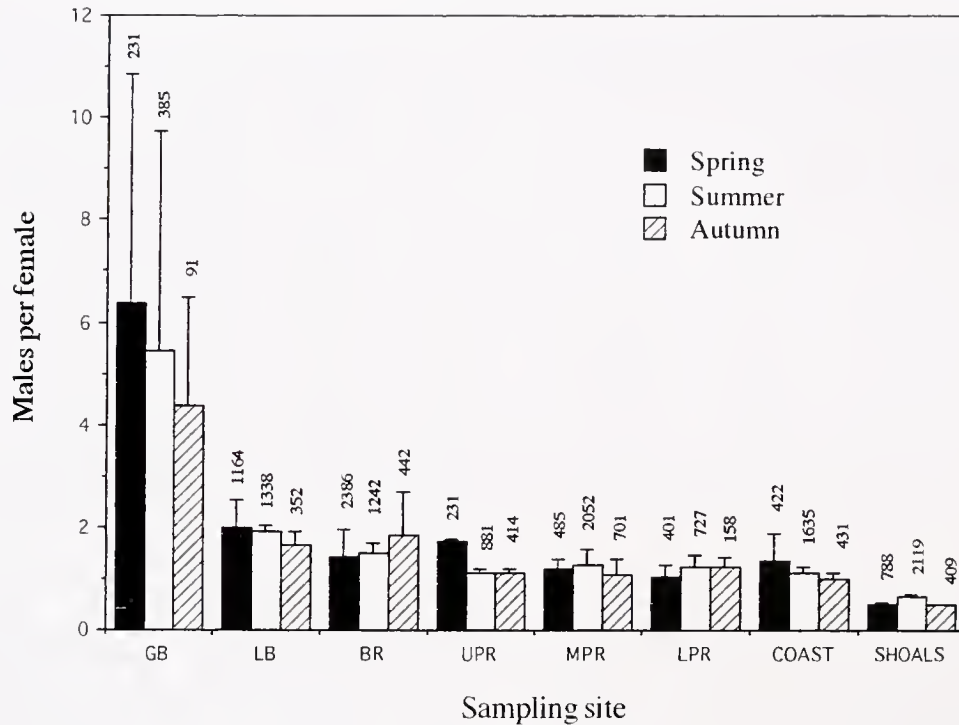


Figure 3. Mean ( $\pm$ SD) number of males per female at each of the sampling sites in the spring, summer, and autumn. Means are based on the 4 years included in the study (1989 to 1992). The number of lobster examined (*n*) is given vertically above each error bar. GB (Great Bay), LB (Little Bay), BR (Bellamy River), UPR (Upper Piscataqua River), MPR (Middle Piscataqua River), LPR (Lower Piscataqua River).

heavily skewed toward males in the largest (>85 mm CL) size classes. This was particularly true at the uppermost riverine site (upper Piscataqua), and, to a lesser extent, the middle Piscataqua site. At the coastal site, there was a less pronounced pattern of change in sex ratio with size class. There tended to be more males than females in the smaller size classes (<70 mm), about equal

numbers of males and females in the 71–90 mm size classes, and about twice as many males as females in the largest size class (>90 mm). At the Isles of Shoals site, however, there were consistently fewer males than females in all of the larger size classes (>65 mm), and the same number, or more, males than females in the smaller size classes.

TABLE 2.

Mean (and standard deviation) carapace length (mm) of male and female lobsters at each site in each year, and in all years combined.

Site	1989		1990		1991		1992		All Years	
	M	F	M	F	M	F	M	F	M	F
Great Bay	80.7*	71.2	81.3*	75.5	83.7*	78.7	77.7	76.7	82.3*	77.4
SD=	9.6	16.7	7.2	7.2	8.1	5.8	7.8	2.8	8.3	7.5
Little Bay	78.1*	74.9	75.3	73.5	81.7*	78.3	78.3*	76.0	80.0*	76.0
SD=	6.7	8.6	6.4	7.2	6.9	6.3	7.7	7.5	7.3	7.3
Bellamy River	73.6*	72.0	74.4*	72.0	80.4*	76.0	79.8*	76.8	76.6*	73.3
SD=	10.7	10.4	8.4	8.4	8.2	7.0	8.5	6.8	9.2	8.7
Upper Piscat. R.	51.7	53.7	53.0	50.9	75.1	74.8	No data	No data	57.6	57.2
SD=	14.7	14.2	13.3	13.4	9.5	6.5			16.2	15.6
M. Piscat. R.	72.2	71.7	74.9*	73.5	77.0*	75.3	78.1	70.4	73.9*	73.1
SD=	7.0	7.7	7.7	7.6	6.7	6.8	8.2	12.2	7.4	7.6
L. Piscat. R.	76.5*	74.3	65.3	63.9	72.2	70.3	78.0	80.9	70.0	68.5
SD=	8.8	10.8	12.4	12.3	11.9	13.2	7.7	8.6	12.4	12.9
Coast	77.7*	76.5	71.9*	74.4	75.5	75.2	75.1	67.5	74.1	74.9
SD=	5.5	5.1	13.2	11.8	9.7	10.4	3.9	16.4	11.3	10.6
Shoals	75.4*	80.1	No data	No data	79.6	80.1	80.1*	81.4	79.5*	80.6
SD=	5.8	11.9			7.5	7.6	8.4	8.7	7.8	8.3

\* Between male and female lengths within a year and site indicates that the mean lengths of the two sexes are significantly different (Mann-Whitney U test,  $p < .05$ ). Sample sizes are given in Table 1.

TABLE 3.

Mean ( $\pm$ SE) number of males per female in different size categories at each sampling size, based on data collected over 4 years.

Carapace Length (mm)	Great Bay	Little Bay	Bellamy R.	Upper Pisc. R.	Middle Pisc. R.	Lower Pisc. R.	Coast	Shoals
= <40	1.00	—	1.21 $\pm$ 0.49	1.52 $\pm$ 0.22	—	0.71 $\pm$ 0.20	0.93 $\pm$ 0.05	—
41–45	—	1.00	0.77 $\pm$ 0.19	1.29 $\pm$ 0.17	0.67 $\pm$ 0.29	0.55 $\pm$ 0.25	0.25 $\pm$ 0.17	—
46–50	—	0.75 $\pm$ 0.25	1.33 $\pm$ 0.47	0.90 $\pm$ 0.28	1.17 $\pm$ 0.96	0.68 $\pm$ 0.31	2.5 $\pm$ 0.90	1.5 $\pm$ 0.35
51–55	1.00	1.85 $\pm$ 1.06	1.14 $\pm$ 0.19	1.25 $\pm$ 0.43	1.45 $\pm$ 0.22	0.69 $\pm$ 0.19	1.31 $\pm$ 0.17	3.50 $\pm$ 1.77
56–60	1.33 $\pm$ 0.29	0.47 $\pm$ 0.22	1.27 $\pm$ 0.14	1.39 $\pm$ 0.23	0.96 $\pm$ 0.31	0.76 $\pm$ 0.15	1.23 $\pm$ 0.14	1.50 $\pm$ 0.35
61–65	1.75 $\pm$ 0.48	1.42 $\pm$ 0.27	1.16 $\pm$ 0.12	1.19 $\pm$ 0.17	0.99 $\pm$ 0.22	1.23 $\pm$ 0.20	1.65 $\pm$ 0.09	0.95 $\pm$ 0.19
66–70	2.38 $\pm$ 0.69	1.45 $\pm$ 0.15	1.34 $\pm$ 0.19	1.14 $\pm$ 0.26	1.21 $\pm$ 0.09	1.02 $\pm$ 0.18	1.35 $\pm$ 0.26	0.75 $\pm$ 0.16
71–75	3.00 $\pm$ 0.75	1.28 $\pm$ 0.15	0.79 $\pm$ 0.09	1.17 $\pm$ 0.23	1.14 $\pm$ 0.32	0.98 $\pm$ 0.25	0.97 $\pm$ 0.10	0.60 $\pm$ 0.02
76–80	3.42 $\pm$ 0.70	1.94 $\pm$ 0.20	1.38 $\pm$ 0.22	1.13 $\pm$ 0.06	1.16 $\pm$ 0.15	1.28 $\pm$ 0.09	1.08 $\pm$ 0.09	0.49 $\pm$ 0.01
81–85	12.82 $\pm$ 5.68	2.74 $\pm$ 0.28	2.53 $\pm$ 0.41	1.69 $\pm$ 0.17	1.11 $\pm$ 0.12	1.38 $\pm$ 0.18	0.98 $\pm$ 0.20	0.62 $\pm$ 0.03
86–90	9.05 $\pm$ 1.78	3.9 $\pm$ 0.34	5.12 $\pm$ 2.03	7.00 $\pm$ 0.00	3.05 $\pm$ 1.04	1.79 $\pm$ 1.21	0.93 $\pm$ 0.17	0.58 $\pm$ 0.04
>90	14.5 $\pm$ 5.48	6.43 $\pm$ 3.05	9.11 $\pm$ 2.41	3.00 $\pm$ 0.00	2.19 $\pm$ 1.28	0.80 $\pm$ 0.14	2.2 $\pm$ 1.08	0.55 $\pm$ 0.03

## DISCUSSION

Results of this study indicate that this estuarine population of lobsters departs, in many ways, from the expected 1:1 sex ratio typical of coastal populations. Among the most consistent of our findings was the observed spatial difference in sex ratio. In each season of each year, the upper estuary had more males than females. This skewed ratio tended to decrease, in a clinal fashion, as one moved down the estuary toward the coast, where the sex ratio approximated the expected 1:1 ratio. Surprisingly, this clinal trend continued outside the estuary, so that at the Isles of Shoals, which is located about 11 km from the mouth of the estuary, there were consistently more females than males (Table 1).

Lobster populations with skewed sex ratios have been reported by others (Skud and Perkins 1969, Briggs and Mushacke 1979, Munro and Therriault 1983, Campbell and Pezzack 1985, Karnofsky et al. 1989, Estrella and McKiernan 1989), and several explanations have been put forth to explain the disparity between numbers of males and females. Fishery-related factors, including differential catchability of males and females (Miller 1990), and regulations that protect some (e.g., ovigerous, V-notched) females (Estrella and McKiernan 1989), can result in skewed sex ratios. Differential catchability of the sexes is an unlikely explanation for our results, because Becker (1994), who also worked in the Great Bay estuarine system, found sex ratios virtually identical to ours using SCUBA sampling. Furthermore, if males and females differed in their trapability, as suggested by Fogarty and Borden (1980), Miller (1990), and Campbell (1992), we would have expected skewed sex ratios in all of our study sites because the same types of traps, including identically sized escape vents, were used at all locations. It is possible, however, that the skewed ratio favoring females at the Isle of Shoals may have resulted from differences in mean size, and therefore, trapability, of the sexes. When data from the 2 years were combined, females were significantly larger than the males (Table 2). It has been suggested that female lobsters in some areas (Rhode Island) have a proportionately wider carapace width than similarly sized males (Fogarty and Borden 1980). This difference in body proportion between the sexes may not be geographically universal, however, because Krouse and Thomas (1975) found no significant differences in the carapace length-width ratios of males and females along the Maine coast. If females in our study area do have a proportionately

wider carapace, they would not move as readily through escape vents, so it is possible that the larger mean size of the females at this site influenced sex ratio in the catch. It is also possible that regulatory protection of certain females may explain the sex ratio observed at the Isles of Shoals, where females outnumbered males. At this location, percentages of ovigerous and V-notched females (5–12%) are relatively high as compared to the estuary (<1%) (Howell, W. H. & W. H. Watson. Dept. of Zoology, Univ. of New Hampshire, Durham, NH 03824. Unpubl. data). Thus, both size and protective management may explain the preponderance of females at the Isles of Shoals, but it is also possible that this site simply has a distinctive physical habitat that has resulted in an aggregation of females such as that reported by Campbell (1990).

Ecological factors may also affect sex ratio. Skud and Perkins (1969) and Briggs and Mushacke (1979) found a segregation of the sexes by depth, while Karnofsky et al. (1989) suggested that intraspecific competition affected sex ratio. Our sampling locations were similar in depth ( $\approx$ 3–10 m), so it is highly unlikely that our observations resulted from segregation of the sexes by depth. It is also unlikely that intraspecific competition was a factor. Karnofsky et al. (1989) found that there were nearly twice as many males as females in a small, shallow cove in Buzzards Bay, MA, USA and that a disproportionately large proportion of the males were missing one or more claws. The authors suggested that the cove may function as a refuge for injured males, and that these individuals had been displaced to this shallow water site by aggressive, intraspecific competition for mating shelters. Moreover, they suggested that the relative paucity of females at their study site resulted from the preference of females for deeper areas where the dominant males held mating shelters. We have no information about where mating occurs in our study area, including the depth of mating shelters and whether or not the spatial distribution of females is affected by the distribution of dominant males. Thus, it is possible that the mechanism described by Karnofsky et al. (1989) may be applicable to this study, but it is doubtful, because we saw no indication that the proportion of males missing claws differed among sites (Howell and Watson).

The spatial and temporal trends of the data in this study indicate that sex ratio may be associated with seasonally changing gradients of salinity and/or temperature that are typical of northern estuaries. In particular, it is likely that male and female lobsters differ in their physiological and behavioral responses to salinity



and/or temperature and that these differences result in the sex ratio patterns we observed.

Water temperature affects many, if not all, aspects of lobster biology. In a laboratory study, Crossin et al. (1998) documented that lobsters are capable of sensing temperature, and that they behaviorally thermoregulate; seeking preferred temperatures and avoiding water that is either too warm or too cool. Results from related studies, also done in our laboratory, further suggest that males and females may respond differently to changing temperature. In one study, 75% of females, but only 50% of males, exited their shelters as shelter temperature was increased (Jury, S. H. Dept. of Zoology, Univ. of New Hampshire, Durham, NH 03824. Unpubl. data); whereas in another study, in which males and females were placed in a thermal gradient tank, males generally preferred warmer temperatures than females, particularly in the spring and fall, when ambient temperatures were seasonally lower (Jury, S. H. The effect of acclimation temperature and sex on the behavioral thermoregulation of the American lobster, *Homarus americanus*. In prep.). Although these data are preliminary, they suggest that males and females differ in their temperature preferences, and that spatial and temporal differences in temperature could thus affect sex ratio.

Although laboratory studies on temperature are relatively scarce, numerous field studies have documented that water temperature affects the temporal and spatial distribution of lobsters, and that males and females differ in their movements in response to seasonally changing temperatures (Munro and Therriault 1983, Roddick and Miller 1992, Lawton and Lavalli 1995, Estrella and Morrissey 1997). It has been suggested, for example, that seasonal onshore-offshore migrations are associated with temperature selection, and are adaptive for accelerating growth and egg development (Saila and Flowers 1968, Cooper and Uzmann 1971, Pezack and Duggan 1986, Estrella and Morrissey 1997). This may also be true, on a geographically smaller scale, for seasonal migrations that occur within New England estuaries, including Great Bay (Watson et al. 1999). Differential migration of the sexes, associated with seasonal changes in water temperature, can also effect sex ratio. Roddick and Miller (1992) found, for example, that males and females arrived at, and departed from, a small embayment in Nova Scotia in different months, and these differences in seasonal movements resulted in skewed sex ratios. Adult females have also been reported to move to deeper water earlier in the autumn than males (Campbell and Stasko 1986, Robichaud and Campbell 1991), which results in temporal and spatial segregation of the sexes. Munro and Therriault (1983) found more males than females in estuarine locations in the Magdelaine Islands, and speculated that this resulted from differential migration of the sexes. Both sexes left the estuaries as temperatures cooled in the autumn, but males were more likely to return in the spring as temperature increased. A similar situation may exist in the Great Bay Estuary. In a study concurrent with this one, Watson et al. (1999) documented that lobsters tended to migrate up the Great Bay Estuary in the spring as temperatures increased, and down the estuary in the summer and autumn. Although Watson et al. saw no marked differences in the movements of males and females, their data were somewhat equivocal on this point, and they suggested that differential movement of the sexes was possible. Munro and Therriault (1983) suggested that the reason for males returning earlier than the females was to take advantage of the warmer temperatures of the estuarine sites for molting. Indeed, they found that all males <75 mm CL molted twice each year.

It has also been suggested that there are seasonal differences in the catchability of males and females, that these differences are caused by the two sexes molting at different times, and that differential catchability results in seasonally changing sex ratios (Tremblay and Eagles 1997). In the Great Bay Estuary, however, we saw no evidence that males and females molted at different times or in different locations (Howell and Watson unpubl. data). We conclude from this that there is no difference between the sexes in location and temperature of molting. Thus, although our skewed sex ratios may indeed be related to temperature-mediated differences in movement between the sexes, it seems unlikely that it is strongly correlated with molting, as suggested by Munro and Therriault (1983) and Tremblay and Eagles (1997).

A number of laboratory and field studies have documented that salinity can also effect the temporal and spatial distribution of lobsters. Lobsters are considered to be poor osmoregulators (Dall 1970), and several previous field studies have shown that lobsters use behavioral mechanisms to avoid low salinities (Munro and Therriault 1983, Reynolds and Casterlin 1985, Maynard 1991, Jury et al. 1995). In a recent laboratory investigation Jury et al. (1994a) measured hemolymph osmolarity, oxygen consumption, heart rate and ventilation rate of lobsters under salinity regimes similar to those found in the Great Bay Estuary under spring runoff conditions. They found that exposure to decreasing salinity (from 20 to 10 ppt) caused an increase in oxygen consumption, heart, and scaphognathite rate. At the lowest salinity (10 ppt), females required more energy than males to maintain the same hemolymph osmolarity. Females also recovered more slowly than males as salinities were subsequently increased. This study has been confirmed by Houchens (1996), and extended to show that female lobsters suffer significantly more mortality than males when held at 5–10 ppt. For this reason, upper estuarine locations where salinities are the lowest, particularly in the spring, probably represent a stressful and potentially lethal environment for females. In a second set of experiments, Jury et al. (1994b) measured the behavioral response of lobsters to reductions in salinity. When given a choice of salinity, females were more selective in their preference for higher salinity, and females found low salinities more aversive than did males. Results from these studies indicate that lobsters respond to changes in salinity, that male and female lobsters differ in their physiological and behavioral responses, so that males find low salinity less aversive and less stressful. It is likely that these differences partially explain the observed skewed sex ratios found in this study. In general, we found an inverse relationship between lobster sex ratio and salinity. Physiological and behavioral differences in the way each sex responds to salinity could also explain the seasonal trends in sex ratio that we observed. The number of males per female was highest in the spring in the upper estuary, when salinities were lowest, and then declined over summer as salinities increased. We believe that the observed reduction in sex ratio was caused by the arrival of more females as salinity increased in these areas.

Aside from the physiological and/or behavioral reasons already discussed, it is possible that the observed spatial pattern in sex ratio may also relate to the reproductive biology of lobsters. Because lobster embryos and larvae are quite vulnerable to low (<14 ppt) salinity (Scaratt and Raine 1967, Charmantier et al. 1998, Forward 1989), relatively low salinity environments, such as those in the upper estuary, may be suboptimal for reproduction. Unpublished data on the distribution of ovigerous females in this study support this view (Howell and Watson). We caught and examined 8,153

female lobsters as part of this study, and 168 of these (2.06%) were ovigerous. Of these 168, only 43 were caught in the estuarine and riverine sites, and the remaining 125 were from the Coast and Shoals. The low incidence of ovigerous females in the estuary is similar to the situation reported for blue crabs in the upper Chesapeake Bay by Hines et al. (1987), and it is likely that ovigerous females avoid the low salinity conditions of the estuary, because salinity is generally too low for larval survival. Note, however, that Munro and Theriault (1983) found a higher percentage of ovigerous females (13–16%) in estuaries than they did at the coast (7%). The difference between their study and ours may have resulted from the fact that our upper estuarine salinities are typically as low as 10–15 ppt in any given year; whereas the lowest reported by Munro and Theriault was 22 ppt.

We also found that sex ratio was more skewed in larger size classes (>80 mm CL) in all of our estuarine and riverine locations. Changes in American lobster sex ratio with size class have also been noted by Karnofsky et al. (1989). They found that females dominated the 50–59 mm CL size class, but that males were more numerous than females in size classes  $\geq 60$  mm CL. As a result, males were not only more common, they were also larger. We believe that the observed changes in sex ratio with size class are related to changes in mobility with size. Wahle and Steneck (1992) suggested that small lobsters ( $\leq 60$  mm CL) are dependent on their shelters to avoid predation, but that this vulnerability is eventually outgrown, and lobsters  $\geq 60$  mm CL are able to move about more freely, because they are virtually immune to predation. Once this release has occurred, mobility generally increases as lobsters continue to increase in size (Campbell and Stasko 1986, Campbell 1989). The fact that both mobility and skewness in sex ratio increase with size class indicates that changes in sex ratio with size may result from differential movement of the sexes. When small, both sexes move little, and sex ratio is approximately 1:1. As size (and mobility) increase, males, which are more tolerant of low salinity than females, may travel further up the estuary, especially in the spring, resulting in the predominance of males in the larger

size classes in this location at this time. Studies are currently underway to determine if the aforementioned differences in the behavior of male and female lobsters exist, even in the smaller size classes, or if they manifest themselves only as they reach sexual maturity. If the latter situation is true, it supports the view that the strongest influence on female migratory behavior in the estuary is related to reproduction and the seeking of appropriate habitats for hatching of larvae. In the Great Bay Estuary, ovary dissections indicate that approximately 50% of females have reached sexual maturity of 80 mm CL (Howell and Watson unpubl. data), and it is in size classes greater than this that we observe the most skewed sex ratios.

In summary, we believe that the skewed sex ratio patterns we observed in this study resulted from differential movement of the sexes; probably in response to salinity and temperature cues. Both sexes tend to move down the estuary in the summer and autumn. Males, which are more tolerant of low salinity and warmer temperatures, return to upper estuarine areas earlier than females in the spring, which accounts for the elevated sex ratio seen in these locations. Although some females move up the estuary as salinity rises, thereby making the sex ratio more nearly equal, more females than males remain in the lower estuary, because they are less tolerant of low salinity and warmer temperatures, and/or because it is a more favorable (higher salinity) location to release their larvae. The fact that sex ratio is most skewed among the largest size classes, which are also the most mobile, supports our contention that skewed sex ratio in our study site results from differential movement of the sexes.

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## PARALYTIC SHELLFISH TOXINS IN MUSSELS AND *ALEXANDRIUM TAMARENSE* AT VALDES PENINSULA, CHUBUT, PATAGONIA, ARGENTINA: KINETICS OF A NATURAL DEPURATION

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**ABSTRACT** Paralytic shellfish toxin profiles of *Alexandrium tamarense* (Lebour) Balech and mussels (*Aulacomya ater*) contaminated by the dinoflagellate, were obtained from eight sampling stations along the Valdés Peninsula, Chubut, Argentina. The samples were collected from November 1995 to May 1996. The data show, that the monitoring began after an outbreak during a bloom of *A. tamarense*. The highest cell densities were found in November 1995 at Bengoa ( $1.81 \times 10^3$  cells/L) and Larralde ( $1.2 \times 10^3$  cells/L), both stations are located in the San José Gulf. Occurrence of other species of phytoplankton are also reported. *A. tamarense* was never more than 2% of the total phytoplankton population. Low temperatures and high salinity were found in November 1995, when the highest *A. tamarense* cell density was observed. Using a postcolumn derivatization high-performance liquid chromatography (HPLC) analysis, the PSP toxin profiles of Patagonian coast phytoplankton and mussel samples were obtained for the first time. The average PSP toxin profile of over 30 mussel samples from all monitoring stations showed the gonyautoxins 1-4 (GTX 1-4) epimers to be the most abundant PSP toxins. These epimers were the most prevalent ones in the *A. tamarense* present in the phytoplankton samples analyzed. Other PSP toxins quantified in mussel samples were: STX, dcSTX, and C1-C4. NeoSTX was never found in mussel or phytoplankton samples. The highest toxicity in the phytoplankton samples was 490 fmol of PSP toxins/cell and mussels 631  $\mu\text{g}$  STX eq./100 g, both of which were obtained in November, 1995. The decrease of the toxicity in the filter feeder *Aulacomya ater*, occurs following an exponential decay of the first order, showing that, in the San José Gulf, Valdés Peninsula, the natural depuration process of *A. ater* can be interpreted by a one-compartment model. According to the detoxification rate determined for *A. ater*, a native South American filter-feeder bivalve, can be classified as a moderate detoxifier.

**KEY WORDS:** PSP, depuration, HPLC toxin profiles, *Alexandrium tamarense*, mussel, Patagonia, Argentina, *Aulacomya ater*

### INTRODUCTION

In the southern part of Argentina, the presence of paralytic shellfish poisoning (PSP) had been associated with the occurrence of *Alexandrium* sp., with outbreaks of PSP attributed to *A. catenella* in the Argentine sector of the Beagle Channel (Benauides et al. 1995) and *A. tamarense* from the southern Atlantic coast (47°S) to Uruguayan shores (34°S) (Carreto et al. 1998). The first recorded toxic bloom attributable to *A. tamarense* on the Argentine coast was documented at the Valdés Peninsula in 1980 (Carreto et al. 1996). Since that time, the phenomenon has occurred periodically in the spring and summer seasons, where the PSP producer *A. tamarense*, which increases its density in coastal waters, had been the causative source of PSP toxins contained in filter-feeder bivalves (Carreto et al. 1996, Carreto et al. 1998).

Because such filter feeders as *Aulacomya ater*, the "cholga," take dinoflagellates as food and concentrate such PSP producers as *A. tamarense*, they constitute a public health problem, as well as cause damage to the commercial shellfish industry worldwide (Hallegraeff 1993, Asakawa et al. 1994, Anderson et al. 1996; Lagos et al. 1996; Compagnon et al. 1998). Along the Patagonian coast, one of the most economically important species of bivalves affected by quarantines because of PSP toxicity is the "cholga," which presents its highest population density in the infralittoral zone and represents a commercial harvest of about 100 tons per year from this area.

The natural depuration of filtering organisms is a process that has been little studied, and toxin kinetic studies are rare (Blanco et al. 1997, Bricelj and Shumway 1998). Very recently, laboratory results of feeding studies and concomitant field monitoring of dinoflagellate concentrations and toxicity in bivalve tissues have been implemented in Galicia Rías, Spain (Moroño et al. 1998). This type of study could, in the future, be used to develop predictive relationships between water column toxin concentrations and peak shellfish toxicities, showing the merits and efficacy of both phytoplankton and shellfish monitoring.

Depending upon their detoxification kinetics, bivalves have been classified into two major groups: slow detoxifiers (e.g., *Saxidomus giganteus*, *Spisula solidissima*, *Placopecten magallanicus*, and *Patinopecten yassoensis*) and rapid-to-moderate detoxifiers (e.g., *Mytilus edulis* and *Mya arenaria*) (Bricelj and Shumway 1998).

The development of dynamic models linking toxic cell concentrations and toxin accumulations in the filter-feeder body could provide valuable tools for predicting the timing and duration of toxic blooms (Blanco et al. 1997, Bricelj and Shumway 1998). Both one- and two-compartment models have been used to describe the detoxification kinetics of PSP toxins in different mussel species (Blanco et al. 1997, Bricelj and Shumway 1998, Moroño et al. 1998). Two depuration rates have been described: the initial one is very fast, followed by a second, slower one (Lassus et al. 1989,

Silvert and Cembella et al. 1995; Blanco et al. 1997; Bricelj and Shumway 1998). The study of natural toxic events is fundamental for the appropriate management of affected areas to avoid health risks from PSP toxins.

This paper describes a toxic event attributable to an outbreak of *A. tamarensis*. Because the precise chemical composition of PSP toxins produced by this dinoflagellate in this area was not known, in this study we show the quantitative high-power liquid chromatography (HPLC)-FDL analyses of PSP toxin contents and PSP toxin profiles of Patagonia coast phytoplankton and mussel samples for the first time. Using these data, the natural depuration kinetics of *A. ater*, one of the native mussel species from the Valdés Peninsula was studied. Using a one-compartment model, it was possible to predict the time when peak toxicity was attained and the detoxification rate for *A. ater* at the San José Gulf, Valdés Peninsula was obtained.

### MATERIALS AND METHODS

Six locations around the San José Gulf and two more in Nuevo Gulf (Chubut, Argentine Patagonian coast) were chosen for a field-sampling program. The presence of PSP toxins was investigated in the samples of phytoplankton and mussel samples collected from each location, from November 1995 to May 1996. The mussel and phytoplankton samples were obtained from principal *A. ater* harvesting areas.

The phytoplankton cell counts were done by filtering 2 L of seawater in a 20- $\mu$ m phytoplankton net. The phytoplankton samples were collected at a distance about 1 m from the natural mussel banks. These banks were generally found at between 10 to 15 m depth. Also, the surface and bottom water temperatures and the salinity of the seawater were measured using a multiparameter Cole-Parmer sensor. The turbidity of the sea water was measured using a Secchi disk.

All mussel samples were extracted as described by the standard AOAC mouse bioassay method (Williams 1984). Pellets of *A.*

*tamarensis* cells were suspended in 0.01N HCl and disrupted by sonication. For clean-up, the mussel and phytoplankton samples were passed through a cartridge column (Millipore Corp., Sep-Pack C18) and filtered using microcentrifuge filters (Millipore Corp., Ultrafree-MC filters Units, 400  $\mu$ L, NMWL: 5,000).

Toxin analyses were carried out on an HPLC with on-line fluorescent detection using ion pair chromatography with postcolumn derivatization, as described previously (Oshima 1995a, Lagos et al. 1996). For HPLC, a Shimadzu LC-10AD liquid chromatograph apparatus, on-line with a Shimadzu RF-551 spectrofluorometric detector was used. A silica-base reversed phase column (Prodigy 5  $\mu$ m C8, 4.6  $\times$  150 mm, Phenomenex, CA, USA) was used for the analytical quantification. The oxidizing reagent and acid were pumped using a dual-head pump (model SP-D-2502, Nihon Seimitsu Kagaku Co., Ltd.). Toxin concentrations were determined by comparing the peak areas for each toxin with those of the standard. As external standard, pure PSP toxin solutions were prepared and calibrated by HPLC-FDL and HPLC-MS used in our laboratory. The PSP toxins were purified using preparative liquid chromatography starting from high PSP-contaminated shellfish collected in the southern fjords of Chile (Lagos et al. 1996, Compagnon et al. 1998; Andrinolo et al. 1998). The phytoplankton cells were counted using the Utermöhl inverted microscope technique (Utermöhl 1958). The taxonomic determinations were made according to E. Balech 1977.

### RESULTS AND DISCUSSION

Until recently, on the Argentine Patagonian coast, all monitoring of PSP toxins by routine survey programs or during PSP outbreaks was carried out using the mouse bioassay to determine the total toxicity in shellfish (Carreto et al. 1996, Carreto 1998). Because of the annual periodicity of this phenomenon, in collaboration with Dr. N. Santinelli and her group (Universidad Nacional de la Patagonia), we resolved to look for a paralytic shellfish toxin



Figure 1. Sampling locations in the San José and Nuevo gulfs, Valdés Peninsula. Bivalve and plankton samples came from the principal mussel harvesting areas. The monitoring stations for samplings were: Riacho (42°25'S, 64°36'W), Punta Logaritmo (42°24'S, 64°29'W), Larralde (42°25'S, 64°22'W), Punta Conos (42°20'S, 64°04'W), Bengoa (42°16'S, 64°05'W), Punta San Román (42°15'S, 64°14'W), Punta Pardelas (42°37'S, 64°16'W) and Cerro Avanzado (42°50'S, 64°53'W).

TABLE I.  
Occurrence of *A. tamarense* and other species of phytoplankton at San José Gulf (Nov. 95–May 96).

Phytoplankton Community	Nov. 95 R	Nov. 95 PL	Nov. 95 L	Nov. 95 PC	Nov. 95 B	Nov. 95 SR	Feb. 96 R	Feb. 96 PL	Feb. 96 L	Feb. 96 PC	Feb. 96 B	Mar. 96 PL	Mar. 96 L	Mar. 96 PC	Mar. 96 B	Mar. 96 SR	May 96 PC	May 96 B	May 96 SR
Total Phytoplankton	150.490	91.223	74.625	193.102	98.314	48.902	413.589	596.583	665.523	366.688	672.025	205.882	14.139	234.909	273.699	179.355	51.754	30.360	112.350
<i>Alexandrium tamarense</i>	633	39	1.243	70	1.824	522			53										
Major species																			
<i>Chaetoceras</i> sp.	88.110	39.300	10.960	23.715	5.296	3.040	137.270	96.144		182.655	93.000	7.619		100.119	42.600		1.356		
<i>Thalassiosira</i> spp.															35.500		2.712		
<i>Asterionella japonica</i>	13.617																		
<i>Skeletonema costatum</i>							211.788		527.916										
<i>Cylindrotheca closterium</i>							11.766												70
<i>Pseudonitzschia</i>																			
<i>pseudodelicatissima</i>	26.433	3.930		70.000		14.440													
<i>Pseudonitzschia</i> spp.	6.408	2.358		83.765		22.800			53										60
<i>Dinophysis acuminata</i>	40											30	99						
<i>Prorocentrum micans</i>	158	236	600	927	250		954	653	427	212		60		126		42			42
<i>Prorocentrum lima</i>																	452		
<i>Gyrodinium</i> sp.	237	78	771						38	159	3.000		99	798			7.910	7.080	
<i>Dinoflagellate</i> spp.				340	1.186			192	232	212		42	99		182		24.860	10.500	27.025
<i>Raphidophyceae</i> spp.																			
<i>Flagellates</i>								7.844		53			2.079						
<i>Rhodomonas</i> sp.							11.766	12.018	21.238	49.815	3.000	68.571	8.514	34.824		72.985			

R, Riacho; PL, Punta Logartitno; L, Larralde; PC, Punta Conos; B, Bengoa; SR, San Román.

producing dinoflagellate bloom in this area, with the idea of studying a natural bloom at the Valdés Peninsula and also for the first time, to do quantitative HPLC-FDL analyses of PSP-toxin contents and obtain the PSP toxin profiles of Patagonia coast phytoplankton and mussels. Accomplishing this, successive samplings were carried out between November of 1995 and May of 1996 at eight monitoring stations around the Valdés Peninsula, located on the southern Atlantic coast of Argentina (Fig. 1).

The qualitative phytoplankton surveys showed mainly diatoms, such as *Chaetoceros* spp., with *Pseudonitzschia* sp. as important components of this phytoplankton. In the San José Gulf, during the months of February and March, a great abundance of nanoplankton was observed as containing unidentified species and cryptoficeae of the genera *Rhodomonas* (Table 1). In May 1996, also in the San José Gulf, dinoflagellates constituted an important fraction of the total phytoplankton population, their main components being small, unidentified dinoflagellates. In the Punta Conos monitoring station, also on the San José Gulf, the presence of *Prorocentrum lima* was detected in only one sampling. *Dinophysis acuminata* and *Prorocentrum micans*, all potentially diarrhetic shellfish poisoning (DSP) species, were normal components of the phytoplankton of both gulfs (Table 2).

The PSP-producing dinoflagellate *A. tamarensis*, was detected in the quantitative phytoplankton samples at six stations during the monitoring of November 1995, in the San José Gulf. The highest cell densities were registered at Bengoa station with  $1.8 \times 10^3$  cell/L. *A. tamarensis* was absent during the rest of the monitoring program. The population of *A. tamarensis* never surpassed 2% of the total phytoplankton cells. In contrast, during the entire monitoring period of this station, a total absence of *A. tamarensis* was observed in the phytoplankton samples collected at the monitoring stations in Nuevo Gulf.

Nevertheless, the analyzed mussel samples collected from this gulf showed toxicities as high as 631  $\mu\text{g}$  of STX equiv./100 gr.

This PSP toxin contamination must have come from another outbreak occurring at least 2 months earlier.

The study of the physicochemical parameters on both sides of the isthmus of Carlos Ameghino (between San José and Nuevo gulfs) showed no significant differences between them. Figure 2 shows the mean variation of temperatures and salinity of seawater on the surface and at the bottom (20-m depth), as well as the depth of the Secchi disk measured in each monitoring station. The lowest temperature values were registered in November 1995, with a minimum of 10°C (early spring) and an average of 14.5°C during the summer months of 1996. The salinity varied from  $40.12 \pm 0.42$  g/L (mean  $\pm$  SEM,  $n = 11$ ) in November 1995 (late spring) to  $33.88 \pm 0.1$  g/L (mean  $\pm$  SEM,  $n = 9$ ) in February 1996 (mid-summer) and remained constant during the entire summer season. The Secchi disk had a average value of  $5.4 \pm 0.3$  m (mean  $\pm$  SEM,  $n = 24$ ) during the monitoring program, showing a constant turbidity in both gulfs during the monitoring period.

The toxin profiles found in phytoplankton samples from San José Gulf showed that the majority of the PSP toxins present were from the gonyautoxins group (GTXs). Among these, GTX 1-4 epimers were slightly predominant. From the group of the saxitoxins (STXs), similar quantities of STX and dcSTX were found (Fig. 3). None of the GTX 5, neoSTX, and C1-C4 toxins were detected in any of the phytoplankton samples. The average total toxicity per cell of *A. tamarensis* was  $336 \pm 142$  fmoles of toxins per cell (mean  $\pm$  SEM,  $n = 7$ ). Phytoplankton samples that showed a complete absence of *A. tamarensis* also showed an absence of PSP toxins.

The average total toxicities per cell shown by *A. tamarensis* collected in the San José Gulf, are within the ranges of cell toxicity reported for other *A. tamarensis* cells and *Alexandrium* strains, from other locations, including the coast of Argentina. For example, *A. tamarensis* kept in culture in the United States showed a cell toxicity of approximately 220 fmoles/cell (66 pg STX eq./cell, Bricelj et al. 1990). Oshima 1992, reported an average toxicity of

TABLE 2.  
Occurrence of *A. tamarensis* and other species of phytoplankton at Nuevo Gulf (Nov. 95–May 96).

Phytoplankton Community	Nov. 95 P	Feb. 96 P	Mar. 96 P	Mar. 96 CA	May 96 P	May 96 CA
<b>Total Phytoplankton</b>	<b>13.412</b>	<b>192.787</b>	<b>215.256</b>	<b>191.384</b>	<b>42.605</b>	<b>25.856</b>
<i>Alexandrium tamarensis</i>						
<i>Chaetoceros</i> sp.		50.952	110.121	12.136	830	3.616
<i>Thalassiosira</i> spp.				54.612		
<i>Asterionella japonica</i>				11.715		
<i>Skeletonema costatum</i>				9.372		
<i>Cylindrotheca clasterium</i>				36.408		
<i>Pseudonitzschia pseudodelicatissima</i>		33.968				
<i>Pseudonitzschia</i> spp.	778			507		
<i>Dinophysis acuminata</i>			158			452
<i>Prorocentrum micans</i>	38		9.372	1.911		
<i>Prorocentrum lima</i>						
<i>Gyrodinium</i> sp.	115			78		
<i>Raphidophyceae</i> spp.		706	2.343			452
<i>Flagellates</i>	1.205					
<i>Rhodomonas</i> sp.				6.068	19.034	
<i>Phaeocystis</i> sp.		13.587				
<i>Dinoflagellate</i> spp.					3.544	452



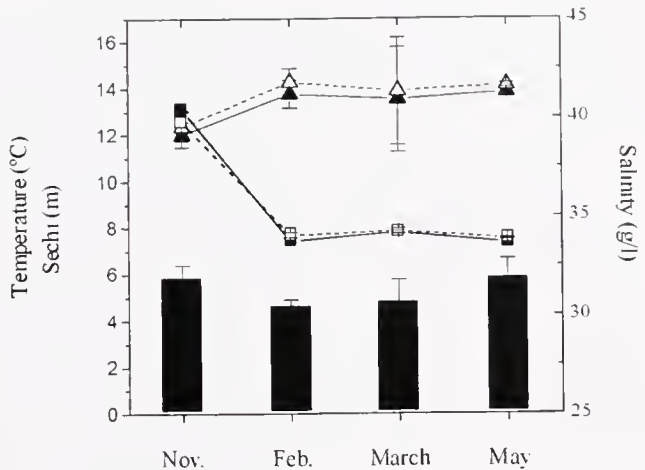


Figure 2. Average physicochemical parameters at Valdés Peninsula (Nov. 95–May 96). Surface temperature (open triangles,  $\Delta$ ); bottom temperature (filled triangles,  $\blacktriangle$ ); Surface salinity (open squares,  $\square$ ); bottom salinity (filled squares,  $\blacksquare$ ), and Secchi disk (filled bars). Errors bar represents SEM (n = 7).

595 fmole/cell in cysts of *A. tamarense* isolated in sediments from ship ballast tanks, they also showed that this amount was sixfold higher than that of the natural population of vegetative cells. In Argentina, Benavides et al. 1995 reported a toxicity of 325  $\mu\text{g}$  STX eq./cell for an *Alexandrium* strain, which corresponds approximately to 1,083 fmol/cell, to now, the highest toxic *Alexandrium* strain reported (Benavides et al. 1995, Bricej and Shumway 1998).

Carreto et al. (1996), describes the presence of large quantities of C1-C2 in *A. tamarense* cells isolated from the Mar del Plata coast and grown in batch cultures in f/2 medium. They also showed that the GTX 1-4 epimers were the predominant ones in the gonyaulax toxins; likewise, Anderson et al. (1996) describes a high proportion of C1-C2 in *A. tamarense* cells collected in the South China Sea and cultured under controlled conditions. Moreover, Oshima 1992 reported that *A. tamarense* in culture showed a larger proportion of C1-C2 than the natural vegetative cells. The absence of C1-C2 in the phytoplankton samples analyzed in this study can be explained as differences among populations of the same strain or to the possibility that *A. tamarense* synthesizes low quantities of C1-C2 under natural conditions, so that these PSP toxins could not be detected because of the low number of cells present in the natural plankton sample extracted. The differences detected in toxin profiles and the quantities of toxins produced per cell between natural and cultured cells reinforces the idea that important differences exist in the PSP production of *A. tamarense*, depending upon on whether it is subject to natural or laboratory conditions.

The PSP toxin profile corresponding to the filter-feeder bivalve *A. ater*, collected in November 1995 (Fig. 4), was practically identical to the profile shown in the natural phytoplankton samples (Fig. 3), where the only PSP toxin producing dinoflagellate unambiguously identified was *A. tamarense* (Lebour) Balech (Balech 1977). A decrease in the relative levels of dcSTX and the definite preponderance of GTX 1-4 epimers, were the changes observed (Fig. 4). The similarities between the PSP toxin profiles of the filter-feeder bivalve *A. ater* and the phytoplankton samples clearly show that the dinoflagellate *A. tamarense* is the source of the PSP toxin contamination in this gulf (Fig. 5).

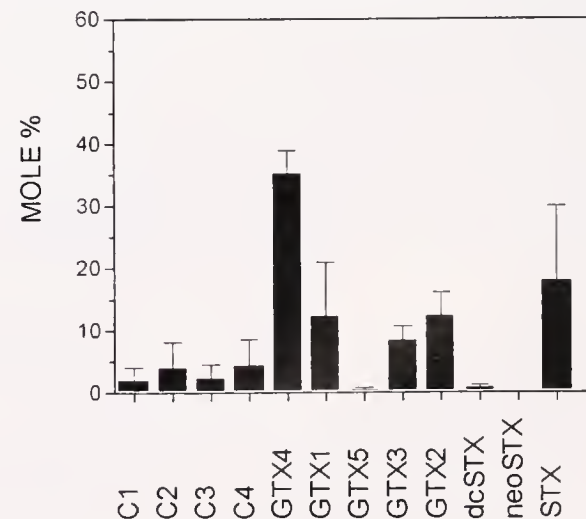
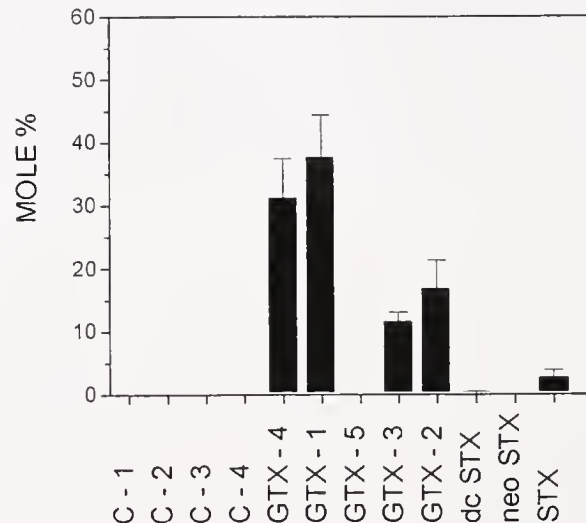
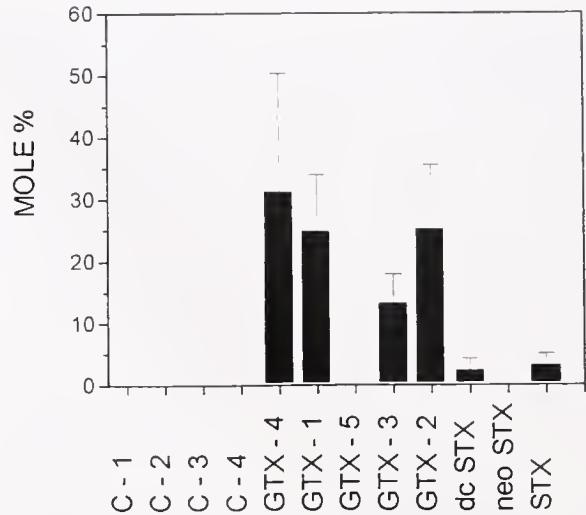


Figure 3. Average PSP toxins profile of *A. tamarense*, San José Gulf, Valdés Peninsula, November 1995 (mean  $\pm$  SEM, n = 7).

Figure 4. Average PSP toxin profiles of the filter-feeder bivalve *A. ater* in November 1995 at San José Gulf, Valdés Peninsula (mean  $\pm$  SEM, n = 9).

Figure 5. Average PSP toxins profile found in *A. ater* throughout the 6 months of monitoring programs (Nov. 1995–May 1996) (mean  $\pm$  SEM, n = 34 profiles).

By the analyses of all bivalve toxin profiles recorded during the monitoring program, it was possible to obtain an average toxin profile of the bivalve *A. ater* from both gulfs. Although the toxic profile preserves the main characteristics of the two previous ones (Figs. 3, 4), the profile obtained from all samples showed the presence of small amounts of N-sulfocarbamoyl-11-hydroxy-sulfate toxins (C1-C4), suggesting that these PSP toxins are probably produced by *A. tamarensis* cells and can be detected only when enough of these cells are concentrated, as normally occurs in filter-feeder bivalves. The GTXs continue being the most prevalent ones. Also, in some samples, trace quantities of GTX 5 were detected. STX was always present in significant quantities (Fig. 5). These data suggest that changes in the toxin profiles would be related to the depuration process by means of specialized and specific processes of transport as well as by metabolic transformations occurring inside the filter-feeders (Shimizu and Yoshioka 1981, Sullivan et al. 1983; Oshima 1995b).

The monitoring program coincided with a toxic event already underway, because toxicity of the samples diminished from an average of  $437.22 \pm 161 \mu\text{g}$  of STX eq/100 g (mean  $\pm$  SEM,  $n = 9$ ) in November 1995 to  $26.00 \pm 20 \mu\text{g}$  of STX eq/100 g (mean  $\pm$  SEM,  $n = 6$ ) in May of 1996 (Fig. 6). This figure shows that the natural depuration process takes place in the form of an exponential decay. This process lasts for about 6 months after the causative dinoflagellate *A. tamarensis* has disappeared from the phytoplankton community.

Natural depuration in the filter-feeder bivalve *A. ater* occurs in the form of an exponential decay of the first order, indicating that the data can be interpreted by following a one-compartment model. This kinetic of natural depuration can be described according to the equation  $C = C_0 * e^{-kt}$ , where  $C$  is the total toxicity of the contaminated filter-feeder at a given moment,  $C_0$  is the initial total toxicity (maximum toxicity reached),  $k$  is the apparent first-order detoxification rate, and  $t$  is time (Bricelj and Shumway 1998). According to this equation, the calculated maximum toxicity of *A. ater* should have been around  $561 \mu\text{g}$  of STX eq./100 g in October 1995. Also, according to this equation, the natural detoxification process occurs at a depuration rate of  $0.017 \text{ d}^{-1}$ , meaning that 50% detoxification occurred every 41 days in this area, under these conditions of bloom intensity, environment, oceanography, and physiology. According to this model, the contaminated *A. ater* in this harvesting area should reach safe toxicity limits (under  $80 \mu\text{g}$  STX eq./100 g) at least 4 months after November 1995, which is, in effect, what occurred. In March 1996, the average total toxicity measured by HPLC in *A. ater* was  $25 \pm 7 \mu\text{g}$  of STX eq./100 g.

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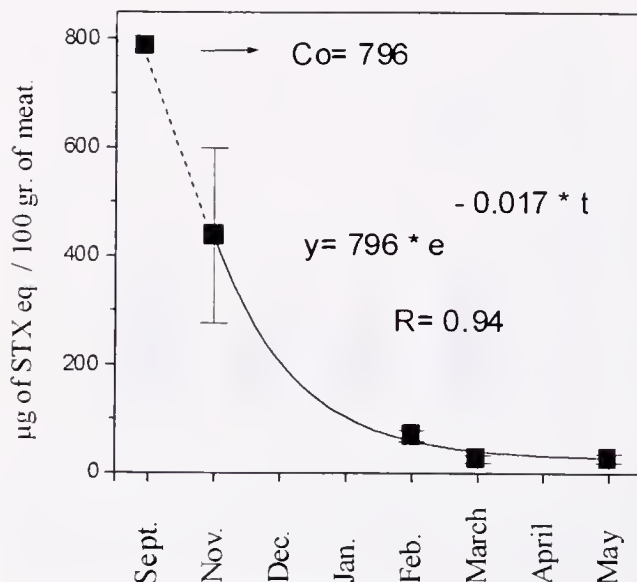


Figure 6. Representative detoxification of *A. ater* throughout the monitoring period in Valdés Peninsula.

(means  $\pm$  SEM,  $n = 7$ ). These findings support the idea that our monitoring began after the outbreak, when the bloom of *A. tamarensis* was in decline and suggests that the toxicity found in *A. ater* in the San José Gulf came from a bloom of *A. tamarensis* that had a higher cell density and must have occurred in September 1995.

The detoxification rate determined for *A. ater* from the San José Gulf, Valdés Peninsula, on the southern Atlantic coast of Argentina, correlated very well with that calculated for *A. ater* in an *A. catenella* bloom that occurred in a southern Chilean fjord (Compagnon et al., 1998). Making a similar analyses, once again, the data for detoxification kinetics were best interpreted by a one-compartment model, showing that 50% detoxification occurred every 33 days in *A. ater* in the Chilean fjord. According to the data shown in this paper and that reported by Compagnon et al. 1998, the filter-feeder bivalve *A. ater* should be considered a moderate detoxifier bivalve.

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## EFFECT OF A CONTINUOUS SUPPLY OF THE TOXIC DINOFLAGELLATE *ALEXANDRIUM MINUTUM* HALIM ON THE FEEDING BEHAVIOR OF THE PACIFIC OYSTER (*CRASSOSTREA GIGAS* THUNBERG)

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**ABSTRACT** The Pacific oyster *Crassostrea gigas* is sporadically subject to summer blooms of the toxic dinoflagellate *Alexandrium minutum* along French coasts. To account for differences in toxin accumulation between oysters and mussels, trials were performed using a recirculating flume. All results were analyzed using two linear models, respectively describing the effect of food change (step function) and comparing the effects of *A. minutum* diet with a control. Feeding oysters with *A. minutum* for 8 to 15 days induced significant inhibition (as compared with a nontoxic dinoflagellate, *Scrippsiella trochoidea*) of shell valve activity, clearance rate, filtration rate and biodeposition rate. Nevertheless, some "tank effect" may have been involved. Regardless of the exposure period of oysters to *A. minutum*, no "compensation" phenomenon was observed; that is, no return to higher shell valve activity comparable to that noted with nontoxic species. When the *A. minutum* diet was followed by one based on flagellates (*Isochrysis galbana*, *Tetraselmis suecica*) or diatoms (*Skeletonema costatum*, *Thalassiosira weissflogii*), most responded with an immediate and significant increase in shell valve activity. A decreasing trend was observed with flagellate- but not with diatom-based diets during a 15-day "detoxification" period. Clearance and filtration rates were unchanged or only slightly modified with flagellates or diatoms; whereas, the biodeposition rate was significantly increased with diatoms but not with flagellates. *Skeletonema costatum* seems to be the most efficient diet if steady increases in both valvular activity and biodeposition rates are considered as positive physiological changes in oyster feeding behavior.

**KEY WORDS:** *Crassostrea gigas*, *Alexandrium minutum*, *Scrippsiella trochoidea*, *Isochrysis*, *Tetraselmis*, *Skeletonema*, *Thalassiosira*, ecophysiology, paralytic shellfish poisoning

### INTRODUCTION

Various studies have shown that edible bivalves react differently to toxic dinoflagellates in their diet. Some species, such as *Mercenaria mercenaria*, retract their siphon and close their valves in the presence of toxic *Alexandrium* spp. (Shumway 1989); whereas, others, such as the mussel or scallop, show varying reactions, depending upon whether clearance rate alone is considered or a wider set of parameters characteristic of feeding physiology (Bricelj et al. 1990, Shumway and Cucci 1987). Moreover, the relative concentrations of toxic and nontoxic algae in the flow of filtered particles are important factors modifying the feeding behavior of shellfish (Bardouil et al. 1996).

Oysters are often cited in the literature as the species most sensitive to ingestion of toxic *Alexandrium* spp. (Shumway et al. 1990), and are considered capable of selective sorting of food (Shumway et al. 1985 a, Shumway et al. b, Ward et al. 1998). For this reason, as well as its economic importance on the French shellfish market, the Pacific oyster *Crassostrea gigas* was the experimental model chosen for this study. The toxic dinoflagellate strain selected was *A. minutum* Halim, a PST-producing species that proliferates occasionally along French coasts, causing toxic events in the Breton abers, Morlaix Bay, the Rance estuary (English Channel), and off Toulon (Erard-Le Denn 1991, Erard-Le Denn and Belin 1997). Previous studies (Bardouil et al. 1993, Bardouil et al. 1996) considered only the initial ecophysiological response; that is, the 6 hours following exposure to a diet either composed or not composed of toxic *Alexandrium* strains. In the present study, feeding behavior was evaluated for up to 30 days in comparison with a nontoxic control offering the same food value as well as microalgal strains currently used in aquaculture. The purpose of these trials was not only to determine whether feeding was inhibited in the presence of a slightly toxic strain but also to

ascertain whether physiological behavior returned to normal within a week or more. The choice of the Pacific oyster and the toxic dinoflagellate *A. minutum* as study materials was also based on field observations (Ledoux et al. 1989, Erard-Le Denn 1991), which showed a faster and higher contamination of mussels (400  $\mu\text{g}/\text{STXeq}/100 \text{ g}^{-1}$ ) than oysters (250  $\mu\text{g}/\text{STXeq}/100 \text{ g}^{-1}$ ). This suggested that the feeding behavior of oysters might account for differences in toxicity.

### MATERIALS AND METHODS

#### Biological Material

Oysters (*Crassostrea gigas* Thunberg) were obtained in March and October 1996–1997 from a breeding farm in Bourgneuf Bay (Atlantic coast) with no history of toxic algal blooms. The individuals were in sexual resting phase and had a mean weight (shell + tissues) of  $51.3 \pm 5.1 \text{ g}$ . Before transfer into the experimental unit, they were acclimated for 5 to 6 days in 35-L tanks supplied with natural seawater kept at  $16 \pm 0.5^\circ\text{C}$ . During the acclimation period, the oysters were fed (discontinuous inputs) daily with *Scrippsiella trochoidea*, a nontoxic dinoflagellate of the same size and nutrient value as *Alexandrium minutum* (Bardouil et al. 1993).

Algae were cultured in thermoregulated rooms ( $16 \pm 1^\circ\text{C}$ ) with a light intensity of  $50 \pm 4 \mu\text{E}/\text{m}^2/\text{s}^{-1}$  and a 12-h light/12-h dark photoperiod. The AM89BM strain of *A. minutum*, isolated in Morlaix Bay in 1989, had a toxicity evaluated at 0.5  $\text{pg}/\text{eq}$ . STX per cell according to the ion-pairing reverse-phase high-performance liquid chromatography (HPLC) method described by Oshima et al. 1989. Toxin quantification was performed with internal standards supplied by MACPS/IMB (Halifax, NS, Canada). The conversion factors for toxicity were those used by Oshima (1995).

The nontoxic strains *S. trochoidea* Paulsen, *Tetraselmis suecica*

Kylin, *Isochrysis galbana* Parke, *Thalassiosira weissflogii* Hustedt, and *Skeletonema costatum* Cleve were cultured, like *A. minutum*, in Provasoli's nutrient medium (1966). Silica ( $\text{Na}_2\text{SiO}_3$ ) was added only to the diatom culture to obtain a final concentration of  $0.1 \text{ mg}\cdot\text{L}^{-1}$ . The cultures were used at the end of exponential growth phase.

#### Experimental System

Because the flow-through system previously used to test individual oysters (Bardouil et al. 1993) was not suitable for long-term experimentation accommodating only five oysters, with flow-rates limited to  $4.5 \text{ L}\cdot\text{h}^{-1}$ , two 100-L flumes were used, each containing 45 oysters and supplied by a recirculated seawater circuit (flow rate:  $800 \text{ L}\cdot\text{h}^{-1}$ ) thermoregulated at  $15.9 \pm 0.4^\circ\text{C}$  (Fig. 1).

A 30-L "buffer" tank placed within the circuit just after the flume outlet contained the heat exchanger and the pumps ensuring circulation of the water and continuous measurement of chlorophyll *a* (10 AU-Turner Designs fluorometer) with 340–500 nm excitation and 665-nm emission filters. The supply of microalgae was provided at the flume inlet by means of an Ismatec peristaltic micropump with a flow adjusted according to the requirements established at the beginning of the experiment. This pump was started and stopped as a function of the threshold set for *in vivo* fluorescence. The continuous measurements provided by the fluorometer were integrated via an acquisition and control card (AD Clone interface) connected to a PC.

The 45 oysters, once their epibionts were removed, were fixed in rows of nine on five polyvinyl chloride (PVC) bars glued to the bottom of each flume and directed toward the water inlet. The PVC bars were 7.5 cm apart, and the oysters were positioned at a height of 2 cm. The biodeposits (feces and pseudofeces) were collected between the bars. Depending upon the filtration activity of the shellfish, the exposure period to the toxic algal strain ranged from 8 to 14 days, while the subsequent nontoxic diet was set arbitrarily at 15 days.

#### Ecophysiological Parameters

The levels of microalgae were kept constant in the experimental tanks to ensure a nutrient input [total particulate matter (TPM)

content] equivalent to  $0.5 \text{ mg}\cdot\text{L}^{-1}$ ; that is, the quantity of *A. minutum* required to induce a toxic concentration in the bivalves greater than the salubrity threshold ( $80 \text{ }\mu\text{g}\cdot\text{eq}\cdot\text{STX}$ ,  $100 \text{ g}^{-1}$  of meat) at the end of the exposure period. This corresponded to  $120 \text{ cells}\cdot\text{L}^{-1}$  for *A. minutum* (Lassus et al. 1994),  $62 \text{ cells}\cdot\text{mL}^{-1}$  for *S. trochoidea*,  $1,900 \text{ cells}\cdot\text{mL}^{-1}$  for *S. costatum*,  $340 \text{ cells}\cdot\text{mL}^{-1}$  for *T. weissflogii*,  $12,000 \text{ cells}\cdot\text{mL}^{-1}$  for *I. galbana* and  $2,000 \text{ cells}\cdot\text{mL}^{-1}$  for *T. suecica*.

These values were kept constant by means of a computer-assisted regulation system (Fig. 1) and controlled by continuous measurement of *in vivo* fluorescence, which was itself calibrated by cell counts performed (according to the species) with a microscope or a Coultronics particle counter. Cell enumerations were done every 20 min during the diurnal period. The TPM levels were measured in 8-L samples obtained every morning, filtered on Whatman GF/C, and weighed after drying at  $60^\circ\text{C}$  for 24 h.

Partial (30 L) or total (130 L) renewal of the water circuit was performed every day and every 4 days, respectively, to avoid high concentrations of ammonium. The ammonium level was checked every morning by colorimetric measurements, according to the method developed by Koroleff (1969).

The feeding behavior of oysters (Hawkins et al. 1996) was monitored using two variables: the clearance rate (CR), expressed in  $\text{L}\cdot\text{h}^{-1}\cdot\text{g}^{-1}$  dry weight and filtration rate (FR), expressed in  $\text{mg}\cdot\text{h}^{-1}\cdot\text{g}^{-1}$  dry weight, so that

$$\text{CR} = \frac{\text{PIM of biodeposits}}{\text{PIM of seawater}} \cdot \text{FR} = \text{CR} \times \text{TPM}_{\text{water}}$$

$$\text{TPM}_{\text{water}} = \text{PIM} + \text{POM}$$

where PIM = particulate inorganic matter in  $\text{mg}\cdot\text{h}^{-1}\cdot\text{g}^{-1}$  dry weight for biodeposits and in  $\text{mg}\cdot\text{L}^{-1}$  for seawater, and POM = particulate organic matter in  $\text{mg}\cdot\text{L}^{-1}$ . The clearance rate represents the volume of seawater per hour "depleted" of these particles, and the filtration rate the mass of particles filtered per hour (Hawkins et al. 1997).

Biodeposits were sampled in the morning (nocturnal production) and the evening (diurnal production) using a mechanical pipette. PIM values were determined after filters were burned at

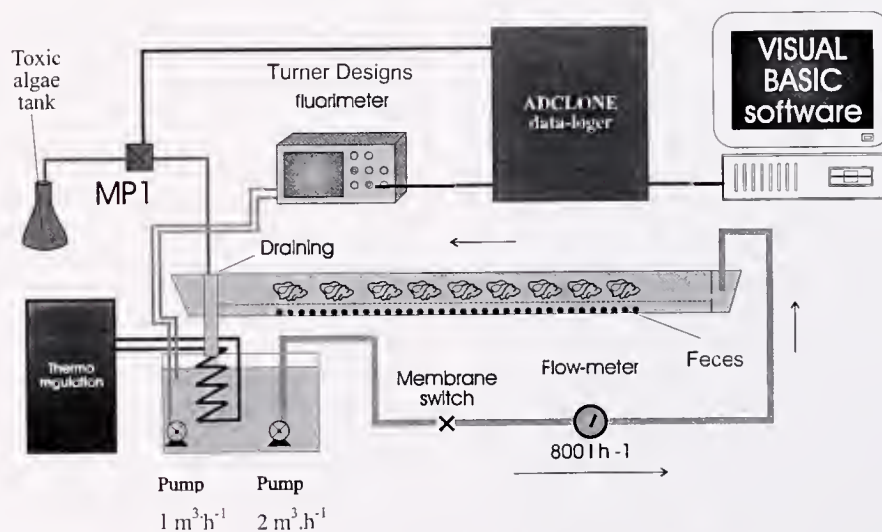


Figure 1. Simplified diagrammatic view of a complete recirculated water system showing the 100-L rearing tank (raceway), the 30-L thermoregulated "buffer" tank, and the algal culture micropump-delivered (MP) system.

450°C for 1 h. The shell valve activity of oysters was recorded every hour during the diurnal period by direct observation of the number of oysters displaying open or closed valves.

#### Data Analysis

Depending upon the availability of materials, the experiments were conducted in three steps, all at 16°C and with preliminary acclimation to temperature when seawater was affected by seasonal variations: (1) exposure to *A. minutum* and then, separately, to two flagellates (*I. galbana* and *T. suecica*); (2) exposure to *A. minutum* and then, according to the same protocol, to two diatoms (*S. costatum* and *T. weissflogii*); and (3) separate exposure to *A. minutum* and *S. trochoidea*.

The first two experiments were intended to compare the feeding behavior of the same oyster populations fed with a toxic strain and then with nontoxic species recognized in aquaculture for their food value. The switch in diets was expected to modify feeding behavior; that is, to speed up feeding activity significantly, provided that the supposed reduction in feeding activity attributable to the *A. minutum* diet was confirmed first. The purpose of the last experiment was to compare the feeding activity to two oyster subgroups, one fed *A. minutum* and the other *S. trochoidea*; that is, "control" nontoxic dinoflagellate. The objective was to elucidate the role of the "toxicity" factor in the observed feeding inhibitions.

The impact of a change of food in a raceway for given experimental conditions was assessed using a general linear model, which allowed testing for three effects: (1) a "food" effect; that is, a constant value modeling a possible shift in mean level for a given parameter when diet is switched; (2) a linear time effect by fitting a simple regression line in the data; and (3) a fixed "day" effect to model the between-day variation over a 3- or 4-day period after each water change in the raceway.

Results for two raceways under different or similar food conditions during the same period were compared by analysis of covariance (ANCOVA), a linear model that includes the food (or raceway) fixed effect and a linear time effect crossed with the previously described effect. This interaction term allows a possible trend in the data to be taken into account by estimating a slope for each raceway.

## RESULTS

The levels of ammonium in the water remained lower than 10  $\mu\text{atg}/\text{L}^{-1}$  for all exposures to *A. minutum*. However, diets composed of diatoms and especially the flagellate *I. galbana* led to occasional increases above 20  $\mu\text{atg}/\text{L}^{-1}$  in morning concentrations, requiring more frequent total renewal of flume water (every other day). However, these brief accumulations of nitrogenous wastes did not modify oyster behavior significantly.

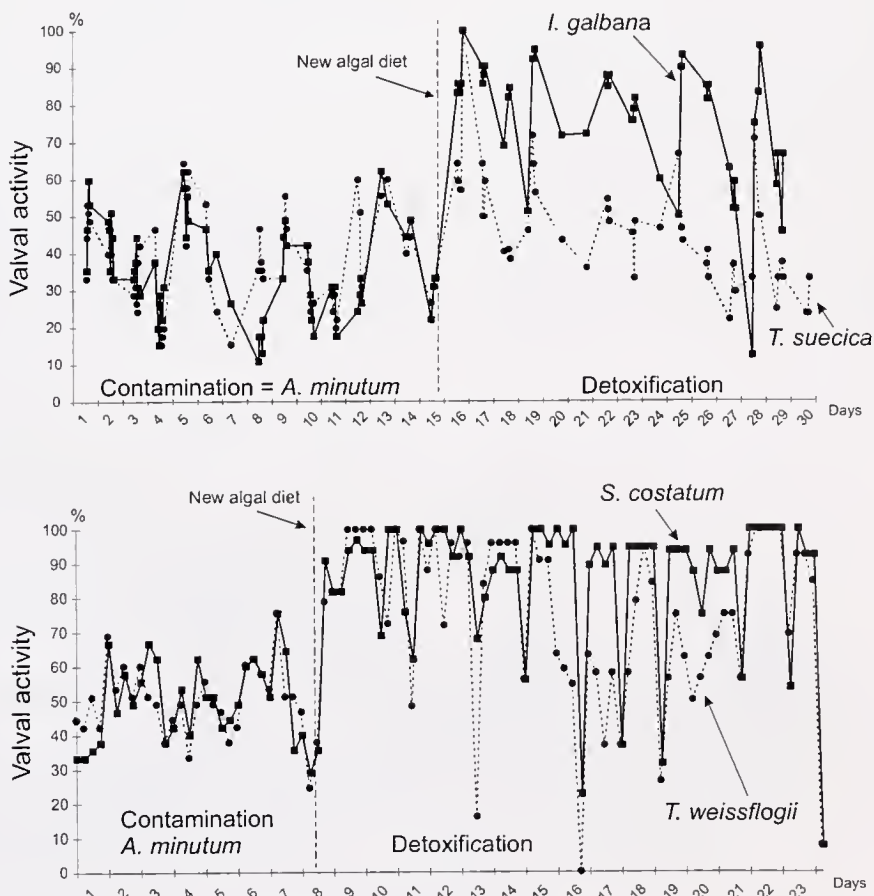


Figure 2. A, B, daily mean shell valve activities (expressed as percentages of oysters displaying open valves) when fed *A. minutum* and then *I. galbana* and *T. suecica* or *S. costatum* and *T. weissflogii*. Parameters used in the linear model are indicated.

TABLE 1.  
Results of a change of algae on parameters monitored in the raceway.

		Oct. 1996		March 1997	
		<i>Isochrysis</i>	<i>Tetraselmis</i>	<i>Skeletonema</i>	<i>Thalassiosira</i>
Shell valve activity	Food	50.0 (<0.0001)	14.7 (.0006)	48.2 (<.0001)	42.2 (<.0001)
	Slope	-0.96 (0.0003)	-0.52 (.02)		
Clearance rate	Food		0.21 (.011)	1.8 (<.0001)	1.6 (.0004)
	Slope		-0.01 (.01)	-0.1 (<.0001)	-0.1 (.002)
Filtration rate	Food		0.26 (.02)	0.9 (<.0001)	1.2 (<.0001)
	Slope		-0.02 (.005)	-0.1 (<.0001)	-0.1 (.001)
Biodeposition	Food		6.4 (.009)	11.1 (.001)	7.5 (.002)
	Slope			-1.3 (<.0001)	-0.9 (<.0001)

For each experimental condition and parameter, the "food" effect value (FEV) is given together with the slope value of the time series indicated below. Only significant values ( $p < .05$ ) are given, with their associated probabilities shown in parentheses.

#### Exposure to *A. minutum*, then to a Nontoxic Diet

Shell valve activities (Fig. 2), showed: (1) variations relating to the period of total renewal of seawater; (2) a mean daily activity involving only 40 to 50% of individuals for a diet composed of *A. minutum*; and (3) variable mean daily activities for the different nontoxic diets experimented after exposure to *A. minutum*: between 80 and 90% of individuals were active with diatoms, around 70% with *I. galbana* and only 50% with *T. suecica*.

This kind of graphical representation is quite valid for the other ecophysiological parameters [CR, FR, and biodeposition rate (BR)]. For this reason, only the food effect and feeding trend during the detoxification phase were considered. Seawater renewal, although integrated into the model to avoid any interference with variations obviously linked to a switch in algal diet, was not a meaningful parameter in terms of physiological variations.

The results (Table 1) clearly show a significant "food effect" when the *A. minutum* diet was replaced by flagellates or any diatom species. The early increase in shell valve activity was less pronounced with *T. suecica* but detectable (food effect value: 14.7). A significant decreasing trend in shell valve activity was observed

( $p = .0003$ ) with *Isochrysis*, and to a lesser extent with *T. suecica* ( $p = .02$ ), but not with any of the diatom species used.

The CR for oysters was low (0.10 to 0.20 L/h<sup>-1</sup>) when they were fed first with *A. minutum* and then with two different flagellate diets. When diets composed of *A. minutum* were replaced with diatoms, the CR differed slightly. Conversely, there was no significant difference between *A. minutum* and *Isochrysis* diets and a very slight effect of *T. suecica* (FEV : 0.21 L/h<sup>-1</sup>). The results were quite similar for FR but not for BR, for which food effect was more or less marked with *T. suecica*, *S. costatum*, or *T. weissflogii*, but not significant with *Isochrysis*. A decreasing trend in BR was observed for each of the diatom diets.

#### Comparison Between Two Populations Fed Toxic or Nontoxic Diets

The two 45 oyster populations experimented in two different raceways were expected to be identical: animals of similar length and weight were randomly distributed in each tank, kept at the same temperature, light intensity, and photoperiod and fed continuously with an identical seston supply (same TPM value). Nevertheless, as in the *S. trochoidea* versus *A. minutum* experiment

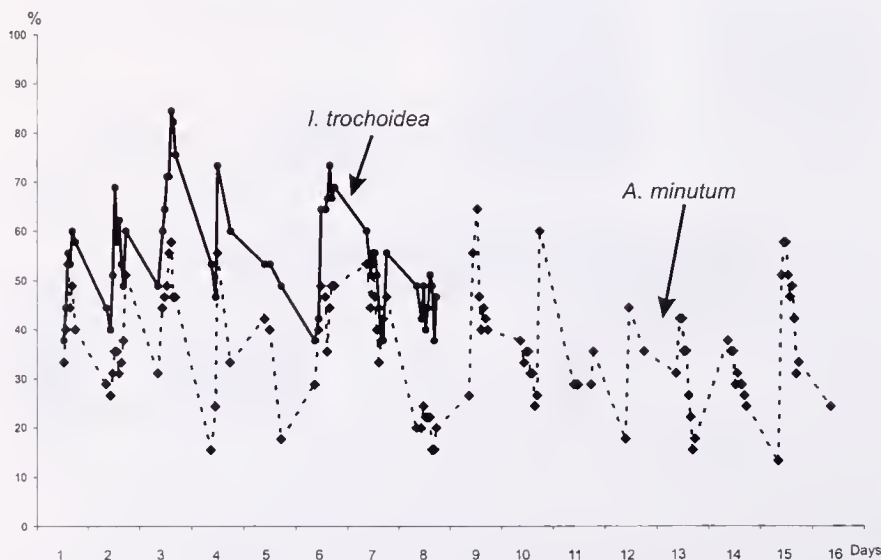


Figure 3. Oyster shell valve activity with *S. trochoidea* and *A. minutum* as food supply. Parameters used in the linear model are indicated.



(Fig. 3), two oyster populations fed only with *A. minutum* were compared. The results (Table 2) show a very significant increase (as compared to *A. minutum*) in shell valve activity or CR and BR when oysters were fed *S. trochoidea*.

However, in terms of a possible "raceway" effect on oysters fed only *A. minutum*, it is noteworthy that shell valve activity and CR were not affected; whereas, FR and BR displayed significant differences, although less pronounced than with the *Scrippsiella/Alexandrium* experiment.

#### DISCUSSION

The first results obtained for short-term experimental contamination (6 h in an open circuit) (Bardouil et al. 1993) showed higher clearance rates in oysters fed with *S. trochoidea* than and *A. minutum*-based diet. Mean time-averaged biodeposition rates and filtration rates were also much higher for *S. trochoidea* than for *A. minutum*. Moreover, for parameters not evaluated here, the authors found that the toxic nature of *A. minutum* seemed to account for a lower absorption of this species than that of *S. trochoidea*. Finally, the high absorption rate of a more toxic strain, such as *A. tamarense* seemed to be attributable to the greater ability of oysters to digest this species than *A. minutum*. Subsequent studies (Lassus et al. 1996) also seemed to demonstrate the resumption of filtering activity (after nearly total inhibition) in oysters exposed to *A. fundyense* for two 6-h periods before and after overnight fasting.

The results obtained here under quite different conditions (higher flow rates, seston input kept constant, long-term exposure to algal foods, larger size of oyster population) showed a significant reduction in shell valve activity as well as CR, FR, and BR when oysters were fed with toxic *A. minutum*, rather than the nontoxic control, *S. trochoidea*. Nevertheless, these results must be considered with caution, especially for FR and BR, because some "raceway" effects may interfere.

When oysters were fed for 8 to 15 days with toxic alga, the reduction in shell valve activity seemed to be stable over time. The transition to a nontoxic diet led to a very marked increase in shell valve activity with both diatoms and flagellates. In fact, an observation that needs to be corroborated is the apparently different feeding behavior (if shell valve activity, as defined in this study, is regarded as a good indicator of feeding activity) between oysters fed diatoms or flagellates as detoxication foods. Whereas shell valve activity remained high throughout detoxication time, regardless of the diatom species used, a decreasing trend was observed with flagellates, particularly with *Isochrysis*, thus foreshadowing a return to the previous lower activity.

The impact of a change of food on other physiological parameters can vary according to the type of algal food and the involved parameter. Generally speaking, FEV are much less pronounced than for shell valve activity. The biodeposition rate, as observed in a previous study (Lassus et al. 1996), seems to be the second most sensible parameter, after an *Isochrysis* diet. CR and FR were only slightly affected by the change of diet, even when FEV shell valve activity was high.

From a practical point of view, the linear model used here not only allows the possible effects of water renewal to be taken into account, but also provides an opportunity to consider some interesting performances for specific diets, especially in terms of en-

TABLE 2.  
Summary of the results, in October 1997, of the comparison between two raceways with the same alga as food (*Alexandrium minutum*), or with two different algae.

Species Raceway	Oct. 1996	March 1997	Oct. 1997
	<i>Alexandrium minutum</i> Raceway 1 vs. 2	<i>Alexandrium minutum</i> Raceway 1 vs. 2	<i>A. minutum</i> (Raceway 1) vs. <i>Scrippsiella trochollidea</i> (Raceway 2)
Shell valve activity			<0 (<.0001)
Clearance rate			<0 (.0005)
Filtration rate	<0 (.004)	>0 (.04)	<0 (.02)
Biodeposition	<0 (.002)	>0 (.01)	<0 (.002)

The sign of the difference between the two raceways is indicated, with the associated probability in parentheses, when significant ( $p < .05$ ).

hanced and steady feeding activity; for example, a diet with high shell valve activities, high biodeposition rates and high FEV. Another interesting feature for the diatom diet is that shell valve activity remained roughly constant during the detoxication period. The negative slope for flagellate diets may be attributed to (1) a temporary effect on either valval activity or BR (*Tetraselmis*), only detectable during the first days of food change; or (2) a more interesting efficiency of diatoms in oyster detoxication. In the case of populations exposed successively to a toxic and then a nontoxic diet, it is likely that the presence of toxins in tissues affects bivalve physiology after the toxic diet is stopped and during the period of nontoxic diet exposure. Any residual traces of toxin in oyster tissues might affect feeding behavior negatively.

*A. minutum* has been used in only one experimental study in the literature (Bricelj and Cembella 1995). Moreover, laboratory of *in situ* studies have generally focused on toxin distribution per organ and the kinetics of accumulation and detoxification in bivalves known to have very long retention times for paralytic toxins, as in the case of the surf clams *Spisula solidissima* (Bricelj and Cembella 1995) and *Paphies subtriangulata* (Mackenzie et al. 1996).

Because of a scarcity of information and considering the current worldwide spread of *A. minutum* (northern and southern European coasts, Australia, New Zealand) it is essential to have a better knowledge of the different effects of this species on commercial bivalve physiology, particularly for oysters.

#### ACKNOWLEDGMENTS

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## SURVIVAL OF TOXIC DINOFLAGELLATES AFTER GUT PASSAGE IN THE PACIFIC OYSTER *CRASSOSTREA GIGAS* THUNBURG

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**ABSTRACT** The oyster *Crassostrea gigas* drastically reduced its clearance rate immediately after exposure to the toxic dinoflagellates *Alexandrium minutum* Halim (AM89BM), *Alexandrium tamarense* MOG835, and two toxic naked strains of *Gymnodinium mikimotoi*. Two nontoxic thecate dinoflagellates: *Alexandrium tamarense* and *Scrippsiella trochoidea* (M93/12) were ingested but with clearance rates below those measured for the diatom *Thalassiosira weissflogii* (control). All of the four thecate dinoflagellates tested survived gut passage. They were usually found intact, immobile, and viable in the feces. These cells generally constituted up to 50% of the fecal material; they recovered their motility 24 h after incubation in favorable conditions. Egestion of such viable cells may influence the recurrence and duration of toxic blooms through reinoculation of the water column. The survival of these algae to passage through the gut of *C. gigas* could also enhance the risk of their transfer to virgin areas through displacement of these bivalves.

**KEY WORDS:** *Crassostrea gigas*, dinoflagellates, thecae, feces, clearance rate, cell viability, gut passage

### INTRODUCTION

It has been shown that the responses of mollusks to dinoflagellates are species-specific (Twarog and Yamaguchi 1974, Cucci et al. 1985, Gainey and Shumway 1988, Shumway 1990, Bricelj et al. 1992). The observed differences in the reaction of bivalves to dinoflagellates have not been well clarified. Differences may be related to toxic profiles of the algae, algal concentrations, cell palatability, and size, or to differences in digestive function (Wikfors and Smolowitz 1995). On the other hand, the physiology of oysters is known to be adversely affected by feeding on toxic dinoflagellates, especially species belonging to the genus *Alexandrium*, which produce paralytic shellfish poisoning (PSP) toxins (Shumway et al. 1985, Shumway and Cucci 1987, Lassus et al. 1996, Bricelj and Shumway 1998). Reduction or complete suppression of clearance rate and/or biodeposition of *Crassostrea gigas* exposed to toxic strains of *Alexandrium* sp. has been reported (Shumway 1990, Bardouil et al. 1993, Bardouil et al. 1996, Lassus et al. 1996). *Gymnodinium* spp. are often associated with large marine faunal kills (Gentien and Arzul 1990, Heinig and Campbell 1992). Also, *Gyrodinium aureolum* has been shown to reduce *C. gigas* larval survival (Helm et al. 1974, Smolowitz and Shumway 1997). In this study, we determined the impact of a toxic diet on feeding habits of *C. gigas*. To this effect, we report here clearance rates of *C. gigas* when fed each of six dinoflagellates of differing toxicity.

Bricelj et al. (1993) hypothesized that the sustained bivalve filtration on toxic strains would enhance their transfer from the upper layer to the bottom, a process that may influence the duration and fate of a bloom in coastal waters. Until now, few studies have addressed the problem of the survival of ingested dinoflagellates after their gut passage in wild or cultured mollusks, important filter feeders. We reported here the fate and survival capacity for thecate and athecate dinoflagellates after gut transit in *C. gigas*

involving extracellular digestion, acidic (pH < 5) and mechanical (crystalline style) actions. Cells surviving gut transit and present in the feces could play an important role in bloom maintenance and transfer in virgin environment (Carriker 1992, Scarrat et al. 1993).

### METHODS

#### *Oyster Preparation and Algal Cultures*

Adult Pacific oysters (*Crassostrea gigas*) ranging in size from 8 to 10 ± 2 (SD) cm were obtained from Normandy (France) from March to early June 1997. The oysters were maintained in filtered (10 µm) running seawater (salinity: 35 psu) at a constant temperature of 18°C for less than a fortnight. Before experiments, animals were placed in 1-µm filtered seawater for 24 h to clear the gut contents. Experiments were conducted with the following unialgal diets: the diatom *Thalassiosira weissflogii*, four thecate dinoflagellates including toxic strains of *Alexandrium minutum* (Halim) Balech (AM89BM), and *Alexandrium tamarense* (MOG 835, Japan), and the nontoxic strains *Alexandrium tamarense* (Tamar Estuary, UK) and *Scrippsiella trochoidea*. We also tested two naked ichthyotoxic dinoflagellate strains, *Gymnodinium mikimotoi* Tinduff 87 and *Gymnodinium mikimotoi* Tinduff 95, isolated in Brest Bay (Tinduff). All phytoplankton cells were cultured without agitation in f/2 medium (Guillard and Ryther 1962) at 17°C on a 12-h light:12-h dark cycle at 90 µE m<sup>-2</sup> s<sup>-1</sup>. Nonaxenic algal cultures were collected in the late exponential to early stationary phase (10 to 15 days after inoculation). Ledoux et al. (1990) and Erard-Le Denn (1991) give mean diameter and toxic profiles for *Alexandrium minutum*, which is often responsible for PSP shellfish contamination along the Brittany coast (France). Toxicity of *Gymnodinium* strains related to free or esterified polyunsaturated fatty acids composition are reported in Bodennec et al. (1995) and Arzul et al. (1995). Characteristics of the species tested and final cell concentrations in the beakers are reported in Table 1.

#### *Measurement of Clearance Rate*

The effect of diet was evaluated by quantifying clearance rate. This parameter was measured indirectly according to Coughlan (1969) by determining the decrease of microalgal concentrations attributable to oyster filtration over time. For simple and rapid

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TABLE 1.  
Characteristics and final cell concentration in the beakers of the tested algae.

Algal Strain	Mean Diameter ( $\mu\text{m}$ )	Toxicity	Final Cell Concentration (Cells/mL <sup>-1</sup> )
<i>Thalassiosira weissflogii</i>	16	Nontoxic	2.892 $\pm$ 421
<i>Alexandrium tamarens</i> (UK)	36	Nontoxic	830 $\pm$ 127
<i>Scrippsiella trochoidea</i>	26	Nontoxic	675 $\pm$ 112
<i>Alexandrium tamarens</i> (MOG 835)	31	Gonyautoxins 1,2,3,4; C toxins	850 $\pm$ 130
<i>Alexandrium minutum</i> Halim	29	Gonyautoxins 2,3; saxitoxins	1.200 $\pm$ 148
<i>Gymnodinium mikimotoi</i> Tunduff 87	26-37	Lipidic haemolysins 18:5n3	586 $\pm$ 79
<i>Gymnodinium mikimotoi</i> Tunduff 95	"	"	1.100 $\pm$ 96

measurements, cell concentrations were determined with a fluorometer (Turner Designs Co.), based on the measurement of *in vivo* chlorophyll fluorescence (Brand et al. 1980). Oyster clearance rates were calculated using the following equation (Coughlan 1969)  $CR = \ln(F_0/F_t) \times M/t$ ; where CR is clearance rate,  $F_0$  is the initial fluorescence value of ambient seawater,  $F_t$  is fluorescence after time  $t$ ,  $M$  is the total volume of ambient seawater, and  $t$  is the time after the start of the experiments. Strong linear relationships were established between cell concentration and fluorescence of the studied phytoplankton species. Correlation coefficient  $R$  ranged from 0.78 to 0.99 with  $p < .05$ , which allowed a routine measurement of cell concentrations using a fluorometer apparatus.

In each experiment, a series of three oysters were exposed to the tested algae. Each bivalve was placed individually in aerated transparent beakers containing 200 mL of microalgal culture diluted with 2 Liters of 1- $\mu\text{m}$  filtered seawater. Control beakers were left without animals to correct for algal cell division during experiments. Mean concentrations (Table 1) of the algae in the beakers fed to the oysters were close to those observed at the moment

of a red tide to simulate bloom conditions (Erard-Le Denn 1991). No aeration was used during feeding experiments with dinoflagellates. When oysters were fed diatoms, the water was gently stirred periodically to avoid sedimentation of the algae. Feces and pseudofeces were removed at regular intervals with Pasteur pipettes. All experiments were carried out in triplicate. Data are expressed as means  $\pm$  SD and statistical analysis were performed with Student's  $t$ -test. Another set of experiments gave an equivalent ratio of 10/90 in volume of tested alga/*Thalassiosira* for measurement of clearance rate. Clearance rate of oysters exposed to *T. weissflogii* was defined as the control. This diatom is known to be a good diet for *C. Gigas* (Lassus et al. 1996).

#### Viability Test and *Alexandrium minutum* Growth Experiment

During this study, we used principally fluorescein diacetate (FDA), a vital stain based on the measurement of intracellular esterase, which colors viable cells green under blue light excitation. Accumulation of fluorescein in cells happens when nonspecific esterases are present in the cell, when membranes are intact

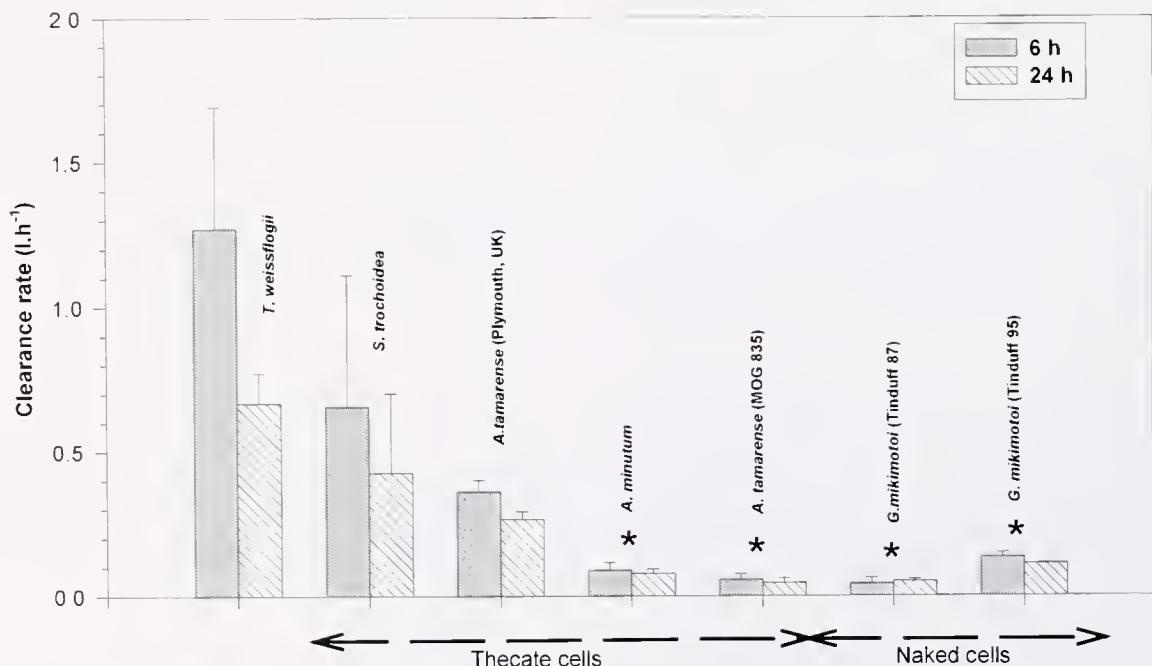


Figure 1. Mean 6- and 24-h clearance rates and standard errors of *Crassostrea gigas* when fed different unialgal diets: the diatom *Thalassiosira weissflogii* (control), four dinoflagellate thecate species including nontoxic strains of *Alexandrium tamarens* (Tamar Estuary, UK) and *Scrippsiella trochoidea* and toxic (\*) strains of *Alexandrium minutum* (Halim) Balech (AM89BM) and *Alexandrium tamarens* (MOG 835 Japan), and two naked toxic (\*) strains of *Gymnodinium mikimotoi* (Tinduff 87 and Tinduff 95).

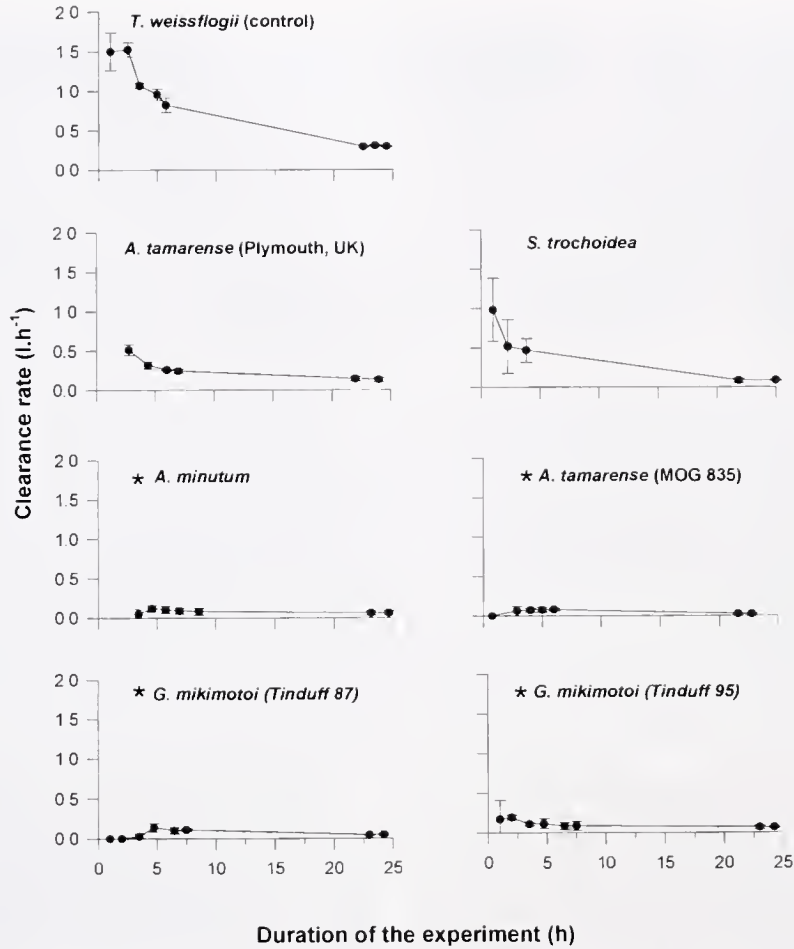


Figure 2. Temporal variation of clearance rate of *Crassostrea gigas* incubated 24 h with algal cultures (same algae as in Fig. 1). Results are means  $\pm$  SD of triplicate samples. \*Denotes toxic strain.

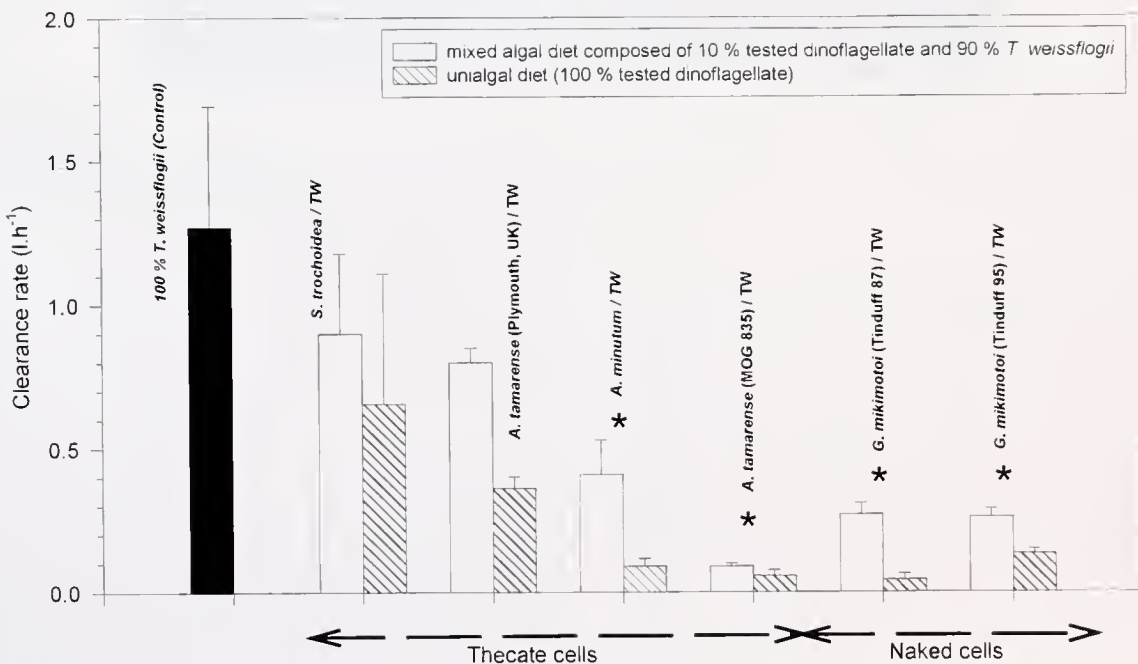


Figure 3. Mean (and standard errors) of 6-h clearance rates of *Crassostrea gigas* exposed to unialgal diet of *Thalassiosira weissflogii* (control) or one of six tested dinoflagellates and to mixed algal diet (tested dinoflagellate/*Thalassiosira*) given in (10/90) ratio. \*Denotes toxic strain.

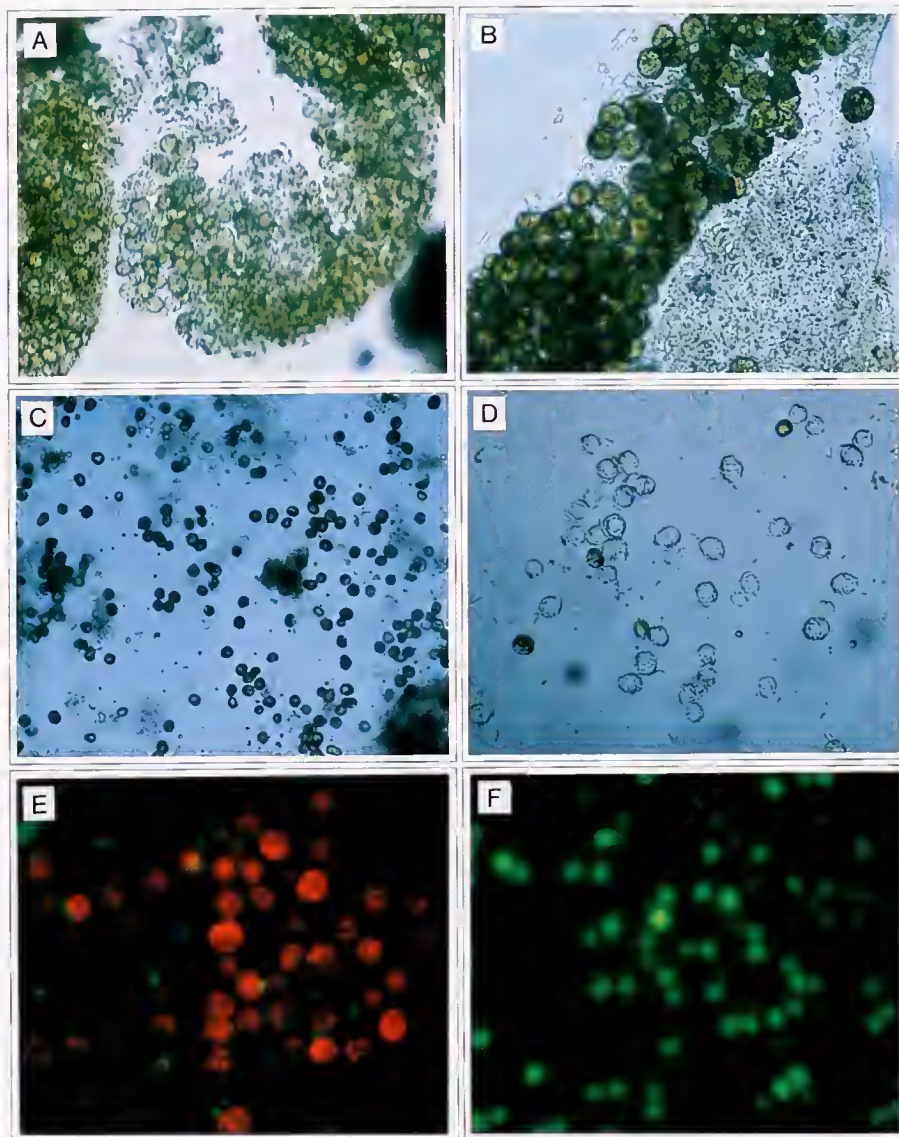


Figure 4. Light microscope photographs of microalgae found in (A, B: nondisrupted, C: disrupted) feces produced by *Crassostrea gigas* fed the dinoflagellates (A) *Alexandrium minutum*, (B) nontoxic strain of *Alexandrium tamarense*, and (C) *Scrippsiella trochoidea*. (D) corresponds to empty thecae of *A. minutum* observed at the end of 24-h incubation period, time necessary to take off the old thecae and to recover the cell motility. Photos in fluorescent light of egested cells dyed with the vital staining Fluorescein diacetate of (E) *A. tamarense* cells (nontoxic strain) colored immediately after their egestion and (F) *A. minutum* stained 24 h after the incubation in filtered seawater.

and when they allow transport. The two former criteria are met when the cell is alive. However, low metabolic activity or a blockage affecting membrane exchange in the case of pellicular cyst formation could be responsible for the failure to stain these cells. Therefore, when egested algae were nonmotile and colored negatively with FDA, several replicate batches of these cells were selected under the dissecting microscope and micropipetted into separate wells of a multiwell plate containing 1- $\mu\text{m}$  filtered natural seawater. We then monitored these cells at regular intervals for 24 h, and the proportion of cells that regained their motility and were colored positively (green cells) with FDA was noted. This proportion of viable cells (number of green cells/number of incubated cells) was estimated by counting at less 40 cells in three batches of egested algae incubated in the multiwell plate. The cells were observed in bright field Nomarsky and counted in fluorescent light using a Zeiss microscope, and photographs were taken routinely. Another set of experiments consisted of monitoring the fate the

egested toxic dinoflagellate *A. minutum* and its capacity to grow. Fresh feces produced by *C. gigas* fed *A. minutum* were pipetted and rinsed several times with 1- $\mu\text{m}$  filtered seawater and 40 mL tubes of *i/2* medium were inoculated with disrupted or intact feces and then monitored.

## RESULTS

### Clearance Rate

When oysters were fed dinoflagellates, clearance rates averaged for 6 or 24 h ranged between 0.05 and 0.65  $\text{L}/\text{h}^{-1}$  regardless of the relative toxicity of each species or strain. It was significantly ( $p < .05$ ) lower than that recorded with *T. weissflogii* (0.67–1.27  $\text{L}/\text{h}^{-1}$ ) (Figs. 1, 2). Nevertheless, the lowest rates (0.02–0.13  $\text{L}/\text{h}^{-1}$ ) were obtained when oysters were fed toxic cells of *A. minutum*, *A. tamarense* (MOG 835), and the two toxic strains of *G. mikimotoi* (Figs. 1, 2). Even the inclusion of a relatively low pro-

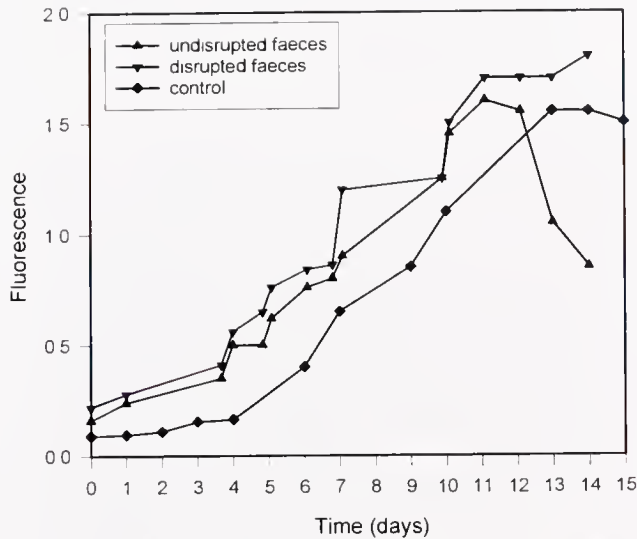


Figure 5. Growth curves of egested *Alexandrium minutum* contained in disrupted and nondisrupted faeces and inoculated in *f/2* medium and of the control culture composed by noningested cells of *A. minutum*.

portion (10%) of these toxic strains in a diet composed of *T. weissflogii* a significant ( $p < .05$ ) decrease of *C. gigas* clearance rate in comparison to the control (100% of the diatom) was observed for all tested dinoflagellates (Fig. 3). Also the inclusion of 10% of a nontoxic dinoflagellate strain induced clearance rates lower ( $p < .05$ ) than that of control. Although fecal pellet production was not quantified, we observed that *C. gigas* produced less material in the case of all dinoflagellate diets, especially with toxic strains of *A. minutum* and *A. tamarensis* (MOG 835). Oysters arrested their defecation 5 to 6 h after exposure to *A. tamarensis* (MOG 835). We noted a negligible production of pseudofeces with all the dinoflagellate diets. In contrast, when *C. gigas* was given *T. weissflogii*, a notable production of pseudofeces was observed.

#### Effect of Gut Passage

Oysters fed *T. weissflogii* produced feces containing intact viable cells (colored positively with FDA) at the beginning of the experiment. Five to six hours after the start of the feeding experiment, the percentage of debris increased greatly to dominate. Oysters that ingested any of the two strains of the naked dinoflagellate *G. mikimotoi* usually produced feces composed of ruptured cells. By contrast when *C. gigas* were fed the thecate algae *A. minutum*, *A. tamarensis*, (toxic and nontoxic strains) and *S. trochoidea* the produced feces were composed mainly of intact immobile cells (50–90%). None of the recently egested intact cells were colored positively with FDA (Fig. 4E). This is not proof that they were dead. When these egested cells were incubated in favorable conditions and monitored for 24 h, we observed that they progressively became motile; 24 h later, nearly all of the cells ( $\geq 90\%$ ) swam normally and responded positively to vital staining (Fig. 4F). We also observed numerous empty thecae (Fig. 4D) at the bottom of the well presumably belonging to the cells that recovered their vitality and had shed their old thecae. When the egested *A. minutum* cells contained in disrupted or intact feces were inoculated in *f/2* medium, they showed normal growth and reached concentrations not significantly ( $p < .05$ ) different from those of noningested control cells (Fig. 5).

## DISCUSSION

Previous workers have shown a variety of responses of bivalves to the presence of dinoflagellates, ranging from total avoidance to normal filtration (Gainey and Shumway 1988, Shumway 1990, Bricelj et al. 1993). In our experiments, clearance rates were generally close to those reported by Bardouil et al. (1993) Bardouil et al. (1996), and Lassus et al. (1996). We observed a significant decrease in *C. gigas* filtration in all of the tested dinoflagellates. Toxic *A. minutum* and *A. tamarensis* were the most avoided species, and the oysters arrested filtration activity by complete shell valve closure within a short time after exposure. Surprisingly, the lack of toxicity of both *A. tamarensis* (Plymouth strain) and *S. trochoidea* did not enhance their clearance rate by the oysters in comparison to control. Bardouil et al. (1993) showed that the inclusion of even 10% of the toxic strain of *A. tamarensis* in the algal diet of *C. gigas* induced a significant decrease in clearance rate equivalent to that induced by toxic *A. tamarensis* alone. These results are confirmed here and extended to other toxic dinoflagellate species: *A. minutum* and two strains of *G. mikimotoi*. Our work showed a high sensitivity of *C. gigas* to PSP-producing dinoflagellates but also to other types of toxins produced by species of the genus *Gymnodinium*.

Bardouil et al. (1993) observed numerous intact cells in feces when oysters were fed different dinoflagellates. Shumway and Cucci (1987) reported that *Protogonyaulax tamarensis* was filtered and rejected in pseudofeces. Bricelj et al. (1993) has demonstrated that cells of toxic *Alexandrium fundyense* ingested by the blue mussel *Mytilus edulis* survived gut passage. In this study, we observed that, a few hours after the start of the ingestion, the diatom *T. weissflogii* and the two naked strains of *G. mikimotoi* were egested principally as ruptured cells. In contrast, all of the four thecate dinoflagellates (*A. minutum*, *S. trochoidea*, and two strains of *A. tamarensis*) were egested intact. Just after their egestion, the cells were immobile and were not colored positively with FDA. This did not imply that these cells were dead. Less than 24 h after their egestion, up to 90% of the cells recovered their motility. Hence, the egested *A. minutum* when cultured in favorable conditions divides normally. Further experiments must study the fate and growth of the other egested viable dinoflagellates. The negative coloration with FDA of freshly egested cells might imply an arrest of their membrane exchange and/or a drastic reduction of their metabolic activity attributable to gut passage. However, these egested cells did not differ morphologically from vegetative ones; they may correspond to temporary cysts becoming active in presence of favorable conditions. The resistance of the ingested dinoflagellates to gut passage may be responsible for the maintenance of blooms (Scarratt et al. 1993). Different oyster species exhibit very low levels of toxicity when exposed to toxic algal blooms (Bricelj et al. 1991). Lassus et al. (1989) reported that the level of paralytic phycotoxin accumulation in *C. gigas* is low as compared to that of other shellfish when fed dense *A. tamarensis* cultures. The present work showed that *C. gigas* fed the tested toxic thecate dinoflagellates produced feces full of intact cells, process that may contribute to the initial detoxification, as was suggested by Bricelj and Cembella (1991) for other mollusks.

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## PHENOLOXIDASE ACTIVITY IN THE HEMOLYMPH OF BIVALVE MOLLUSKS

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**ABSTRACT** The oxidative enzyme phenoloxidase has been proposed as a component of internal defense in a variety of organisms. We measured the phenoloxidase activity of hemolymph plasma and hemocytes from a selection of marine and freshwater bivalve mollusks. Phenoloxidase activity is present in the hemolymph and hemocytes of *Placopecten magellanicus*, *Argopecten irradians*, *Geukensia demissa*, *Mercenaria mercenaria*, *Lampsilis teres*, and *Lampsilis claibornensis*. The enzyme activity in the freshwater species was 5 to 10 times higher than that in the marine animals. Phenoloxidase activity was not consistently stimulated by pre-incubation of hemolymph plasma or extracts of hemocytes with trypsin, bacterial cell wall lipopolysaccharides and fungal cell wall components (zymosan). The role of this enzyme, if any, in internal defense remains to be elucidated.

**KEY WORDS:** bivalves, phenoloxidase, internal defense, hemolymph

### INTRODUCTION

Mollusks possess a variety of mechanisms that are involved in internal defense against pathogenic organisms. The internal defenses consist of both cellular and humoral components (Simina and van der Knaap 1986, Bachere et al. 1995). The hemolymph of all mollusks contains a variety of hemocytes that protect the animal from a wide spectrum of harmful organisms (Fisher 1986, Lopez et al. 1997). In addition, the hemolymph contains a variety of molecules that have been implicated in internal defense. These molecules include cytotoxic factors, lectins, hemagglutinins, and oxidizing enzymes (Renwrautz 1986, Leippe and Renwrautz 1988, Lopez et al. 1997). Phenoloxidase is an enzyme ubiquitous in the hemolymph of many animals; this enzyme has been proposed as a component of the internal defenses of invertebrates (Smith and Soderhall 1991). The activation of phenoloxidase by foreign proteins has been documented for a variety of arthropod species (Soderhall 1982, Soderhall et al. 1994). In decapod crustaceans, the phenoloxidase is secreted by hemocytes into the hemolymph in an inactive form; activation occurs in the presence of pathogens or pathogenic cellular components and involves the action of a serine protease on the inactive prophenoloxidase (Aspan and Soderhall 1991).

In mollusks, the enzyme occurs in mantle tissues and is involved in shell growth and repair (Waite and Wilber 1976, Jones and Salueddin 1978). Phenoloxidase activity has also been reported in both hemocytes and hemolymph from several bivalves (Coles and Pipe 1994, Renwrautz et al. 1996, Asokan et al. 1997, Carballal et al. 1977, Lopez et al. 1977). The presence of phenoloxidase in the hemolymph of mollusks and stimulation of the activity of the enzyme by bacterial and fungal cell wall components have led to interest in the possible role of the protein in internal defense. We have examined the activity of phenoloxidase in the hemolymph of several species of mollusks. Phenoloxidase occurs in both plasma and hemocytes in all of these species. The activity varies widely among the species and among individuals within a species. Neither bacterial lipopolysaccharides nor fungal cell wall extracts consistently stimulated the activity of phenoloxidase.

### MATERIALS AND METHODS

#### Animals

Specimens of the Atlantic ribbed mussel, *Geukensia demissa* were collected from salt marshes either in St. John's County,

Florida or Jasper County, Mississippi. Scallops (*Placopecten magellanicus* and *Argopecten irradians*) and clams (*Mercenaria mercenaria*) were obtained from the Marine Biological Laboratory, Woods Hole, Massachusetts. The freshwater mussels *Lampsilis claibornensis* and *Lampsilis teres* were collected from the Ochlockonee River near Tallahassee, Florida, and from drainage ditches on the USL experimental farm in Cade, Louisiana, respectively. The marine species were maintained in recirculating, filtered seawater (30‰) at room temperature (23°C). The freshwater animals were maintained in aquaria containing pondwater at room temperature. The animals were not fed and were used within 2 weeks of collection.

#### Collection of Hemolymph and Hemocyte Extracts

Hemolymph was collected from *G. demissa* by prying the valves apart and inserting a syringe with a 22-gauge needle into the adductor muscle sinus. The hemolymph (usually 1–1.5 mL from each animal) was centrifuged in a microcentrifuge (Beckman microfuge E) for 2 min. The supernatant plasma was removed with a Pasteur pipette, and the cellular pellet was resuspended in buffer (10 mM Tris HCl, pH 7.5) and agitated. This preparation was then centrifuged, and the supernatant (hemocyte extract) removed.

#### Phenoloxidase Assays

We compared several assays for detection of phenoloxidase activity. Initially, we used a modification of the method of Horowitz and Shen (1952). This assay uses L-3,4-dihydroxyphenylalanine (L-DOPA) as a substrate. Our assay mixture contained 50  $\mu$ L of L-DOPA (3 mg mL<sup>-1</sup>), 50  $\mu$ L Tris HCl (50 mM, pH 7.5) and 50–100  $\mu$ L enzyme preparation in the wells of a flat-bottomed 96-well microplate. We used an automated plate reader set to a wavelength of 492 nm or 615 nm to measure the absorbance increase caused by the formation of melanin. The L-DOPA rapidly oxidized producing a high background absorbance in the blank wells. The low activity of phenoloxidase in molluscan hemolymph necessitated long (15–60 min) incubation times, and it was difficult to quantitate phenoloxidase activity consistently with this assay.

Next, we adapted the method of Pye (1974) to our microplate reader protocol. This assay employs 4-methylcatechol as a substrate; the oxidized catechol forms a colored product when complexed with either the methyl or benzyl ester of hydroxyproline. The

rate of spontaneous oxidation of catechol was much slower than that of L-DOPA, and we used the catechol assay with benzylhydroxyproline ester for all data reported here. The assay mixture contained 50  $\mu$ L Tris HCl (50 mm, pH 7.5), 100  $\mu$ L ester, 10  $\mu$ L 4-methylcatechol, and 50–100  $\mu$ L of hemolymph plasma or hemocyte extract in the wells of a 96-well flat-bottom microplate. In blank wells, deionized water was substituted for the plasma or hemocyte extract. For the assays of preparations from marine species, the Tris buffer contained 900 mm NaCl. All components of the assay mixture except catechol were added and the absorbance at 492 nm of each well was measured with the microplate reader immediately after addition of the catechol. The mixtures were then incubated, and additional measurements to the absorbance were made after 15, 30, 60, and 120 min. The effects of trypsin, lipopolysaccharides from *Vibrio cholerae*, and the fungal cell wall extract zymosan were investigated by adding 10  $\mu$ L of stock solutions to achieve the desired concentration in the assay well. Ten  $\mu$ L of water were added to the blank wells for these assays.

The protein content of the hemolymph plasma and hemocyte extracts was determined by a modified Lowry assay (Miller 1950), with bovine serum albumin as a standard. Phenoloxidase activity is reported as the change in absorbance units  $\times 10^3 \text{ min}^{-1} \text{ mg protein}^{-1}$ .

#### Statistics

Student's *t*-tests were used to assess whether changes in activity were different from zero; we used  $\alpha = 0.05$ . All percentage data were transformed by the arcsin transformation before statistical analysis.

### RESULTS

Phenoloxidase activity occurs in the hemolymph plasma and hemocyte extracts of all of the species studied. Phenoloxidase activity in the hemolymph plasma and in the hemocyte extract is shown in Figures 1 and 2, respectively. The variability among species and among individuals of the same species is large. The specific activity is highest in both plasma and cell extract from the two unionid mussels. The concentration of protein in the plasma of the animals examined in this study ranged from 0.4 to 4  $\text{mg mL}^{-1}$ .

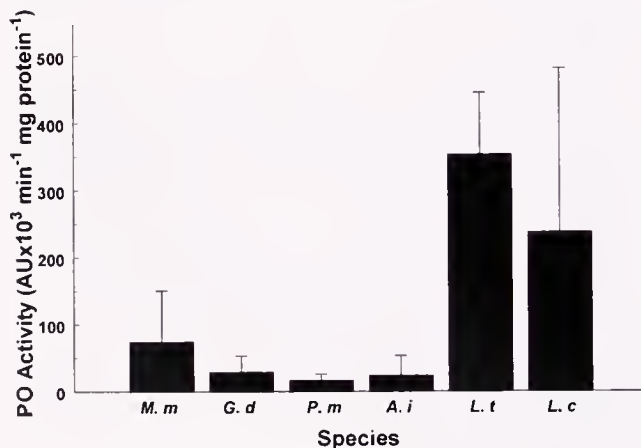


Figure 1. Phenoloxidase activity in bivalve hemolymph plasma. Each bar is the mean  $\pm$  standard deviation ( $n = 8\text{--}12$ ). Mm = *Mercenaria mercenaria*; Gd = *Geukensia demissa*; Pm = *Placopecten magellanicus*; Ai = *Argopecten irradians*; Lc = *Lampsilis claibornensis*; Lt = *Lampsilis teres*.

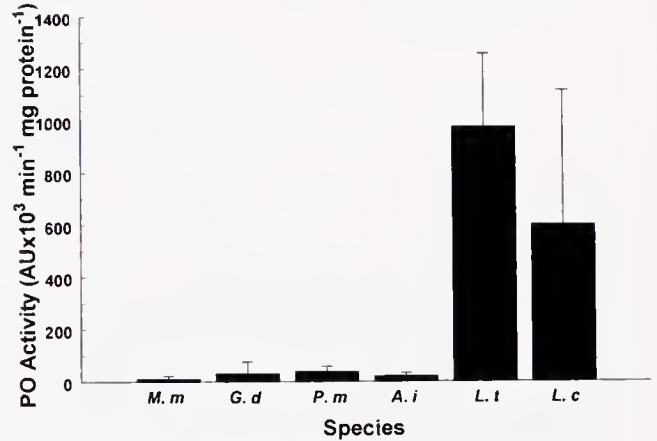


Figure 2. Phenoloxidase activity in bivalve hemocyte extracts. Each bar is the mean  $\pm$  standard deviation ( $n = 8\text{--}12$ ). Mm = *Mercenaria mercenaria*; Gd = *Geukensia demissa*; Pm = *Placopecten magellanicus*; Ai = *Argopecten irradians*; Lc = *Lampsilis claibornensis*; Lt = *Lampsilis teres*.

The effects of trypsin on phenoloxidase activity are summarized in Table 1. There is no consistent stimulation of activity by trypsin. In fact, many of our plasma or hemocyte extract preparations were inhibited by trypsin. This accounts for the large standard deviations. Of the samples tested, only the hemocyte extract preparations from *L. claibornensis* were all stimulated by the trypsin treatment. We assayed the phenoloxidase activity of hemolymph plasma and hemocyte extracts from *P. magellanicus* after incubation with different concentrations of trypsin; the results are shown in Figure 3. There was no consistent stimulation or inhibition of these preparations in a dose-dependent manner by trypsin.

The effects of increasing concentrations of the fungal cell wall extract, zymosan, and of lipopolysaccharides from the cell wall of the bacterium *Vibrio cholerae* on phenoloxidase activity of hemolymph plasma and hemocyte extracts from *P. magellanicus* are shown in Figures 4 and 5, respectively. Neither agent produced consistent stimulation of enzyme activity. As with trypsin, the activity of some preparations was stimulated and the activity of others inhibited by preincubation with these agents. None of the means of percentage change is significantly different from zero. Additional experiments with hemolymph plasma and hemocyte extracts from *A. irradians* produced similar results—neither zymosan (0.01–1.0  $\text{mg mL}^{-1}$ ) nor LPS ( $10^{-3}$ – $10^{-1}$  m) stimulated phenoloxidase activity (Figs. 6, 7). Phenoloxidase activity in the two

TABLE 1.

The percentage change in phenoloxidase activity in bivalve hemolymph plasma and hemocyte extracts preincubated with trypsin (2.5  $\mu\text{g mL}^{-1}$ ) for 30 min.

Species	Plasma	Hemocyte Extract	n
<i>Mercenaria mercenaria</i>	51.9 $\pm$ 55.3	38.0 $\pm$ 80.2	8
<i>Geukensia demissa</i>	17.7 $\pm$ 124.1	238.4 $\pm$ 243.3	8
<i>Lampsilis teres</i>	-17.5 $\pm$ 22.7	106.7 $\pm$ 91.1*	9
<i>Lampsilis claibornensis</i>	-10.0 $\pm$ 23.2	9.9 $\pm$ 25.8	7

Values are mean  $\pm$  standard deviation.

\* Increase significantly different from 0 ( $p < .05$ ).

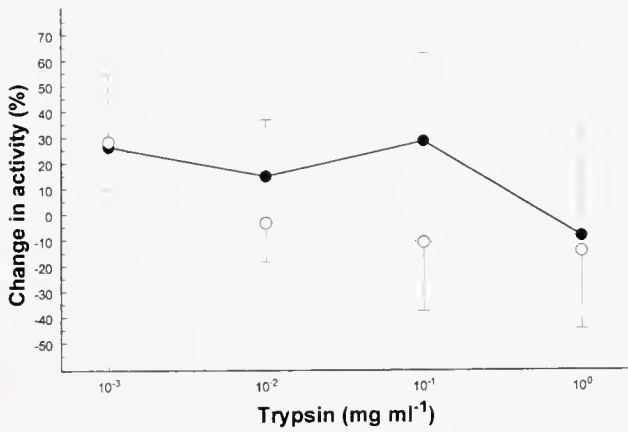


Figure 3. The effect of increasing concentrations of trypsin on phenoloxidase activity in hemolymph plasma (solid circles) and hemocyte extracts (open circles) from *Placopecten magellanicus*. Each point is mean  $\pm$  standard deviation (n = 6).

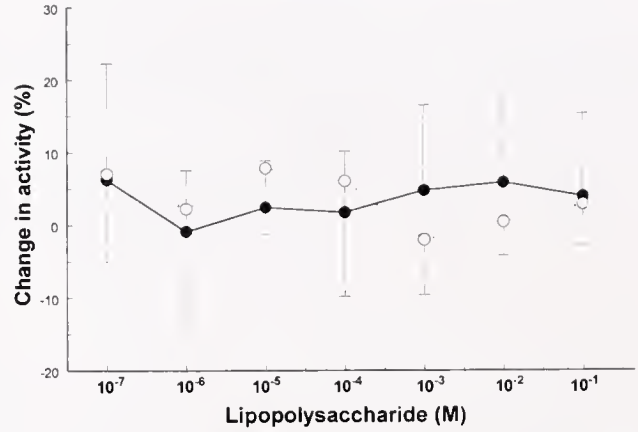


Figure 5. The effect of increasing concentrations of lipopolysaccharide from *Vibrio cholerae* on phenoloxidase activity in hemolymph plasma (solid circles) and hemocyte extracts (open circles) from *Placopecten magellanicus*. Each point is mean  $\pm$  standard deviation (n = 6).

species of *Lampsilis* is not consistently stimulated by either zymosan or LPS (Table 2).

DISCUSSION

Phenoloxidase activity has been found in hemocytes of the marine mussels *Mytilus galloprovincialis*, *Mytilus edulis*, *Perna viridis* and the clam *Ruditapes decussatus* (Coles and Pipe 1994, Renwranz et al. 1996, Asokan et al. 1997, Carballal et al. 1997, Lopez et al. 1997). Phenoloxidase activity is not found in hemocytes from the arcid clam *Scapharca inequivalvis* (Holden et al. 1994). The oxidation of L-DOPA by phenoloxidase in *M. edulis* hemolymph is not stimulated by trypsin and zymosan, but 0.25% zymosan supernatant stimulates the enzyme activity 10-fold (Coles and Pipe 1994). We did not find zymosan to be an effective activator of phenoloxidase activity; it is possible that differences in experimental protocol account for the difference in results.

It should be noted that the concentrations of zymosan and LPS reported to activate phenoloxidase in mollusks are easily 4 or more orders of magnitude higher than the threshold for activation of mammalian white blood cells. It is possible that components of

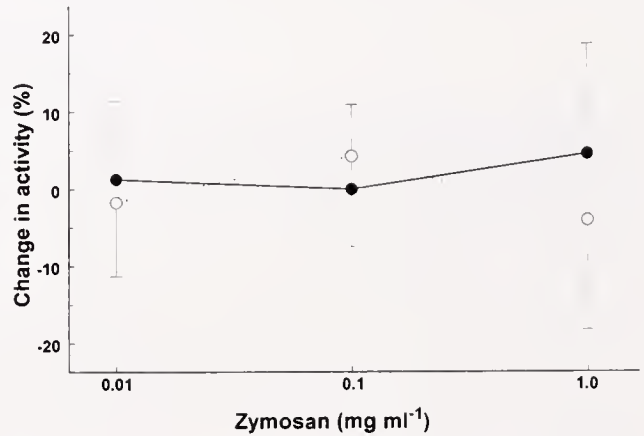


Figure 6. The effect of increasing concentrations of zymosan on phenoloxidase activity in hemolymph plasma (solid circles) and hemocyte extracts (open circles) from *Argopecten irradians*. Each point is mean  $\pm$  standard deviation (n = 9).

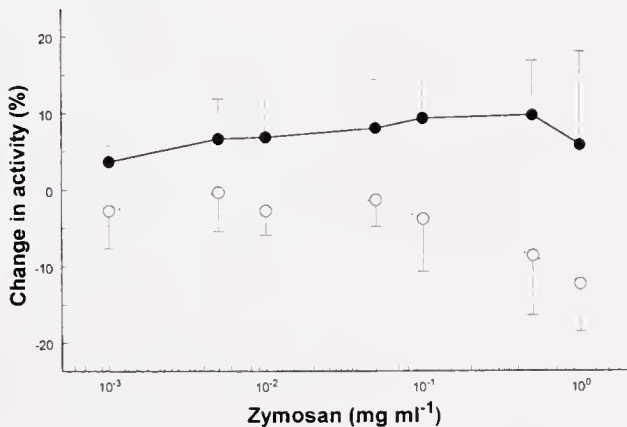


Figure 4. The effect of increasing concentrations of zymosan on phenoloxidase activity in hemolymph plasma (solid circles) and hemocyte extracts (open circles) from *Placopecten magellanicus*. Each point is mean  $\pm$  standard deviation (n = 6).

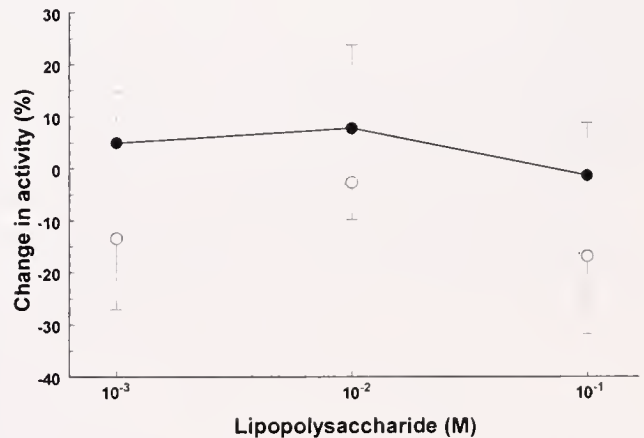


Figure 7. The effect of increasing concentrations of lipopolysaccharide from *Vibrio cholerae* on phenoloxidase activity in hemolymph plasma (solid circles) and hemocyte extracts (open circles) from *Argopecten irradians*. Each point is mean  $\pm$  standard deviation (n = 5-11).

TABLE 2.

Percentage change in phenoloxidase activity in hemolymph plasma and hemocyte extracts from two species of *Lampsilis* preincubated with either zymosan ( $10^{-5}$  M) or bacterial lipopolysaccharides (LPS;  $0.1 \mu\text{g mL}^{-1}$ ).

Species	Treatment	Plasma	Hemocyte Extract	n
<i>L. teres</i>	LPS	$-1.2 \pm 35.6$	$-13.2 \pm 43.2$	8
	Zymo	$3.5 \pm 27.3$	$-5.5 \pm 22.1$	7
<i>L. claiibornensis</i>	LPS	$6.9 \pm 19.2$	$-17.8 \pm 37.1$	9
	Zymo	$39.3 \pm 29.2$	$-17.1 \pm 29.9$	8

Values are mean  $\pm$  standard deviation.

molluskan pathogenic organisms will be more potent stimulators of phenoloxidase activity.

Phenoloxidase activity seems to be a common feature of the hemocytes of bivalve mollusks. The enzyme is secreted into the hemolymph, and the activity varies widely among individual animals. This variability may reflect the relative proportions of different kinds of blood cells in the hemolymph of individual animals.

The granulocytes of *Mytilus edulis* can be separated into basophilic and eosinophilic cells by cytochemistry (Moore and Lowe 1977). Phenoloxidase activity is present in granulocytes but not in basophilic cells (Bayne et al. 1979, Coles and Pipe 1994). The proportion of hemocytes that exhibit phenoloxidase activity varies among individual animals (Renwranz et al. 1996), and the proportion of granulocytic cells that contain phenoloxidase is also highly variable (Coles and Pipe 1994). The phenoloxidase activity of an individual mollusk may be determined by the environmental and pathological stresses that the animal has been subjected to, and may be a component of internal defense. However, unlike the phenoloxidase system in many arthropods, the activity of the enzyme in bivalves is not consistently stimulated by trypsin or by bacterial and fungal components. A clearer understanding of the function of phenoloxidase in bivalve hemolymph depends on further experiments. It is particularly important to demonstrate that the enzyme activity is affected by natural pathogens of mollusks.

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## PROTEIN CONTENT DETERMINES THE NUTRITIONAL VALUE OF THE SEAWEED *ULVA LACTUCA* L FOR THE ABALONE *HALIOTIS TUBERCULATA* L. AND *H. DISCUS HANNAI* INO

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**ABSTRACT** The nutritional value to abalone of *Ulva lactuca* L. with different tissue nitrogen levels was studied. The seaweed was cultured at two levels of ammonia-N enrichment. Cultures receiving 0.5 g ammonia-N m<sup>-2</sup> d<sup>-1</sup> ("low-N") yielded 164 g m<sup>-2</sup> d<sup>-1</sup> of fresh thalli containing 12% crude protein in dry matter and 12 kJ g<sup>-1</sup> energy; cultures receiving 10 g ammonia-N m<sup>-2</sup> d<sup>-1</sup> ("high-N") produced 105 g of fresh thalli m<sup>-2</sup> d<sup>-1</sup> containing 44% protein and 16 kJ g<sup>-1</sup> energy. High-N and low-N algae and a "standard" mixed diet of 75% *U. lactuca* and 25% *Gracilaria conferta* (w/w) containing 33% protein and 15 kJ g<sup>-1</sup> energy were fed to juvenile (0.7–2.1 g) and adult (6.9–19.6 g) *Haliotis tuberculata* and *H. discus hannai* in a 16-week feeding trial. Voluntary feed intake of the high-N and standard diets were significantly lower than the low-N diet in all the cases. Clear differences in performance between treatments were found in the juvenile and adult abalone of both species. Juveniles fed high-N and standard diets grew significantly faster (specific growth rate of *H. tuberculata* was 1.03% day<sup>-1</sup> on high-N algae as compared to 0.72% on low-N algae; *H. discus hannai* grew 0.63 and 0.3% day<sup>-1</sup> on high- and low-N algae, respectively) and showed much better food conversion ratios. The nutritional value of *Ulva lactuca* to abalone is greatly improved by a high protein content, attainable by culturing the seaweed with high supply rates of ammonia.

**KEY WORDS:** integrated mariculture, abalone, seaweed, protein content, nutrition, FCR

### INTRODUCTION

Reduced fishery landings and increasing global demand for abalone have resulted in major market opportunities for cultured abalone (Oakes and Ponte 1996). The European abalone, *Haliotis tuberculata* L., and the Japanese *H. discus hannai* Ino, are valuable shellfish (Mgaya and Mercer 1994, Oakes and Ponte 1996), stimulating considerable effort into the development and optimization practices of their culture.

The supply of sufficient amounts of adequate food throughout the abalone's long growout phase continues to limit the development of intensive abalone culture; suitable artificial diets are generally too expensive (Fleming and Hone 1996) unless fed in conjunction with macroalgae (Uki and Watanabe 1992). Traditionally, cultured abalone have been fed wild-gathered macroalgae; however, the increasing intensity of abalone culture, the unreliable and seasonal nature (availability and quality) of the food harvest (Uki and Watanabe 1992, Mai et al. 1995), tougher environmental legislation, and the establishment of cultures in areas with no access to wild seaweed populations (Shpigel et al. 1996) have lead to increasing interest in the use of cultured macroalgae as abalone food (Tenore 1976, reviewed in Uki 1989, Marsden and Williams 1996, Shpigel and Neori 1996).

The robust chlorophyte *Ulva lactuca* L. has been successfully adapted to vegetative tank culture (DeBusk et al. 1986). It has been a highly effective biofilter species, removing dissolved nutrients from mariculture effluents and sustaining rapid production of seaweed biomass (Tenore 1976, Vandermeulen and Gordin 1990, Cohen and Neori 1991, Neori et al. 1991, 1996). Cultured and wild *Ulva lactuca* have been successfully used as diets for abalone (Tenore 1976, Shpigel et al. 1996); but to obtain commercially acceptable abalone growth rates, it has been necessary to feed other algal species in conjunction with *U. lactuca* (Mercer et al.

1993, Stuart and Brown, 1994). Given the worldwide distribution, ease of culture, biofiltration potential, and high productivity of *Ulva lactuca*, combined with its high palatability to abalone (Stuart and Brown 1994), learning to manipulate the dietary value of this seaweed to the nutritional requirements of abalone and understanding these requirements are of considerable value.

The availability of suitable quantity and quality of dietary protein are considered to be a prime factor governing the growth of abalone fed natural diets (Mai et al. 1995, Britz 1996). Previous studies have noted that the relative amounts of tissue nitrogen, predominantly in the form of protein and free amino acids (Duke et al. 1989b, Pedersén 1994), can vary in *Ulva* spp., depending upon the alga's growth conditions and the availability of inorganic nitrogen in the growth medium (DeBusk et al. 1986, Duke et al. 1989a, Vanderneulen and Gordin 1990, Cohen and Neori 1991, Neori et al. 1991). However, such studies have not considered possible subsequent effects upon the macroalgivores. Attempts to manipulate the chemical composition of a seaweed by adjusting the culture environment have so far been restricted to macroalgae of direct commercial value, such as the agarophyte genus *Gracilaria* spp. (e.g., Lapointe and Ryther 1979, Lignell and Pedersén 1987). Some important progress has, however, been made in enhancing the dietary value of certain phytoplankton species fed to bivalves, by adjusting the nutrient composition of the algal culture medium (Engirt et al. 1986, Herrero et al. 1991).

The present study was established with two main objectives: (1) to develop *Ulva lactuca* cultures of substantially different and stable tissue nitrogen levels; and (2) to determine the nutritional values of these *U. lactuca* cultures for the abalone *Haliotis tuberculata* and *H. discus hannai*.

### MATERIALS AND METHODS

#### Abalone

Fifty juvenile European Abalone, *Haliotis tuberculata* (18.2–24.7 mm shell length, 0.8–2.1 g individual live weight; 76–89 g total biomass) or Japanese Ezo Awabi, *H. discus hannai* (17.9–

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24.4 mm, 0.7–1.8 g; 56–63 g biomass) were stocked to 28-L aquaria. Each aquarium contained two half-pipe shelters, and received 50 volume exchanges  $d^{-1}$  of 10  $\mu m$  filtered seawater at 21.7–23.1°C. An airline suspended in the center of each aquarium supplied vigorous aeration to circulate food material, which was retained by a 1-mm mesh covering the outlet pipe. Aquaria were also established with adult animals: 10 *H. tuberculata* (37.0–50.2 mm, 6.9–19.6 g each; 123–125 g total biomass) or 10 *H. discus hamai* (34.0–50.8 mm, 5.0–16.1 g; 79–81 g biomass) individually identified by Dymo™ tags. All animals had previously received a mixed diet of *U. lactuca* and *G. conferta*.

#### Diets and Algal Cultures

Three distinct live seaweed diets were produced for the abalone. Two were monospecific *Ulva lactuca*, grown at two different levels of nitrogen enrichment. A "standard" diet consisted of a mixture of medially enriched *U. lactuca* together with *Gracilaria conferta*. This mixed diet had been found in our preliminary observations to support good growth in weaned abalone juveniles.

Macroalgal cultures were established in November 1995 in the land-based facilities of the National Center for Mariculture, Eilat, Israel. The cultures were supplied with 5–6 volume exchanges  $d^{-1}$  of 10- $\mu m$  filtered water pumped from the Red Sea at 20-m depth (41 ppt, 19.5–25.3°C). Vegetative *Ulva lactuca* thalli, isolated from the Red Sea as in Vandermeulen and Gordin (1990), were grown in 1m<sup>2</sup>, 600-L tanks agitated with vigorous aeration. The technique has been described in Vandermeulen and Gordin (1990) and in Cohen and Neori (1991). Inorganic nutrients were added to the media in a concentrated solution, containing disodium phosphate (DSP, at a flux of 0.6 g P m<sup>-2</sup> d<sup>-1</sup>), and ammonium sulphate (at fluxes determined by the experimental treatment). The solution was dripped into the cultures over a 4-h period every morning, this being considered ample time for *Ulva* spp. to take up its daily ammonia-N requirement (Fujita et al. 1988). The ammonia-N was added at two levels, 0.5g N m<sup>-2</sup> d<sup>-1</sup> ("low-N" *U. lactuca* culture) and 10 g N m<sup>-2</sup> d<sup>-1</sup> ("high-N" culture). These levels were chosen based on Cohen and Neori (1991), who suggest that these N-fluxes would produce *U. lactuca* of considerably different tissue-nitrogen levels, while sustaining sufficient production to permit harvesting for feeding. Several cultures of high-N and low-N *U. lactuca* were established. Every week, the vessels were emptied and cleaned, the algae were centrifuged (500 r.p.m. for 3 min) to remove surface water, weighed to assess biomass production, and restocked at the original density. The mixed standard diet was obtained by harvesting *U. lactuca* and *Gracilaria conferta* grown in simulation bio-filters (Cohen and Neori 1991) receiving 0.6 g DSP m<sup>-2</sup> d<sup>-1</sup> and 4 g ammonia-N m<sup>-2</sup> d<sup>-1</sup>.

#### Feeding and Growth

A preliminary test was conducted to determine whether the N content of *Ulva lactuca* was decreasing after days of immersion in the abalone tanks. High-N and low-N *U. lactuca* were stocked to separate aquaria without animals. Subsamples were removed after 0, 12, 18, 24, and 48 h, rinsed in deionised water, freeze dried, and the nitrogen content was determined.

High-N and low-N *ulva lactuca* dietary treatments were supplied to triplicate aquaria with juveniles and duplicate aquaria with adult abalone of either species; in addition, a single aquarium for each abalone size class and species was fed the mixed diet. Animals were first stocked to the experimental aquaria on 8 February 1996 after individual live weights (following 2 min drying on

absorbent paper  $\pm$  0.01 g) and shell lengths had been measured ( $\pm$ 0.05 mm). An additional 25 juveniles and 10 adults were taken from the source populations of either species, weighed and held without food for 48 h; the soft body and shell were then separated and freeze dried.

Food algae were added to the aquaria in excess (equivalent to approximately 20% of the resident abalone biomass) at dusk and removed 16 h later, because abalone are assumed to show minimal daytime feeding activity (Barkai and Griffiths 1987, Uki and Watanabe 1992, Mgaya and Mercer 1994). This period was considered representative of total daily feeding. Feed intake rate assessments began following a 2-week acclimation to the diets; harvested algae were centrifuged (as described above), and a known weight ( $\pm$  0.01 g) was supplied to each aquarium. The mixed diet consisted of *U. lactuca* and *G. conferta* offered in a wet weight ratio of 3:1. It was assumed that the abalone showed no preferential feeding behavior. Uneaten algae were collected by siphoning aquarium contents through a 1-mm mesh, allowing feces and detritus to be washed out; collected algae were centrifuged and weighed.

A control aquarium, identical to the experimental vessels, but without animals, was supplied with the effluent water of a randomly selected abalone aquarium from each treatment and was stocked with algae corresponding to the dietary treatment being offered. Change in algal wet weight was assessed, as described above, and the mean percentage weight change was calculated for each treatment (three dietary treatments, juveniles and adults, total  $n = 6$ ) and used as a correction factor (C) of the initial weight of algae fed (see in Definitions, below).

On the occasion of each feed intake rate trial, algal samples were taken from each food-stock culture to produce samples of approximately 20 g wet weight per fortnight of each diet. The samples were weighed, freeze-dried, and reweighed ( $\pm$  0.01 g) to determine water content. The three dried samples of each treatment collected during each 2-week period were combined and stored at  $-20^{\circ}C$  for subsequent analysis of chemical composition.

At the end of each trial, abalone were removed from the experimental vessels, and individual weights and lengths were re-measured; all of the 10 adult abalone and 25 randomly selected juveniles from each vessel were then shucked and freeze dried for subsequent assessment of condition and soft body composition. The juveniles were not tagged. Thus, growth (in weight and length) in each aquarium was estimated by the difference between the average values of the population at the beginning of the experiment and the values of 10 randomly selected juveniles at the end of the experiment.

#### Analytical Procedures

The freeze-dried samples of algae and bodies of the abalone were homogenized in a mill before being subjected to analyses. Water content was calculated by weight loss after 24-h drying at 105°C. Crude protein was measured using the Kjeldahl technique and multiplying N by 6.25. Crude lipid was measured after chloroform-methanol extraction (Folch et al. 1957). Samples were homogenized with a high-speed homogenizer for 5 min, and lipid was determined gravimetrically after separation and vacuum drying. Crude carbohydrate was determined using the phenol-sulphuric acid method (Dubois et al. 1956) after boiling the sample in 1N H<sub>2</sub>SO<sub>4</sub> for 1 h. The resulting color was measured by spectrophotometer against a glucose standard at 490 nm. Ash-free dry weight was calculated from the weight loss after incubation of

samples for 24 h at 550°C in a muffle furnace. Heat of combustion was measured in a Parr bomb calorimeter using benzoic acid as a standard.

#### Definitions

Net feed intake (I) was determined for each aquarium according to the equation

$$I = (A_{IN} \times C) - A_{OUT}$$

where  $A_{IN}$  and  $A_{OUT}$  represent, respectively, the measured weights of algae placed in, and removed from an aquarium and C is the treatment-specific correction factor used to compensate for endogenous changes in fresh weight. Daily feed intake was assessed in this way at 3 to 4 day intervals.

$$\text{Specific growth rate (SGR\%, \%d)} = 100 \cdot (\ln W_1 - \ln W_0) / t$$

( $W_0$  is the weight of an animal at the beginning of each monitoring interval, and  $W_1$  is the weight after t days of growth at the end of the interval);

$$\text{Shell growth (\mu m/day)} = (L_2 - L_1) / t$$

( $L_1$  is the wet length of an animal at the beginning of each monitoring interval, and  $L_2$  is the length at the end of the interval);

$$\text{Condition index (CI)} = \text{soft flesh (g wet)/shell (g wet)}$$

$$\text{Feed intake rate (mg algae/g abalone/day)} = I / \text{abalone standing stock}$$

$$\text{Food conversion ratio (FCR)} = \text{total feed intake (g wet)/total weight gain (g wet)}$$

$$\text{Protein productive value (PPV)} = 100 \cdot \text{protein gain (g)/protein consumed (g)}$$

$$\text{Energy productive value (EPV)} = 100 \cdot \text{energy gain/gross energy consumed}$$

#### Statistical Analyses

The responses of each abalone species and varied sizes to the various diets were analyzed separately. Growth and condition parameters examining the response to the treatments of individual animals were compared by analysis of variance (ANOVA) (Sokal and Rohlf 1995) and Duncan multiple range test. Feed intake and FCR parameters were analyzed for entire aquarium populations using *t*-test. All analyses were carried out with SPSS software.

## RESULTS

#### Algal Production and Composition

By the second week of culture, *Ulva lactuca* production had stabilized in all low-N and high-N cultures. From November 1995 to the end of feeding trials in May 1996, low-N cultures yielded a mean of  $164 \pm 6$  g (SE) fresh *U. lactuca*  $m^{-2} d^{-1}$  ( $n = 57$ ). High-N cultures yielded  $105 \pm 7$  g  $m^{-2} d^{-1}$  ( $n = 38$ ) and showed evidence of "perforation disease," described by Colomi (1989). High-N thalli were also considerably darker than low-N, but morphologically similar, flat, and sheet-like.

Nitrogen (expressed as crude protein), energy, water, and ash content all remained stable in the algal samples collected during the feeding trials. Water content was similar in all three diets, but the ash content of high-N *U. lactuca* was 30–35% lower than that of the low-N or mixed diet (Table 1). The mixed and high-N diets had similar calorific values, but the mixed diet had 25% less crude protein. Both the energy and the nitrogen content of low-N *U.*

*lactuca* were low as compared to the other two diets. Samples of high-N *U. lactuca* placed in nonenriched seawater (data not shown) lost approximately 18% of their tissue N in 24 h, falling from  $5.8 \pm 0.08\%$  N in dry tissue to a stable  $4.8 \pm 0.04\%$  N ( $n = 3$ ); whereas, low-N tissue nitrogen remained constant over 48 h.

#### Abalone Fced Intake and Performance

##### Juvenile Abalone

After 15.5 weeks, mean growth rate of juvenile *Haliotis tuberculata*, expressed in terms of SGR% or shell length increment, was significantly higher in aquaria receiving high-N diet as compared to those fed low-N diet (Table 2). Growth in the single aquarium that continued to receive a standard mixed diet was significantly faster than in the monospecific *Ulva lactuca* treatments. It was also noted that new shell growth during the feeding trials appeared light green in juvenile *H. tuberculata* fed low-N *U. lactuca*, in contrast to the characteristic red-brown shell increments of those animals fed high-N or mixed diets. Voluntary feed intake as apparent daily intake (mg alga per g of abalone biomass) also showed significant differences between the two *U. lactuca* treatments, with the feed intake rate of high-N *U. lactuca* being 68% that of low-N. The combined effects of relatively low feed intake rates and fast growth of juvenile *H. tuberculata* fed high-N *U. lactuca* resulted in a significantly lower (i.e., more efficient) FCR in the high-N treatment.

Although the growth and feed intake rates in juvenile *Haliotis discus hannai* after 16 weeks (Table 3) were considerably lower than in juvenile *H. tuberculata* (Table 2), similar patterns were apparent for the two species: overall feed intake rates of high-N algae were significantly lower ( $p < .001$ ) than for low-N, and use of high-N or mixed diets resulted in significantly higher ( $p < .01$ ) SGR% than with low-N *U. lactuca*. However, only the mixed diet treatment sustained significantly greater shell length growth, when compared to low-N, in *H. discus hannai*. Feeding high-N diet to juvenile *H. discus hannai* also resulted in a significantly more efficient ( $p < .001$ ) FCR. However, only the mixed diet produced a significant effect on the condition (wet flesh: wet shell weight,  $p < .001$ ) of juveniles of both species (Tables 2, 3), as compared to the high-N and low-N treatments. The loss of condition in juvenile *H. discus hannai* fed low-N seaweed was clearly apparent during the experiment as a progressive atrophying ("withering") of the foot muscle; two of the most reduced individuals in one replicate eventually died, these representing the only mortalities during the course of the experiment.

TABLE 1.

Composition of the three experimental diets, low N-*Ulva*, high-N *Ulva*, and the control diet intermediate N-*Ulva* and *Gracilaria* in relation of 3:1 (w/w).

	Low N- <i>Ulva</i>	High N- <i>Ulva</i>	Control
Dry matter	13.41 ± 2.37	16.86 ± 1.79	16.69 ± 2.72
Crude protein	1.66 ± 0.29	7.38 ± 1.02	5.54 ± 1.09
Carbohydrate	3.62 ± 0.67	4.05 ± 0.51	3.81 ± 0.87
Lipid	0.14 ± 0.03	0.13 ± 0.03	0.14 ± 0.04
Ash	4.49 ± 0.86	2.91 ± 0.31	4.23 ± 0.91
Gross energy (kJ/g)	1.60 ± 0.29	2.63 ± 0.40	2.43 ± 0.43

Average values during the whole experimental period are given and components are expressed as % of fresh weight (±SD).

TABLE 2.

Growth of juvenile *Haliotis tuberculata* fed three algal diets (108 days).

	Low-protein <i>Ulva</i>	High-protein <i>Ulva</i>	Control
Initial wt (g)	1.74	1.57	1.71
	± 0.258	± 0.176	± 0.236
Final wt (g)	3.81 <sup>a</sup>	4.79 <sup>b</sup>	6.50 <sup>c</sup>
	± 0.880	± 1.678	± 1.805
SGR% <sup>1</sup>	0.725 <sup>a</sup>	1.028 <sup>b</sup>	1.234 <sup>c</sup>
	± 0.216	± 0.288	± 0.164
Shell growth (µm/day)	80.72 <sup>a</sup>	121.47 <sup>b</sup>	160.30 <sup>c</sup>
	± 20.38	± 33.9	± 35.8
Feed intake (mg algae/g abalone/day)	127.53 <sup>a</sup>	86.19 <sup>b</sup>	98.60
	± 11.07	± 0.77	
FCR <sup>2</sup>	18.17 <sup>a</sup>	7.81 <sup>b</sup>	7.70
	± 1.30	± 0.794	
Condition index <sup>3</sup>	0.586 <sup>a</sup>	0.570 <sup>a</sup>	0.682 <sup>b</sup>
	± 0.073	± 0.077	± 0.088
PPV (p) <sup>4</sup>	24.81 <sup>a</sup>	15.06 <sup>b</sup>	22.71
	± 2.69	± 1.31	
EPV (%) <sup>5</sup>	11.17 <sup>a</sup>	13.38 <sup>a</sup>	15.58
	± 1.34	± 1.11	

Average value ± SD.

<sup>1</sup> Specific growth rate =  $(\ln W_2 - \ln W_1) / \text{days} * 100$ .<sup>2</sup> Food conversion ratio = feed intake (g wet)/weight gain (g wet).<sup>3</sup> Soft flesh (g wet)/shell (g wet).<sup>4</sup> Protein productive value = protein gain (g)/crude protein consumed (g) \* 100.<sup>5</sup> Energy productive value = energy gain (kJ)/gross energy consumed (kJ) \* 100.<sup>a,b,c</sup> Values with the same superscript are not significantly different ( $p < .05$ ) using ANOVA and Duncan multiple range test (SPSS).

Juvenile *Haliotis tuberculata* fed low-N seaweed made more efficient use of the ingested protein (PPV) compared to individuals receiving high-N treatment (Table 2). The reverse was observed in juvenile *H. discus hannai*, where significantly less of the protein ingested as low-N diet was incorporated into abalone tissue, as compared to high-N diet (Table 3). On the other hand, the juveniles of both species utilized energy (EPV) better in the high-N diet, but only in *H. discus hannai* was this trend statistically significant.

#### Adult Abalone

Adults of both *Haliotis tuberculata* and *H. discus hannai* grew significantly better when fed with the mixed and high-N diets than with the low-N diet (Tables 4, 5, respectively). Adults of both species also voluntarily ate significantly more low-N *Ulva lactuca*, as compared with the high-N seaweed. As a mathematical consequence of these two observations, the FCRs for both species fed high-N *U. lactuca* were significantly and strikingly better (lower) than in the animals fed the low-N *U. lactuca*. In *H. tuberculata* the reduction in FCR by feeding high-N seaweed was by 64% and in *H. discus hannai* by 77% (Tables 4, 5).

### DISCUSSION

#### Performance in Response to Dietary Treatment

Culturing *Ulva lactuca* at high- and low-ammonia fluxes yielded thalli that were of considerably different nutritional value

TABLE 3.

Growth of juvenile *Haliotis discus hannai* fed three algal diets (112 days).

	Low-protein <i>Ulva</i>	High-protein <i>Ulva</i>	Control
Initial wt (g)	1.205	1.172	1.132
	± 0.148	± 0.013	± 0.082
Final wt (g)	1.680 <sup>a</sup>	2.367 <sup>b</sup>	2.571 <sup>b</sup>
	± 0.601	± 0.650	± 0.785
SGR% <sup>1</sup>	0.296 <sup>a</sup>	0.627 <sup>b</sup>	0.732 <sup>b</sup>
	± 0.201	± 0.267	± 0.262
Shell growth (µm/day)	31.70 <sup>a</sup>	44.47 <sup>a,b</sup>	54.93 <sup>b</sup>
	± 22.75	± 25.61	± 21.30
Feed intake (mg algae/g abalone/day)	85.0 <sup>a</sup>	32.97 <sup>b</sup>	44.23
	± 18.22	± 4.6	
FCR <sup>2</sup>	31.50 <sup>a</sup>	5.54 <sup>b</sup>	5.93
	± 5.83	± 1.12	
Condition index <sup>3</sup>	0.42 <sup>a</sup>	0.45 <sup>a,b</sup>	0.48 <sup>b</sup>
	± 0.069	± 0.070	± 0.08
PPV (%) <sup>4</sup>	9.15 <sup>a</sup>	20.4 <sup>a</sup>	22.32
	± 7.08	± 4.93	
EPV (%) <sup>5</sup>	5.99 <sup>a</sup>	19.59 <sup>b</sup>	17.70
	± 1.21	± 1.28	

Average value ± SD.

<sup>1</sup> Specific growth rate =  $(\ln W_2 - \ln W_1) / \text{days} * 100$ .<sup>2</sup> Food conversion ratio = feed intake (g wet)/weight gain (g wet).<sup>3</sup> Soft flesh (g dry)/shell (g dry).<sup>4</sup> Protein productive value = protein gain (g)/crude protein consumed (g) \* 100.<sup>5</sup> Energy productive value = energy gain (kJ)/gross energy consumed (kJ) \* 100.<sup>a,b</sup> Values with the same superscript are not significantly different ( $p < .05$ ) using ANOVA and Duncan multiple range test (SPSS).

to both abalone species. The animals fed N-enriched seaweed subsequently grew significantly faster, while consuming significantly less seaweed than the animals fed N-depleted seaweed. The above observations agree with the broad principles of herbivorous grazing. Feed intake rate is the main compensatory mechanism for diet quality in herbivores (Bowen et al. 1995), including abalone (Koike et al. 1979, Mgaya and Mercer 1994). Nevertheless, when fed low-quality food, a herbivore feeding to capacity may still be undernourished (White 1978). Bowen et al. (1995) and Britz (1996) suggested that dietary energy content also regulates abalone feed intake rate. In the present study, differences in feed intake rates of the abalone juveniles correlate numerically more closely with differences in energy content of *Ulva lactuca* than with its N content. Low-N and high-N feed intake rates are separated by a factor of 1.5 in juvenile *H. tuberculata* and 2.6 in *H. discus hannai*; high-N *U. lactuca* has 1.6 × energy and 4.4 × N of the low-N *U. lactuca*. Therefore, at least the *H. tuberculata* juveniles eating both diets had about the same energy intakes but very different N intakes. These results suggest that in addition to crude protein content, different energy contents in the *U. lactuca* diets may contribute to the observed differences in feed intake. To corroborate this explanation, it would be necessary to assess the gut capacity of the abalone to determine whether the animals were simply feeding to capacity.

Abalone somatic growth is considered to depend upon the amount of crude dietary protein (Uki and Watanabe 1992, Fleming 1995b, Mai et al. 1995, Britz 1996). Maximum abalone growth



TABLE 4.

Growth of adult *Haliotis tuberculata* fed three algal diets (106 days).

	Low-protein <i>Ulva</i>	High-protein <i>Ulva</i>	Control
Initial wt (g)	12.56 ± 3.17	12.26 ± 4.49	12.34 ± 3.73
Final wt (g)	15.47 <sup>a</sup> ± 3.52	16.05 <sup>a</sup> ± 5.15	18.27 <sup>a</sup> ± 5.64
SGR% <sup>1</sup>	0.202 <sup>a</sup> ± 0.107	0.271 <sup>a,b</sup> ± 0.193	0.371 <sup>b</sup> ± 0.154
Shell growth (µm/day)	43.95 <sup>a</sup> ± 16.33	53.77 <sup>a</sup> ± 23.09	59.88 <sup>a</sup> ± 23.06
Feed intake (mg algae/g abalone/day)	77.0 <sup>a</sup> ± 2.61	36.16 <sup>b</sup> ± 0.94	36.15 9.71
FCR <sup>2</sup>	39.18 <sup>a</sup> ± 1.7	14.20 <sup>b</sup> ± 3.96	9.71
Condition index <sup>3</sup>	0.54 <sup>a</sup> ± 0.106	0.51 <sup>a</sup> ± 0.084	0.52 <sup>a</sup> ± 0.102
PPV (%) <sup>4</sup>	-2.0 <sup>a</sup> ± 9.87	2.32 <sup>a</sup> ± 0.24	7.79
EPV (%) <sup>5</sup>	2.85 <sup>a</sup> ± 1.91	1.91 <sup>a</sup> ± 1.94	4.59

Average value ± SD.

<sup>1</sup> Specific growth rate = (lnW<sub>2</sub>-lnW<sub>1</sub>)/days\*100.

<sup>2</sup> Food conversion ratio = feed intake (g wet)/weight gain (g wet).

<sup>3</sup> Soft flesh (g dry)/shell (g dry).

<sup>4</sup> Protein productive value = protein gain (g)/crude protein consumed (g) \*100.

<sup>5</sup> Energy productive value = energy gain (kJ)/gross energy consumed (kJ) \*100.

<sup>a,b</sup> Values with the same superscript are not significantly different (*p* < .05) using ANOVA and Duncan multiple range test (SPSS).

was typically attained from diets of 35% protein (by dry weight; see in Uki 1989, Uki and Watanabe 1992, Mai et al. 1995, Britz 1996). These findings of previous researchers are corroborated in the present study by the rapid growth and efficient energy use (EPV) of abalone juveniles fed high-N (44% protein) *Ulva lactuca*, as compared to the apparent N-limitation in low-N (12% protein) fed abalone. The substantially more efficient food conversion ratios with high-N diet as compared with low-N diet in juveniles and adults of both species are typical of herbivorous nutrition (Mattson 1980). This has been shown to apply to juvenile *Haliotis discus hannai* with dietary crude protein up to 28–30% of dry matter (Uki 1989, Uki and Watanabe 1992) and to at least 47% for *H. midae* (Britz 1996). Britz (1996) and Uki and Watanabe (1992) found that the efficiency with which ingested protein was utilized for growth (PPV) in abalone increased with decreasing dietary protein level, as corroborated here by the significantly higher PPV of juvenile *H. tuberculata* fed low-N *U. lactuca*. The reverse is seen in juvenile *H. discus hannai*. It is suggested that the loss of condition of low-N fed *H. discus hannai*; that is, reduction in the soft tissue fraction of total dry weight, resulting in low (occasionally negative) levels of protein utilization, has caused this anomaly.

Growth rates of *Haliotis discus hannai* in the present study were lower than in *H. tuberculata* receiving the same diets. Faster *H. discus hannai* growth has been recorded elsewhere (e.g., Uki 1989 reports shell growth of up to 270 µm d<sup>-1</sup> for young juvenile *H. discus hannai* grown at similar temperatures to those used here). However, this study concurs with the results of Mercer et al. (1993), who recorded *H. discus hannai* growth to be consistently

TABLE 5.

Growth of adult *Haliotis discus hannai* fed three algal diets (106 days).

	Low-protein <i>Ulva</i>	High-protein <i>Ulva</i>	Control
Initial wt (g)	7.81 ± 2.22	8.06 ± 3.53	7.98 ± 3.94
Final wt (g)	9.16 <sup>a</sup> ± 3.05	10.47 <sup>a</sup> ± 3.98	11.24 <sup>a</sup> ± 5.71
SGR% <sup>1</sup>	0.143 <sup>a</sup> ± 0.109	0.264 <sup>b</sup> ± 0.163	0.337 <sup>b</sup> ± 0.227
Shell growth (µm/day)	17.68 <sup>a</sup> ± 12.61	29.13 <sup>b</sup> ± 18.14	43.18 <sup>c</sup> ± 24.3
Feed intake (mg algae/g abalone/day)	54.15 <sup>a</sup> ± 4.17	20.82 <sup>b</sup> ± 2.32	19.01 5.8
FCR <sup>2</sup>	36.2 <sup>a</sup> ± 6.76	8.4 <sup>b</sup> ± 1.24	5.8
Condition index <sup>3</sup>	0.43 <sup>a</sup> ± 0.086	0.46 <sup>a</sup> ± 0.15	0.50 <sup>a</sup> ± 0.104
PPV (%) <sup>4</sup>	-3.53 <sup>a</sup> ± 7.21	9.58 <sup>a</sup> ± 6.66	24.84
EPV (%) <sup>5</sup>	-0.77 <sup>a</sup> ± 3.34	4.19 <sup>a</sup> ± 2.85	14.27

Average value ± SD.

<sup>1</sup> Specific growth rate = (lnW<sub>2</sub>-lnW<sub>1</sub>)/days\*100.

<sup>2</sup> Food conversion ratio = feed intake (g wet)/weight gain (g wet).

<sup>3</sup> Soft flesh (g dry)/shell (g dry).

<sup>4</sup> Protein productive value = protein gain (g)/crude protein consumed (g) \*100.

<sup>5</sup> Energy productive value = energy gain (kJ)/gross energy consumed (kJ) \*100.

<sup>a,b</sup> Values with the same superscript are not significantly different (*p* < .05) using ANOVA and Duncan multiple range test (SPSS).

inferior to that of *H. tuberculata* in comparative feeding experiments. They also found, as we have, that for optimal growth *H. discus hannai* required higher diet protein content than *H. tuberculata*.

*Value of Ulva lactuca as a Dietary Alga for Abalone*

The adults of both abalone species showed similar feeding behavior, with mean feed intake of high-N being less than half that of low-N *Ulva lactuca*. Food conversion ratios of *Haliotis discus hannai* adults resemble those found for juveniles, becoming more efficient in high-N fed animals. Spawning activity was occasionally observed in adult *H. tuberculata* vessels, affecting individual feeding rates (as in Mgaya and Mercer 1994), as well as flesh weight and shell deposition (as in Mercer et al. 1993), and causing a negative protein utilization at the low-N treatment.

It seems that if high-N and low-N *Ulva lactuca* were offered as diets to abalone throughout the growout period, the high-N diet would sustain significantly faster growth in both species. Using high-N *Ulva*, both species can sustain high mean growth rates with less than half the food intake. However, previous studies have implied that the extrapolation of short-term abalone feeding trials may be unrepresentative. Day and Fleming (1992) found that abalone fed monospecific algal diets stopped growing after 50 to 200 days. They suggested it was unlikely that a single algal species could supply all essential nutrients.

The performance of juvenile abalone fed the mixed diet suggests that using only gross N and energy measurements to assess

the value of an algal diet has its limits. The values of gross N and energy in the mixed diet were intermediate between those of low-N and high-N *Ulva lactuca*, but abalone performance with the mixed diet was usually superior to that produced even by high-N diet. Problems also arise when using only energy and crude protein to assess relative nutritional values of a single algal species. In the current study, the N and energy content of low-N and high-N *U. lactuca* at the time of stocking to the abalone vessels is used as an indication of dietary value for the animals. These parameters may not be representative of the relative amounts that are ultimately available to the abalone. *Ulva* sp. in a rich medium where N does not limit growth will carry out luxury N uptake, storing excess N in intracellular pools of organic N, predominantly amino acids, and  $\text{NH}_4^+$  (Fujita et al. 1988, Lundberg et al. 1989). Pedersén (1994) notes that if *Ulva* spp. is transferred from an N-rich medium to an N-starved medium, there is a fall in tissue N, as observed in the present study when *U. lactuca* is stocked to the abalone vessels. The  $\text{NH}_4^+$  pool is highly soluble and considered physiologically impossible to maintain if external concentrations fall (Fujita et al. 1988). The release of intracellular  $\text{NH}_4^+$  by *U. lactuca* has been recorded by Vandermeulen and Gordin (1990) and is likely to be the cause of decline in high-N nitrogen content found in the current study.

The quality of the digestible protein, expressed in terms of amino acid composition, may also vary between low-N and high-N *Ulva lactuca*. The amino acid profile of an alga may vary, depending upon the level of N available (Lignell and Pedersén 1987, Miyashita and Miyazaki 1993), hence affecting the dietary value of the alga for abalone (Mai et al. 1995). Other components of the algal composition might affect its nutritional value and vary according to the seaweed's nutrient status. For example, N-starved *Ulva* spp. has increased levels of high energy soluble carbohydrates (cf. DeBusk et al. 1986); whereas, Mercer et al. (1993) noted considerable variation in total lipid and carbohydrate levels in wild gathered *U. lactuca* used as an experimental abalone diet.

Morphologically simple, fast-growing opportunistic seaweeds that lack specific chemical defenses, such as *Ulva* spp., *Enteromorpha* spp., and *Porphyra* spp., are considered to be the most palatable taxa for abalone (Stuart and Brown 1994, Fleming 1995a). Such algae also tend to show the greatest range in tissue-N levels in field-gathered specimens and in culture (Kudoh 1987, Björnsäter and Wheeler 1990, Wheeler and Björnsäter 1992). Considerable intraspecific variations in tissue N have also been noted in other macroalgal species commonly fed to abalone, such as *Gracilaria* spp. (Friedlander et al. 1987, Lignell and Pedersén 1987, Jones et al. 1996).

The demonstration that abalone performance when fed a mono-specific diet of *Ulva lactuca* can vary considerably, depending upon the nutrient status of the alga, explains conflicting conclusions reached in other reports considering the nutritional value of *Ulva* spp. Pickering (1990, reviewed in Stuart and Brown 1994) found differences in abalone growth when fed different ecotypes of *Gracilaria sordida*. However, other researchers have tended to consider only interspecific differences between macroalgae offered as diets for abalone (e.g., Day and Fleming 1992, Shepherd

and Steinberg 1992, Mercer et al. 1993, Marsden and Williams 1996). The documented relative dietary value of *U. lactuca*, as compared to other seaweeds, provides a useful indication of the importance of considering the alga's nutritional status: *Ulva* spp. has been considered a good (Uki 1989) and preferred food species for *Haliotis discus hannai* (Shepherd and Steinberg 1992); whereas, Mercer et al. (1993) noted *H. discus hannai* growth rates when fed their *Ulva* sp. (13% crude protein) to be significantly lower than for any other algae tested; results using *H. tuberculata* tend to be in closer agreement with the present study, the seaweed being considered a preferred diet (Shepherd and Steinberg 1992) of intermediate nutritional value (Koike et al. 1979, Mercer et al. 1993). In other abalone species, Tenore (1976) found good growth performance for juvenile *H. discus* and *H. rufescens* fed biofilter grown *Ulva* sp. (30% protein); whereas, *Ulva* sp. (13.2% protein) was the only diet tested by Stuart and Brown (1994) that produced no significant growth in juvenile *H. iris*.

#### Implications for Commercial Abalone Production and Future Research

In nature, macrophyte development tends to be N-limited (Lignell and Pedersén 1987, Duke et al. 1989a); therefore, protein-N availability is suggested as being the main factor limiting field abalone growth (Fleming 1995b). The protein component of artificial diets represents the most costly ingredient of feeds (Mai et al. 1995) that are often prohibitively expensive (Fleming and Hone 1996). The culture of macrophytes in ammonia-enriched seawater, either by chemical supplement or by mariculture effluents, seems to be a logical procedure for removing N-limitation from the food chain. Our results show that enrichment of N content in *U. lactuca* in this way significantly improves all indices of growth and feed use in juvenile and adult abalone.

The present study suggests a considerable scope for modification of the nutritional value of the algal species commonly used as fresh diets for abalone and also a need for caution when considering interspecific differences between seaweeds without examining intraspecific variations in composition. Seasonal variability of wild seaweed populations with respect to protein content is proposed as critical for farmers harvesting natural stocks, in the selection of optimum sites for stock enhancement projects, and as an ecological tool to help elucidate the factors governing the food selection and population dynamics of abalone. There remains a need to investigate the long-term affects of algae cultured at a range of nutrient enrichment levels. It is also necessary to determine the nutritional requirements of developing abalone, particularly at the critical stages of weaning, rapid juvenile growth, and sexual development in adults.

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## THE UTILITY OF APPARENT DIGESTIBILITY COEFFICIENTS FOR PREDICTING COMPARATIVE DIET GROWTH PERFORMANCE IN JUVENILE GREENLIP ABALONE *HALIOTIS LAEVIGATA*

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**ABSTRACT** The utility of using apparent amino acid and energy digestibility coefficients of individual ingredients to formulate diets for juvenile greenlip abalone, *Haliotis laevigata*, to specified digestible protein and energy levels, and rate of food consumption as a function of body size was studied. Five diets were formulated to have the same total digestible protein and energy contents, based on the apparent amino acid and energy digestibility coefficients of their ingredients. Despite similar nutrient profiles, the diets differed in their level of inclusion and combinations of four ingredients: semolina, soyflour, casein, and fishmeal. Diets were offered at two levels, subsatiation (0.01 g/d/abalone) and excess (0.1 g/d/abalone). Specific growth rates of shell length and wet weight of abalone were not significantly different among diets between feed levels. Thus, it seems that amino acid and energy digestibility coefficients of individual ingredients can be used effectively to formulate diets for abalone to desired levels of digestible protein and energy. The rate of food consumption of an abalone increased allometrically with its size  $dC(t)/dt = a(t)W(t)^b$ , where  $C(t)$  and  $W(t)$  are, respectively, its total food consumption during a certain period of time up to time  $t$ , and its wet body weight at time  $t$ ;  $a(t)$ s and  $b$  are its allometric parameters to be estimated. If  $a(t)$ s were assumed to vary with time, 1% increase in body weight required  $(0.5368 \pm 0.1183)\%$  increase in food consumption. If  $a(t)$ s were assumed to be constant over time, 1% increase in body weight required  $(0.6861 \pm 0.1404)\%$  increase in food consumption.

**KEY WORDS:** digestibility coefficients, protein, energy, growth, abalone

### INTRODUCTION

Commercial nutritionists formulate animal diets to maximize production efficiency (Fleming et al. in press) by defining nutritional requirements, assessing the nutritive value of individual ingredients to be used in the compound diets, and formulating least-cost diets. The current dataset for Australian feed manufacturers formulating diets for greenlip abalone, *Haliotis laevigata* consists of the following:

- (1) specifications for minerals and vitamins (Uki et al. 1985, Coote et al. 1996);
- (2) lipid inclusion level (van Barneveld et al. in press);
- (3) soft body tissue amino acid profile and requirement for the assumed limiting amino acid-lysine (Coote 1997);
- (4) apparent digestible protein and energy values for ingredients commonly available in Australia (Coote 1997); and
- (5) optimal digestible protein level and optimal digestible protein:digestible energy ratio (Coote 1997).

To formulate the diets to match the available information on ingredient specifications with the nutritional requirements for juvenile greenlip abalone on a least-cost basis, feed manufacturers must be confident that altering ingredient levels while maintaining nutritional specifications will not affect the animals' growth. Fleming et al. (in press) found for the greenlip abalone, no significant difference in the digestibility of diets that vary in fiber but contain equal amounts of digestible protein and energy level, based on the individual digestibility coefficients of the component ingredients. No data are available, however, on the additive effects of various ingredients on growth of the greenlip abalone. Growth experiments

are essential for determining whether there are interactions among nutrients within a diet that can influence the proportion of nutrients retained by an animal and used for its growth.

In evaluating diets, it is equally essential that both food consumption and growth rates of experimental abalone be measured to determine whether or not there is diet preference. This is because a higher rate of growth of abalone on one diet may be attributable to its higher rate of consumption but not to its better nutritional quality. The rate of food consumption is, however, a function of the size of an abalone. Unfortunately, the rate of food consumption of individual abalone as a function of their size is rarely calculated, because food consumption is usually observed for a group of abalone (mainly because of experimental constraints) rather than for each individual, and because of a lack of appropriate models for analyzing the resulting data. In this study, we examine the utility of using the apparent digestibility coefficients of ingredient nutrients in formulating diets for the greenlip abalone and propose a method for calculating the rate of food consumption of individual abalone as a function of their size.

### MATERIALS AND METHODS

#### *Abalone*

Hatchery-derived juvenile (12-month-old) greenlip abalone, *Haliotis laevigata*, were used in this experiment. When the experiment started, the mean size and weight of abalone were  $20.43 \pm 0.22$  mm and  $1.17 \pm 0.04$  g, respectively. All abalone were anaesthetised with a 4% stock solution of benzocaine (Ace Chemicals, Camden Park, South Australia) (Hahn 1989), weighed to the ac-

curacy of 0.001 g in wet weight, and measured to the accuracy of 0.1 mm in total shell length. Before being assigned to tanks, each animal was tagged following Coote et al. (1996).

#### Diet Formulation

Five isonitrogenous and isoenergetic diets of different combinations of semolina, Peruvian fishmeal, casein, and defatted soyflour (Baker's Nutrisoy; Ace Chemicals) were formulated, based on previously determined apparent digestible amino acid and energy coefficients of the ingredients for *H. laevigata* (Coote 1997) (Table 1). An "ideal" protein ratio was used in these formulations based on the amino acid profile of the soft body tissues of the abalone (Fleming et al. 1996, Coote 1987). This required inclusion of the free amino acids L-arginine monohydrochloride, L-threonine, L-lysine monohydrochloride, and DL-methionine (Ace Chemicals). Identical amounts of vitamins E (DL-alpha tocopheryl acetate) and C (ascorbic acid), sodium alginate (binder), abalone vitamin and mineral premixes, and phosphate were added to each diet. Although it was not possible to have identical levels of supplemented oil (Jack Mackerel Oil, Triabunna Fish Oils, Triabunna, Tasmania) within all diets (to make the diets isoenergetic), its levels of inclusion varied by  $\leq 10$  g between any two diets. Diet

1 served as a control and contained only semolina and soyflour. All diets were fed to subsatiation or to excess, giving a total of 10 treatments (5 diets  $\times$  2 feeding levels). Each treatment had four replicates (40 tanks in total). All diets were stored at  $-30^{\circ}\text{C}$  before feeding.

#### Experimental Conditions

Four replicates of each diet to be tested were randomly assigned to an 8-L plastic tank of the dimensions  $18 \times 21 \times 31$  cm. Each tank contained 10 abalone randomly chosen from a group of 480 and had a piece of PVC stormwater pipe as a hide. Tanks were on a flow through water system. The seawater was filtered to  $30 \mu\text{m}$  by primary sand filters and then to  $10 \mu\text{m}$  by secondary composite sand filters. Temperature was maintained at  $18.0^{\circ}\text{C}$ , measured on day 29 of the 45-day experimental period, and ranged from  $17.5$  to  $20.0^{\circ}\text{C}$ , except for 2 days when it dropped to  $15.0^{\circ}\text{C}$  because of a system failure. Salinity was  $36.5\text{‰}$  throughout the experiment. The flow rate was maintained at  $300\text{--}500$  mL/min, which gave a water exchange rate of 15 min. Aeration was provided at  $300\text{--}600$  mL/min. All tanks were housed behind a black polyethylene sheet in a room with a 12-h light: 12-h dark regime (lights turned off at 19:00). Abalone were kept in darkness to minimize disturbance and stop bacterial growth. Experimental conditions are summarized in Table 2.

#### Determination of Feeding Levels

Animals were preconditioned for 21 days on the test diet to determine their subsatiation and excess feeding levels. For a water temperature of  $18^{\circ}\text{C}$ , the recommended daily feeding rate is  $1.9\text{--}3.1\%$  of animal wet weight (Hahn 1989). We used this as a guide to set the subsatiation and excess feeding levels. Initially, animals subject to subsatiation treatment were fed  $75\%$  of  $2\%$  of their body weight per day. This corresponded to each tank of abalone being fed  $0.15$  g/d, with an assumed 10 abalone per tank and an assumed average abalone weight of 1 g. Specifically, abalone in each tank were fed  $0.3$  g on Mondays,  $0.3$  g on Wednesdays, and  $0.45$  g on Fridays. Animals in the "fed-to-excess" treatment were fed  $2\%$  of their body weight per day. Thus, animals in each tank were fed  $0.2$  g/d; that is,  $0.4$  g on Mondays,  $0.4$  g on Wednesdays, and  $0.6$  g on Fridays. The amount of diet given to the abalone in the subsatiation treatment was gradually lowered to a level at which they consumed all the given feed (i.e., no feed was left in any of

TABLE 1.  
Ingredient composition (% inclusion on an air dry weight basis) and proximate components of diets.

Ingredients	Diets				
	1	2	3	4	5
Semolina	69.5	75.4	68.6	74.9	71.5
Fishmeal	0	0	10	5	10
Casein	0	10	0	10	5
Soyflour	23.6	7.6	15.6	3.6	7.6
Na alginate	0.5	0.5	0.5	0.5	0.5
Mineral mix <sup>a</sup>	0.3	0.3	0.3	0.3	0.3
Vitamin mix <sup>a</sup>	0.2	0.2	0.2	0.2	0.2
Vitamin E	0.01	0.01	0.01	0.01	0.01
Vitamin C	0.05	0.05	0.05	0.05	0.05
Na phosphate	0.5	0.5	0.5	0.5	0.5
Jack Mackerel oil	4.00	3.85	3.00	3.34	2.92
L-arginine HCL	0.52	0.74	0.55	0.76	0.664
L-threonine	0.18	0.16	0.16	0.15	0.15
L-lysine HCL	0.09	0.09	0.02	0.06	0.025
DL-methionine	0.11	0.09	0.06	0.06	0.05

#### Proximate Analysis

CP%	20.99	22.91	23.02	23.84	23.66
GE MJ kg <sup>-1</sup>	17.8	18.0	17.7	18.1	18.0
DP%	17.15	17.15	17.15	17.15	17.15
DE MJ kg <sup>-1</sup>	10.75	10.75	10.75	10.75	10.75
NDF%	8.88	6.39	11.07	5.91	10.86
ADF%	3.13	2.20	3.32	2.99	2.71
CF%	1.91	1.79	1.53	1.00	0.96
ASH%	3.04	2.22	4.04	3.13	3.56
DM%	93.03	92.65	93.03	93.34	93.81
Crude Fat%	4.74	4.49	4.95	4.54	4.91

CP, crude protein; GE, gross energy; DP, apparent digestible protein; DE, apparent digestible energy; NDF, neutral detergent fiber; ADF, acid detergent fibre; CF, crude fiber; DM, dry matter.

<sup>a</sup> Vitamin and mineral mix (Uki et al. 1985).

TABLE 2.  
Experimental conditions.

Temperature	$18^{\circ}\text{C}$
Salinity	$36.5\text{‰}$
Flowrate	$300\text{--}500$ mL/min
Seawater	Filtered to $5 \mu\text{m}$ nominal
Aeration	$300\text{--}600$ mL/min
Tank volume	8 L
Light regime	12 h L:12 hr D
Feeding levels	Subsatiation, $0.1$ g/d/tank Excess, $1.0$ g/d/tank
Feeding and cleaning times	Tanks were cleaned between 09:00 and 10:00, and abalone were fed between 16:00 and 17:00 every Monday, Wednesday, and Friday.

the subsatiation tanks on the next day when test tanks were cleaned, and abalone were fed). In the fed-to-excess treatment, the amount of diet given was altered until leftover feed was observed in all fed-to-excess tanks on the next day, when test tanks were cleaned, and abalone were fed. The final feeding amounts were estimated at 0.1 g/d for abalone fed to subsatiation and 1.0 g/d for those fed to excess.

The subsatiation feeding level was examined to ensure that abalone fed each of the different diets had consumed the same amount of feed. This meant that any changes in growth rates could be attributed to the interaction between their ingredients but not to differences in consumption. The excess feeding level was tested to determine the maximum growth rates attainable on each of the diets and a possible preference of abalone for one diet and, hence, its greater consumption than others, given unrestricted amount of feed. Such determinations are important, because, regardless of whether or not there are any interactions among ingredients, certain of their combinations should not be used in a diet, because they would result in lower consumption and, consequently, lower growth rates of the abalone. Tanks were cleaned by siphoning between 09:00 and 10:00, and abalone were fed between 16:00 and 17:00 during both the preconditioning and experimental periods.

**Determination of Feed Consumption**

Feed consumption was determined in each of 24 fed-to-excess tanks by siphoning out the remaining feed onto labeled, pre-weighed filter papers (Whatman qualitative size 1). Siphoning was done six times randomly during the experimental period via a vacuum filtration system consisting of a Buchner flask and an electric pump. The filter papers were dried to a constant weight in an oven at temperature 55.0°C and then reweighed. To correct for feed loss from leaching, the procedure above was repeated by placing feed in tanks without abalone. The weight loss was then corrected. The four replicate measurements of the consumption of each diet were averaged for each of the 6 days of measurements (Peterson and Renaud 1989). The resulting six values for each diet were then averaged to give an average consumption rate of (mg/d/abalone). Feed conversion ratios (i.e., feed intake divided by the average wet weight gain) per abalone in the 45-day experimental period were calculated for each diet, using the wet weight gain for each tank and the calculated daily feed consumption rates.

Food consumption of an individual abalone as a function of its size is calculated from that of a group of individuals, by assuming that the amount of food consumed by individual *i* is scaled allometrically with its size (Peters 1983), so that

$$\frac{dC_i(t)}{dt} = f(t)a(t)L_i(t)^b$$

where  $L_i(t)$ ,  $f(t)$ , and  $C_i(t)$  are, respectively, its size at time *t*, its feeding activity function at time *t*, and its total food consumption during a certain period of time up to time *t*;  $a(t)s$  and  $b$  are its allometric parameters to be estimated. The feeding activity function  $f(t)$  specifies its feeding patterns. Although more realistic functional forms of  $f(t)$  can be considered, we assume here that  $f(t) = 1$  if that animal feeds at time *t*;  $f(t) = 0$  otherwise, because of lack of data. The greenlip abalone have been observed to feed for approximately 10 hours every day from 20:00 to 06:00 ( $f(t) = 1$ ) and be relatively sedentary during other hours ( $f(t) = 0$ ) (unpublished data, Corrales et al.). Parameters  $a(t)s$  are a function of time *t*, because an abalone may have a small meal after a big meal.

The size of individual *i*  $L_i(t)$  at time *t* is assumed to satisfy

$$\frac{dL_i(t)}{dt} = G_i L_i(t)$$

solution of which as an initial value problem with  $L_i(t)|_{t=t_0} = L_i(t_0)$  yields

$$L_i(t) = L_i(t_0)e^{G_i(t-t_0)}$$

where  $G_i$  is the (constant) specific growth rate of that individual, and  $L_i(t_0)$  is its size at the start of the experiment at time  $t_0$ . Now,  $G_i$  can be calculated from any two measurements of its size, say,  $L_i(t_0)$  and its size at the end of the experiment  $L_i(t_f)$  at time  $t_f$  as

$$G_i = \frac{1}{t_f - t_0} \log_e \frac{L_i(t_f)}{L_i(t_0)}$$

Because only the total food consumption of a group of 9 to 10 abalone in a single tank was measured

$$\begin{aligned} \frac{dC(t)}{dt} &= \sum_{i=1}^n \frac{dC_i(t)}{dt} \\ &= \sum_{i=1}^n f(t)a(t)L_i(t)^b \\ &= \sum_{i=1}^n f(t)a(t)[L_i(t_0)e^{G_i(t-t_0)}]^b \\ &= \sum_{i=1}^n f(t)a(t)[L_i(t_0)]^b e^{bG_i(t-t_0)} \end{aligned}$$

where  $n$  is the number of abalone in a tank that were alive and contributed to the total food consumption  $C(t)$  at time *t*. Of course, this equation gives the instantaneous rate of total food consumption. However, any feeding experiment can only be effected during a certain period of time. To estimate  $a(t)s$  and  $b$  from data from such an experiment, including ours, we must integrate  $dC(t)$  over a biologically meaningful period of time to yield

$$\begin{aligned} \Gamma(\tau_i, \tau_{i+1}) &= \int_{\tau_i}^{\tau_{i+1}} dC(s)ds \\ &= \int_{\tau_i}^{\tau_{i+1}} \sum_{i=1}^n f(s)a(s)[L_i(t_0)]^b e^{bG_i(s-t_0)} ds \\ &= \sum_{i=1}^n a(\tau_i)[L_i(t_0)]^b \frac{1}{bG_i} e^{bG_i(\tau_{i+1}-t_0)} [e^{bG_i(\tau_i-t_0)} - 1] \end{aligned}$$

where  $\Gamma(\tau_i, \tau_{i+1})$  is the total food consumption of all  $n$  abalone in a tank during the *i*th observational period  $[\tau_i, \tau_{i+1}]$ , and we have assumed that  $a(t)s$  remain constant during that observational period. Six measurements of  $\Gamma(\tau_i, \tau_{i+1})$  were made in the course of our experiment. The size of all test abalone was, however, measured only at the start and end of the experiment. Fortunately, the values of  $L_i(t)$  can be calculated (see above) as

$$L_i(t) = L_i(t_0)e^{G_i(t-t_0)} = L_i(t_0) \left( \frac{L_i(t_f)}{L_i(t_0)} \right)^{\frac{t-t_0}{t_f-t_0}}$$

where  $L_i(t_0)$  is the size of individual *i* at the start of the experiment at time  $t_0 = 0$  d;  $L_i(t_f)$  that at the end of the experiment at time  $t_f = 45$  d. Both  $L_i(t_0)$  and  $L_i(t_f)$  were calculated very precisely, and their errors are negligible. Now, parameters  $a(t)s$  and  $b$  can be

estimated from  $\Gamma(\tau_p, \tau_{t+1})$  and  $L_t(t)$  for abalone from different tanks, by making appropriate assumptions about the distributions of the errors in  $\Gamma(\tau_p, \tau_{t+1})$ . We assumed that the errors in  $\Gamma(\tau_p, \tau_{t+1})$  follow independent normal distributions, with a mean of  $\hat{\Gamma}(\tau_p, \tau_{t+1})$  and a constant variance of  $\sigma^2$ , and estimated all parameters by the standard nonlinear least squares method (SAS, Inc. 1988). The parameters thus estimated are those for shell length. A simple replacement of shell length of an abalone by its body mass yielded estimates of  $a(t)s$  and  $b$  for body mass.

**Measurements**

Both shell length (SL) and wet body weight (BW) were measured at the start and end of the experiment to estimate their specific growth rates. Shell length was measured to the nearest 0.1 mm using an electronic digital caliper, and wet body weight was measured to the nearest 0.001 g. Excess water was dried off the abalone using a paper towel before weighing. The experiment lasted 45 days. The specific growth rate (SGR) was calculated as

$$SGR.100\% = \frac{\ln G(f) - \ln G(i)}{\Delta t}$$

where  $G(i)$  is the SL (mm) or BW (g) at the start of the experiment,  $G(f)$  is the SL (mm) or BW (g) at its end, and  $\Delta t$  is the experimental duration (d) (see above).

**Chemical Analyses**

Proximate analyses of the diets (i.e., dry matter, crude protein, crude, neutral detergent and acid detergent fiber, ash and crude fat) were undertaken using the methods of the Association of Official Analytical Chemists (1984). Gross energy was determined using a Parr 1,281 bomb calorimeter.

**Statistical Analyses**

Analysis of variance (ANOVA) (SAS Institute 1988) was used to determine whether there are differences in mean size of abalone among tanks at the start of the experiment, in mean SGRs among diets for both BW and SL and in mean feed consumption.

**RESULTS**

No significant difference was found in mean length of abalone among tanks at the start of the experiment ( $F_{39,361} = 1.16, p = .2436, n = 400$ ). Because of a failure of the water circulating system, one tank of abalone fed diet 1 to excess died. Also dead were one abalone fed diet 1 to subsatiation, one fed diet 3 to subsatiation, one fed diet 5 to excess, two fed diet diet 4 to subsatiation, and three fed diet 2 to excess.

Mean and standard errors of SGR for both SL and BW are given in Table 3 and Figure 1. No significant differences occurred among the five diets for either SL ( $F_{4,364} = 0.78, p = .5371$ ) or BW ( $F_{4,364} = 0.42, p = .7934$ ). In contrast, significant differences existed between feeding levels for both SL ( $F_{1,364} = 112.02, p = .0001$ ) and BW ( $F_{1,364} = 144.14, p = .0001$ ). The abalone fed to excess had achieved much greater SGRs of both SL and BW than those fed to subsatiation for the same diets (Fig. 1). Significant diet by feeding level interactions were found for BW ( $F_{4,364} = 3.11, p = .0156$ ) but not for SL ( $F_{4,364} = 1.85, p = .1184$ ).

No significant differences were detected in feed consumption among the five diets for any of the 6 days of measurement (day 1,  $F_{4,14} = 1.37, p = .2929$ ; day 2,  $F_{4,14} = 2.76, p = .0699$ ; day 3,  $F_{4,14} = 0.84, p = .5197$ ; day 4,  $F_{4,14} = 1.60, p = .2289$ ; day 5,  $F_{4,14} = 1.13, p = 1.13, p = -.3822$ ; day 6,  $F_{4,14} = 1.89, p =$

TABLE 3.

Mean and standard error (in parentheses) of the specific growth rates (SGR) of shell length and body weight of abalone for both feeding levels for each diet (A, fed to subsatiation; B, fed to excess).

Diet	Feeding Level	N	SGR of shell Length (d <sup>-1</sup> )	SGR of Body Weight (d <sup>-1</sup> )
1	A	4	0.40 (0.04)	0.89 (0.10)
	B	3	0.61 (0.03)	1.54 (0.07)
2	A	4	0.43 (0.04)	1.02 (0.12)
	B	4	0.60 (0.04)	1.52 (0.12)
3	A	4	0.42 (0.04)	0.98 (0.06)
	B	4	0.56 (0.02)	1.40 (0.05)
4	A	4	0.42 (0.05)	1.03 (0.13)
	B	4	0.57 (0.01)	1.40 (0.07)
5	A	4	0.44 (0.07)	1.10 (0.16)
	B	4	0.53 (0.02)	1.34 (0.04)

.1682). The mean consumption rates varied from 0.024 g/d/abalone for diets 1 and 3 to 0.027 g/d/abalone for diet 5 (Table 4). In subsatiation treatments, abalone ate all the offered feed, approximately 0.012 g/d/abalone. In comparison, those fed to excess ate approximately 0.025 g/d/abalone. The feed conversion ratio (FCR) ranged from 0.97 for diet 4 to 1.17 for diet 1 in the subsatiation treatments, and 1.04 for diet 4 to 1.28 for diet 2 in the excess treatments (Table 4). For diets 2, 4, and 5, the FCRs for the subsatiation treatments were all lower than those for the excess treatments for the same diet.

Fitting of the above model to the abalone data by the standard nonlinear least-squares method, assuming that the errors in  $\Gamma(\tau_p, \tau_{t+1})$  follow independent normal distributions, with a mean of  $\hat{\Gamma}(\tau_p, \tau_{t+1})$  and a constant variance of  $\sigma^2$ , yielded the amount of food consumption by individual abalone as a function of their size (Table 5). A likelihood ratio test suggests that the model for constant  $a(t)s$  is significantly different from that for time-varying  $a(t)s$  for either shell length ( $F_{5,107} = 17.0734, p < .0001$ ) or wet body weight ( $F_{5,107} = 17.9178, p = .0171$ ). If  $a(t)s$  were assumed to vary with time, 1% increase in shell length required  $(1.5148 \pm 0.3540)\%$  increase in food consumption. The variation of allometric parameters  $a(t)s$  with time did not have a clear pattern and had very large estimates of standard errors (Table 5). If  $a(t)s$  were assumed to be constant over time, 1% increase in shell length required  $(2.0103 \pm 0.4041)\%$  increase in food consumption. In general, however, the large estimates of standard errors for shell length-based allometric parameters render predictions of food consumption from shell lengths of abalone imprecise. Such a difficulty is partly attributable to the known difficulty and unreliability in choosing an appropriate linear measurement (dimension) for prediction of a three-dimensional (3-D) quantity.

In contrast, reliable predictions can be made of the food consumption of an abalone as a function of its size in body (wet) weight. If  $a(t)s$  were assumed to vary with time, 1% increase in body weight required  $(0.5368 \pm 0.1183)\%$  increase in food consumption. Again, the change of allometric parameters  $a(t)s$  with time did not seem to have a clear pattern but had relatively small estimates of standard errors (Table 5). If  $a(t)s$  were assumed to be constant over time, 1% increase in body weight required  $(0.6861 \pm 0.1404)\%$  increase in food consumption. Thus, models for both constant and time-varying  $a(t)s$  are adequate for describing the data on abalone food consumption and are useful for manipulating levels of feed for aquacultured abalone. Selection between them is



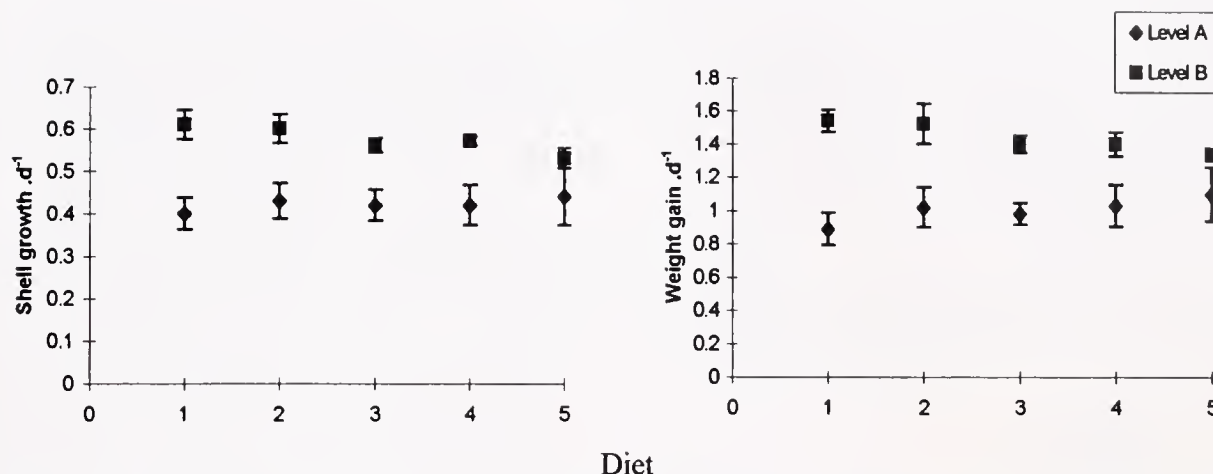


Figure 1. Specific growth rates of shell length and body weight of experimental abalone. Level A, subsatiation (0.1 g/d); level B (1.0 g/day); n = 4.

difficult, because of a lack of a biologically meaningful interpretation of time-varying  $a(t)s$ .

#### DISCUSSION

Diets with different ingredients or ingredient combinations, but formulated to be both isonitrogenous and isoenergetic, based on the apparent digestibility coefficients of nutrients in the ingredients, should yield equal growth rates. In this instance, differences in growth rate of test animals would suggest that ingredient components interact to influence their utilization and that the apparent digestibility coefficients are not additive and, hence, are unsuitable for use in diet formulation. The digestibility coefficients of individual ingredients seem to be additive, and the nutrients from each diet are equally utilized by abalone. This is because similar SGRs were found in abalone fed diets consisting of different ingredients but formulated to have the same levels of digestible protein and energy. Thus, ingredient apparent digestibility coefficients can be used effectively for formulating diets to specific digestible energy and protein levels.

Preference of abalone for the diets containing fishmeal was not observed in this study. This is not surprising, considering that abalone are herbivorous and the levels of fishmeal in the three

diets were probably too low (a maximum of 10%) for abalone to show significant preferential selection. By comparison, rainbow trout, *Oncorhynchus mykiss*, fed diets containing 100% replacement of fishmeal by plant protein, had a significantly reduced weight gain and specific growth rate, although the diets had the same amount of digestible protein and energy (Gomes et al. 1995). This was attributable to a significant reduction in intake of the 100% plant protein diet, as compared with fishmeal-based diets. Such a reduction of voluntary feed intake in plant meal-based diets by rainbow trout was also observed by Gomes and Kaushik (1992), who suggested that this was probably because of their carnivorous nature and hence not being adapted for utilizing plant ingredients in diets.

The lower FCR of diets 2, 4, and 5 for abalone fed to subsatiation than those fed to excess is readily explained. The growth-rate relationship is characterized by an initial increase in growth rate with ration, followed by a leveling off. In general, the optimal ration is attained when fish are fed at restricted ration levels (Talbot 1994). When feed intakes are reduced below the maintenance level, animals, such as limpets (Branch 1992), tend to become more efficient in digesting feed and utilizing its nutrients (Maynard et al. 1969). Similarly, species with an abundant food supply generally have a lower absorption efficiency than those with a

TABLE 4.

Mean and standard error (in parentheses) of wet body weights at the start (initial) and end (final) of the experiment, total weight gain, feed consumption, and feed conversion ratios (FCR) for both feeding levels of each diet. (A, subsatiation; B, excess).

Diet	Feeding level	Mean Initial Weight (g)	Mean Final Weight (g)	Mean Total Weight Gain (g)	Mean Feed Consumption (g/d <sup>-1</sup> /abalone <sup>-1</sup> )	FCR
1	A	1.10 (0.13)	1.56 (0.10)	0.46 (0.03)	0.012	1.17
	B	1.16 (0.07)	2.14 (0.15)	0.98 (0.09)	0.024	1.08
2	A	1.00 (0.10)	1.53 (0.09)	0.53 (0.06)	0.012	1.01
	B	1.05 (0.15)	2.00 (0.14)	0.94 (0.04)	0.027	1.28
3	A	0.94 (0.09)	1.43 (0.99)	0.48 (0.01)	0.012	1.10
	B	1.19 (0.12)	2.15 (0.21)	0.97 (0.09)	0.024	1.10
4	A	1.09 (0.13)	1.66 (0.15)	0.57 (0.04)	0.012	0.97
	B	1.38 (0.14)	2.46 (0.14)	1.08 (0.04)	0.025	1.04
5	A	0.93 (0.13)	1.46 (0.15)	0.53 (0.06)	0.012	1.02
	B	1.50 (0.16)	2.61 (0.25)	1.11 (0.09)	0.027	1.08

TABLE 5.

Estimates of the mean and standard errors (in parentheses) of parameters in models for food consumption as a function of an individual's size by the nonlinear least squares method.

<i>i</i>	Independent Variable	$\tau_i$ (d)	$a(t) \times 10^{-3}$	<i>b</i>	F	R <sup>2</sup>
	Length (mm)					
	Constant $a(t)$		1.0450 (1.3600)	2.0103 (0.4041)	$F_{2,112} = 518.7876$	0.9026
1	Variable $a(t)$	7	3.0866 (3.4626)	1.5148 (0.3540)	$F_{7,107} = 266.7813$	0.9458
2		9	3.9809 (4.4720)			
3		11	5.9518 (6.7825)			
4		21	6.0085 (6.8684)			
5		23	5.0358 (5.7764)			
6		25	1.5148 (0.3540)			
	Weight (mg)					
	Constant $a(t)$		455.5007 (39.0080)	0.6861 (0.1404)	$F_{2,112} = 514.2863$	0.9018
1	Variable $a(t)$	7	606.1055 (41.0125)	0.5368 (0.1183)	$F_{7,107} = 270.7141$	0.9466
2		9	295.5456 (33.2396)			
3		11	381.7449 (35.4795)			
4		21	576.1663 (47.1054)			
5		23	582.8623 (48.6964)			
6		25	489.0853 (44.3772)			

$p < .0001$ ,  $n = 114$ .

The unit of constant or variable  $a(t)$  is  $g/mm^b/d$  for length-dependent food consumption, and  $g/g^b/d$  for weight-dependent food consumption.

more limited food supply (Branch 1982). These observations may reflect more metabolism of animals than digestibility alone (Maynard et al. 1969). For species with an abundant supply of food, energy conservation by reducing metabolic losses may not be necessary or even desirable; whereas, for species suffering from a shortage of food, metabolic adjustments that reduce energy losses may be critical (Newell and Branch 1980).

In this work, we have proposed a novel method for calculating the rate of food consumption of an individual abalone as a function of its size and have demonstrated its utility in data analysis. The relationship thus established between food consumption and abalone size allows abalone farmers to adjust feeding levels accordingly. Indeed, feeding the "correct" amount of food on a commercial farm is economically important. This is because overfeeding of abalone results in food wastage and increases cost; whereas, underfeeding leads to their slower growth and increases time to reach the market size, which in turn increases production cost.

In summary, the growth of abalone growth is unaffected by varying ingredient combinations of their diet, provided that the diets have the same DP:DE ratio. Thus, the apparent digestible protein and energy coefficients are sufficient to define the nutritional quality of their ingredients and can be used effectively in formulating diets. Future experiments should, therefore, determine

the amino acid and energy digestibility of alternate, cheaper ingredients. These could then be substituted into existing diet formulations, provided that the DP:DE ratio is kept constant, equal growth rates are achieved, and the cost of the diet is reduced. These ingredients would, however, have to be assessed for their maximum inclusion levels, because they may contain antinutritional factors, which, at above certain levels within a diet, are detrimental to the growth of the abalone. In addition, future experiments for greenlip abalone should examine how food consumption changes with temperatures that are experienced on commercial farms and construct a temperature- and size-dependent feeding table. This would assist abalone farmers in seasonally adjusting their feeding protocols.

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## GROWTH AND SURVIVAL OF POSTLARVAL ABALONE (*HALIOTIS IRIS*) IN RELATION TO DEVELOPMENT AND DIATOM DIET

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**ABSTRACT** Postlarval abalone (*Haliotis iris*) were reared on five unialgal diatom diets from 3 to 68 days postsettlement. Diatom strain affected both survival and growth, which were positively correlated ( $r = 0.88$ ,  $p = 0.05$ ,  $n = 5$ ). The digestibility and ingestibility of diatoms were both important. Survival ranged from low (<10% on day 37 postsettlement) on *Pleurosigma* sp. diet, to high (>70% on day 68) on *Cocconeis scutellum*, *Cylindrotheca closterium*, and *Navicula ramosissima* diets. Diet had little effect on growth and survival in the first 16 days after settlement, provided postlarvae were ingesting adequate food. Growth rates during the week to day 10 were relatively high (means of 20–29  $\mu\text{m}$  shell length per day). Growth rates from days 10 to 16 were lower than in the first week ( $t = 7.33$ ,  $p < 0.001$ ) and again similar among all diets (means 15–20  $\mu\text{m}/\text{day}$ ) except *Pleurosigma* sp. (2  $\mu\text{m}/\text{day}$ ), which was not ingested by larvae <1 mm shell length. After day 17, postlarvae grew fastest on the strains that were most efficiently digested (*C. scutellum* and *C. closterium*). The digestion efficiency of two diatom strains increased markedly during the experiment, because of changes in diatom condition. Postlarvae were feeding on small diatoms (12  $\times$  4  $\times$  3  $\mu\text{m}$ ) by day 2 postsettlement, and digestive gland development became visible on day 3. Fecal volume increased approximately cubically in relation to shell length, indicating rapidly increasing food consumption.

**KEY WORDS:** benthic diatom, abalone development, growth, survival, postlarval abalone, digestion efficiency

### INTRODUCTION

Even after decades of abalone farming, consistent and efficient postlarval culture remains elusive (Leighton 1989, Roberts et al. 1998). Survival rates through the few months postsettlement are generally low and variable (Searcy-Bernal et al. 1992). Many hatchery operators report mortality peaks 3 to 8 weeks after settlement (Leighton 1989, Roberts et al. 1998). These events are often thought to be food related (Roberts et al. 1998), but the precise mechanisms involved are unknown. The timing of mortality events suggests that the cause may lie in postlarval developments or changes in the biofilm on which postlarvae feed.

Postlarvae in hatcheries feed on a biofilm that is usually dominated by diatoms and their extracellular products. The composition of the biofilm can affect growth and survival of postlarvae. One critical factor is the efficiency with which diatoms are digested. Many diatom strains pass through the abalone gut alive and intact, but efficiently digested strains can produce better growth and survival of postlarvae (Kawamura et al. 1998b). The “digestion efficiency” of a diatom strain has been defined as the proportion of live diatoms that are ruptured during ingestion/digestion (Kawamura et al. 1995). Digestion efficiency has previously been assessed at only one time in an experiment (Kawamura and Takami 1995, Kawamura et al. 1995, Kawamura et al. 1998a). However, digestion efficiency may vary with the condition of the algal culture, or as postlarvae develop.

Another important feature controlling the food value of diatoms is the ease with which they are removed from the substrate and swallowed (their “ingestibility”). Postlarval abalone grazing favors certain diatom species (Norman-Boudreau et al. 1986, Matthews and Cook 1995). Passive selection can arise through such diatom characteristics as cell size, stalk length, or attachment strength

(Matthews and Cook 1995, Kawamura et al. 1995, Kawamura et al. 1998a). Few such examples have been quantified or related to postlarval abalone development.

Most previous studies on the growth of postlarval abalone on specific diatom diets have covered only a few weeks of the postlarval period (Ohgai et al. 1991, Ishida et al. 1995, Kawamura and Takami 1995, Kawamura et al. 1995, Kawamura et al. 1998a). This has limited the ability of those studies to demonstrate changes in growth rates related to postlarval development. For example, previous data suggest that a diatom diet made little difference to the growth rate of small abalone postlarvae (Kawamura and Takami 1995); whereas, larger postlarvae (>800  $\mu\text{m}$  shell length [SL]) grew more quickly on diatoms with high digestion efficiency (Kawamura et al. 1995, Kawamura et al. 1998a). This suggestion was based on separate short-term experiments with different ages of postlarvae. Studies covering the whole postlarval period would be more conclusive and valuable.

Postlarval developments that affect growth and survival are likely to involve the feeding or digestive apparatus. Information about the onset of diatom feeding in postlarval abalone is variable. Seki and Kan-no (1981) reported that postlarval *Haliotis discus hamai* Ino 1953 in hatcheries consume diatoms within 2 days of settlement. In similar conditions, Norman-Boudreau et al. (1986) found that feeding on diatoms was initiated 2 to 6 days after settlement. In contrast, Kitting and Morse (1997) reported that postlarval *H. rufescens* Swainson 1822 on coralline algae did not ingest cellular solids in the first 10 days after settlement, although postlarvae did show feeding movements. This issue is important for hatchery management, because it will affect decisions about the type of food suitable for very young postlarvae and the time at which it should be added.

A related issue is the need to balance food supply with the increasing food consumption of growing postlarvae. Abalone farmers report difficulty in re-establishing suitable diatom films in areas cleared by postlarval grazing (Roberts et al. 1998). A better knowledge of food consumption rates would help in balancing food supply to avoid overgrazing. Despite widespread recognition of this difficulty, there are few quantitative data on abalone grazing rates. Martinez-Ponce and Searcy-Bernal (1998) reported that grazing (measured by diatom clearance) was minimal during the first week but increased abruptly at ~470  $\mu\text{m}$  SL (2–3 weeks postsettlement).

In this paper, we follow the growth and survival of a single batch of abalone throughout the postlarval period on five diatom diets. This lets us examine ways in which postlarval development and diet interact to influence postlarval growth and survival. Diatom diet emerges as a critical factor, although not for very young postlarvae. We show that the food value of some diatoms changes as postlarvae grow or diatom characteristics change.

## MATERIALS AND METHODS

### Abalone Rearing

Adult abalone (*Haliotis iris* Gmelin 1791) were induced to spawn by adding hydrogen peroxide (5 mM final concentration) to alkaline (pH 9.1) seawater (Morse et al. 1977). Larvae were hatched and reared in 1- $\mu\text{m}$  filtered, UV treated, flowing, natural seawater ( $15 \pm 1^\circ\text{C}$ ) at Island Hatcheries Ltd. on Stewart Island, or at the Glenhaven Aquaculture Centre Ltd. in Nelson. Operculate larvae from Stewart Island were transported to Nelson in wet mesh (Tong and Moss 1992) then reared as above. Competent larvae were transferred to tissue culture dishes (Falcon 3046) with 10 mL of 0.2  $\mu\text{m}$  filtered natural seawater (FSW,  $17.5 \pm 1^\circ\text{C}$ , dark) containing 150  $\mu\text{g}/\text{mL}$  each of Penicillin G sodium (Biochemie) and Streptomycin sulphate BP (Sigma). These larvae were induced to attach and metamorphose by the addition of 2  $\mu\text{M}$  GABA (Roberts and Nicholson 1997). Two days after settlement induction, a diatom culture [*Cylindrotheca closterium* (Ehrenberg) Reimann and Lewin 1964] was added as a food supply, and the trays were thereafter incubated in the light ( $35\text{--}70 \mu\text{E}/\text{m}^2/\text{s}^{-1}$  on a 12:12

light/dark cycle) at  $17.5 \pm 1^\circ\text{C}$ . These postlarvae were maintained as a source of experimental animals, by adding supplementary diatom (*C. closterium*) as necessary and replacing the water each 3 to 4 days with new FSW without antibiotics.

### Diatom Cultures

Five strains of benthic diatom were used as diets (Table 1). Four were isolated from Nelson coastal waters, and the fifth (*Pleurosigma* sp.) was obtained from the Tohoku National Fisheries Research Institute in Japan. Cultures were uni-algal but not axenic. Diatoms were grown in tissue culture dishes (Falcon) in the light ( $35\text{--}70 \mu\text{E}/\text{m}^2/\text{s}^{-1}$  on a 12:12 light/dark) at  $17.5 \pm 1^\circ\text{C}$ . Growth medium was Jørgensen's (1962) recipe, supplemented with 0.05  $\mu\text{g}/\text{L}$  vitamin B<sub>12</sub>. For experiments, growth medium was replaced with FSW. Diatom subcultures were established regularly so that postlarvae could be transferred to a new, healthy culture at weekly intervals. Postlarvae were manipulated onto their foot after all transfers. Diatom cell density was quantified at the beginning and end of each week. An excess of food was maintained at all times, except as discussed in Results.

### Growth and Survival of Postlarvae

Three days after settlement induction, postlarvae were dislodged with fine needles and individually pipetted through six FSW washes to remove external diatoms, then placed into diet treatments (three larvae in each of six replicate wells). The *C. closterium* fed to "spare" postlarvae had consistently high digestion efficiency (Fig. 1A), so there was negligible contamination of diets by live diatoms in feces.

We measured SL of individual postlarvae at approximately weekly intervals for 9 weeks. Postlarvae less than ~2 mm SL were measured on an inverted compound microscope video linked to a high-resolution monitor (accuracy  $\pm 10 \mu\text{m}$ ). Larger postlarvae were measured with an eyepiece graticule on a dissecting microscope (accuracy  $\pm 50 \mu\text{m}$ ). Survival was measured as the percentage of postlarvae surviving each week. Postlarvae that died as a result of handling damage, loss, or emersion were excluded from survival calculations. "Specific daily growth rate" was calculated

TABLE 1.  
Details of the diatom cultures used in feeding experiments.

Diatom Species and Authority	Cell Dimensions: Length $\times$ Width $\times$ Depth ( $\mu\text{m}$ ) and [Volume <sup>a</sup> ( $\mu\text{m}^3$ )]	Median Density in Food Cultures (Cells/ $\text{cm}^2$ )	Adhesive Strength <sup>b</sup>	Growth Form <sup>b</sup>	Collection Reference
<i>Cocconeis scutellum</i> Ehrenberg 1838	29 $\times$ 17 $\times$ 2.8 [1,105]	$1.6 \times 10^5$	+++	B	CAWZ F
<i>Cylindrotheca closterium</i> (Ehrenberg) Reimann and Lewin 1964	22 $\times$ 3.5 $\times$ 3.5 [228 <sup>a</sup> ]	$1.1 \times 10^5$	+	A	CAWZ I
<i>Navicula ramosissima</i> (Agardh) Cleve 1895	12 $\times$ 4.1 $\times$ 3.0 [115]	$2.2 \times 10^5$	++	A	CAWZ C
<i>Navicula britannica</i> Hustedt et Aleem 1951	30 $\times$ 8.0 $\times$ 7.6 [1,452]	$1.0 \times 10^5$	++	A	CAWZ M
<i>Pleurosigma</i> sp.	115 $\times$ 16 $\times$ 8.5 [12,193]	$2.0 \times 10^4$	+	A	—

<sup>a</sup> Volumes calculated assuming that all species were ellipsoidal prisms. For *C. closterium*, the thin distal extensions were ignored, and only the swollen section of the cell was used for volume calculations.

<sup>b</sup> According to Kawamura and Hirano (1992). Growth form A are solitary, mobile diatoms of low-to-moderate adhesive strength. Growth form B are highly adhesive, solitary diatoms.

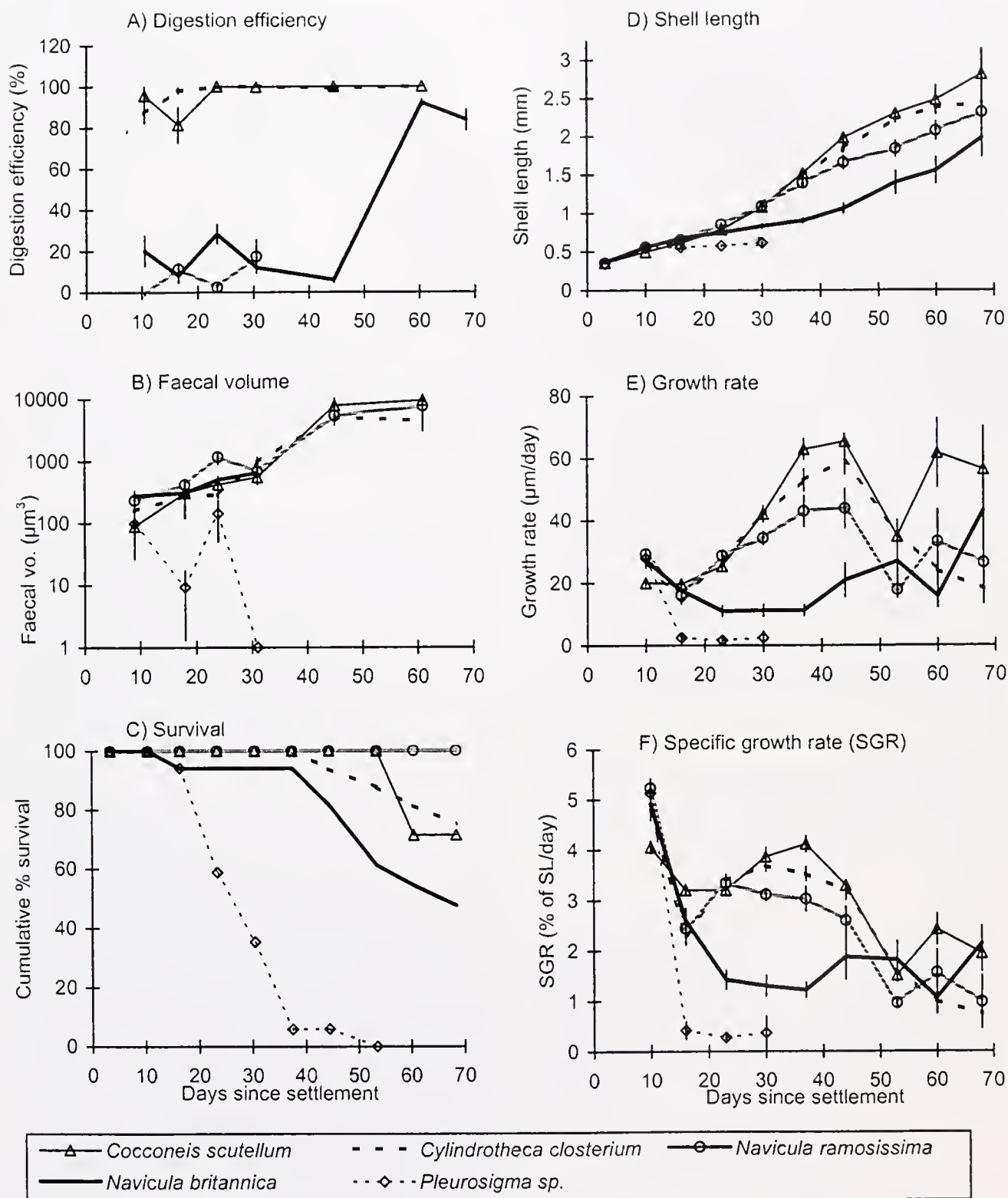


Figure 1. Comparison of diatom digestion efficiency (A) and abalone fecal volumes (B), survival (C), and growth (D-F) for postlarvae fed five diatom diets. Growth rate data (E, F) apply to the period since the last measurement. Other data are instantaneous. Data are mean ± SE; n = 5 for graphs A-B, n = 6 for D-F. Graph C represents survival across all replicates. In graph A, there are no data for *Pleurosigma*, because postlarvae did not ingest cells. In Graph B, the y-axis is logarithmic.

as the percentage of SL grown per day (using final SL for each week).

#### Digestion Efficiency, Fecal Production, and Feeding Observations

In this paper, we use the term "digestion efficiency" to describe the ability of postlarvae to remove the cell contents of diatoms passed through the gut (Kawamura et al. 1995). The term "ingestibility" refers to the ease with which a food item is detached and swallowed by a postlarva. To measure digestion efficiency, individual postlarvae (1 per well,  $n = 5$ ) were removed from the growth experiment, and pipetted through six wells of FSW (to remove external diatoms) before being placed in a clean tissue culture dish with FSW. Recently released fecal material was observed with an inverted microscope, and the proportion of intact versus ruptured diatom cells in the fecal pellet was counted. A parallel count was made of live versus dead cells in the diatom culture from which the fecal material was grazed. Digestion efficiency (%) was calculated as:  $(1 - L/L_0) * 100$ , where  $L_0$  is the proportion of live cells in the source culture, and  $L$  is the proportion of live cells in the fecal pellet.

In most instances, feces were sufficiently translucent that accurate counts of live/ruptured cells could be made, either on intact fecal pellets or after live cells had migrated out. However, when *Navicula ramosissima* developed high digestion efficiency this method was unsatisfactory, because the core of the fecal pellet contained many damaged cells. Such feces were transferred to glass slides by capillary-pipette in a drop of FSW. A cover slip was applied, then gently moved from side to side, spreading the diatoms in the pellet. Checks confirmed that this technique did not damage intact cells.

Fecal volume was quantified by measuring the diameter and length of all fecal pellets released within an hour of the postlarva being removed from its diatom diet. We assumed that feces were cylindrical and that fecal production provided an indication of ingestion rate.

Feeding behavior and gut developments were observed directly with an inverted compound microscope. Observations of feeding in the first few days after settlement used a diet of *Navicula ramosissima* (cells 12- $\mu$ m long and 5- $\mu$ m wide across the valve) added 20 hours postsettlement.

#### Data Analysis

Differences between growth rates were tested using analysis of variance (ANOVA) with Tukey's HSD. Homoscedasticity was assessed using Levene's test, and normality by probability plots of residuals (Kirby 1993). In testing the correlation between survival and growth rate, the growth curve for the *Pleurosigma* sp. diet was extrapolated assuming that postlarval growth in days 30 to 68 continued at the rate observed from days 10 to 30. In fitting the regression curve to fecal volume data, a power relationship was specified.

## RESULTS

#### Early Postlarval Development and Feeding

By 24 hours after the addition of GABA, many larvae had commenced metamorphosis (Table 2). The velum had been shed, and the heart had begun beating. The mouth had opened and the initial processes had fused anteriorly to form the snout. Peristomal shell growth had commenced in some postlarvae, and feeding

TABLE 2.

Timing of the physical changes associated with metamorphosis of *H. iris* larvae.

Developmental Milestone	Age (Days Since Settlement Induction)			
	0.25	1	2	3
Heart beating?	No	Yes	Yes	Yes
Velum shed?	No	Yes	Yes	Yes
Peristomal shell growth?	No	Yes	Yes	Yes
Mouth formed?	No	Yes	Yes	Yes
Feeding movements?	No	Yes <sup>a</sup>	Yes <sup>a</sup>	Yes
Ingestion visible?	No	No	Yes	Yes
Feces visible?	No	No	Yes <sup>b</sup>	Yes
Rotation in gut?	No	Yes	Yes	Yes
Ginger color of digestive gland?	No	No	No	Yes <sup>c</sup>

Larvae were ~160°C days old when induced to settle with 2  $\mu$ M GABA. Data relate to the postlarvae that responded rapidly to the cue. Some larvae showed slower metamorphosis, or failed to metamorphose.

<sup>a</sup> Feeding movements were weak and intermittent in some postlarvae on day 1, but strong and regular on day 2.

<sup>b</sup> Feces contained diatoms (*Navicula ramosissima*) on day 2.

<sup>c</sup> Ginger color of the future digestive gland began to develop on day 3, lateral to the yolk.

movements were visible, although weak and intermittent. No ingestion or feces were observed, but ciliary rotation was visible in the gut between the heart and yolk. By 2 days postsettlement, feeding movements were strong and regular, material was being ingested and rotated in the gut, and fecal pellets were being produced. The feces were dominated by the diatom food supplied (*Navicula ramosissima*). By 3 days postsettlement, the ginger color of the future digestive gland had begun to develop, lateral to the yolk. The ginger color spread throughout the yolk by day 7, progressively obscuring and/or replacing the yolk.

#### Diatom Ingestion/Digestion/Egestion

The five diatom strains showed four different patterns with respect to digestion efficiency and ingestibility. *Cylindrotheca closterium* represented the simplest situation. This diatom was common in fecal pellets by day 9 and had high digestion efficiency throughout the experiment (Fig. 1A). For all other strains, the ingestibility or digestion efficiency changed in relation to postlarval development or diatom culture condition.

*Cocconeis scutellum* was digested very efficiently by all ages of postlarvae (Fig. 1A). However, young postlarvae were inefficient at ingesting *C. scutellum*, and were observed grazing repeatedly and smoothly over the same diatoms without detaching or rupturing the cells. Occasional *C. scutellum* cells were present in some fecal pellets of postlarvae on day 10, but they contributed a very small proportion of the fecal volume. The postlarvae became progressively more efficient at ingesting *C. scutellum* as they grew to ~1 mm SL at day 30 (Fig. 2). The fecal pellets of postlarvae >1 mm SL were densely packed with *C. scutellum* frustules.

Both *Navicula britannica* and *Navicula ramosissima* were common in fecal pellets throughout the experiment. Their digestion efficiency was initially low, but increased during the experiment. The change in digestion efficiency was accompanied by a change in the integrity of fecal pellets. When digestion efficiency was low, live cells moved rapidly out of fecal pellets, and pellets dispersed within ~10 to 30 minutes. When digestion efficiency was



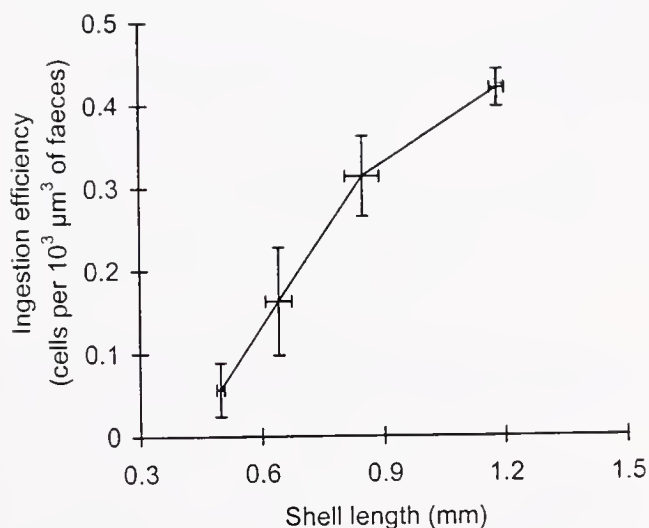


Figure 2. As postlarvae grew, they became more efficient at ingesting *Cocconeis scutellum* cells. Ingestion efficiency was measured as the number of diatom cells per unit volume of feces. Data are mean  $\pm$  SE,  $n = 5$ .

high, the pellets retained their shape. For *N. britannica*, digestion efficiency rose sharply between day 45 and day 61 (Fig. 1A). *Navicula ramosissima* feces showed a similar pattern, with fecal pellets largely dispersing on days 9, 18, 24, and 31 but persisting in subsequent observations. On the latter occasions, accurate digestion efficiency counts were not possible because of the compact nature of the fecal pellets and the small cell size of *N. ramosissima*. A modified technique was developed (see Methods) and used in a follow-up experiment. This showed that young postlarvae (14–16 days postsettlement, 600–700  $\mu\text{m}$  SL) had high digestion efficiencies for both *N. britannica* (mean = 89%, SE = 4.5,  $n = 5$ ) and *N. ramosissima* (mean = 66%, SE = 6.2,  $n = 5$ ). This suggests that the change in digestion efficiency was attributable to a change in the diatom culture rather than a postlarval development.

The postlarvae fed *Pleurosigma* sp. grazed less actively than those fed on other diets, and ~60% did not produce feces when transferred to clean containers. Fecal volumes were low and variable (Fig. 1B), and *Pleurosigma* cells were not present in feces on days 9 to 31. Parallel experiments with spare postlarvae transferred weekly to *Pleurosigma* sp. showed that *Pleurosigma* cells were rare in feces on days 18 (~700  $\mu\text{m}$  SL), 24 (~800  $\mu\text{m}$  SL), and 31 (~1 mm SL), but common on days 45 (~1.55 mm SL) and 61 (~1.6 mm SL). Even these larger postlarvae ingested *Pleurosigma* sp. inefficiently. The large and loosely attached *Pleurosigma* cells tended to be deflected by the snout, or lost by the radula before being swallowed. Some cells entered the mouth, but reappeared on the next stroke of the radula, and were dislodged. Most of these cells had been ruptured and lost some of their cell contents.

Faecal volume increased rapidly as postlarvae grew, but was highly variable among individuals. The relationship between shell length and faecal volume was approximately cubic (Fig. 3).

#### Postlarval Growth

Initial postlarval SL (day 3 postsettlement) was ~0.35 mm (Fig. 1D). Growth rate from days 3 to 10 was similar on all diets (means of 20–29  $\mu\text{m}/\text{day}$ ). Growth rate was significantly lower on *C. scutellum* (Table 3), although the actual difference was small (Fig.

1E). Growth rate then dropped for four of the five diets between days 10 and 16 and, with all diets combined, was significantly lower in the second week than the first (paired sample  $t$ -test,  $t = 7.33$ ,  $p < .001$ ,  $n = 30$ ). Growth rates from days 10 to 16 were again similar among diets (means 15–20  $\mu\text{m}/\text{day}$ ), with the exception of *Pleurosigma* sp. (2  $\mu\text{m}/\text{day}$ ) (Fig. 1E, Table 3).

After day 16, growth rates varied markedly between diets (Fig. 1E), giving rise to diverging growth curves (Fig. 1D). The three best diets produced significantly higher growth rates than the two poorest diets by day 23 (Table 3). By day 37, the growth rate on the *C. scutellum* and *C. closterium* was significantly higher than that on *N. ramosissima* (Table 3).

Growth on *Pleurosigma* sp. remained very low until day 30, when data collection was discontinued because of inadequate survival. Growth rate on *N. britannica* was low until day 37, then showed a moderate increase in subsequent weeks (Fig. 1E). The *N. ramosissima* diet produced intermediate growth rates, which did not increase markedly after day 30, when the digestion efficiency of this diet increased. *C. scutellum* produced rapid postlarval growth, with a dip at day 53. A dip in growth rate at day 53 was also observed for *N. ramosissima* and *C. closterium* when a longer-than-usual interval between food renewals led to food depletion in these diets.

*Cylindrotheca closterium* produced rapid growth up to day 45. The growth rate then dropped sharply for the following 3 weeks (Fig. 1E), when postlarvae repeatedly depleted their food supply. A follow-up experiment with larger experimental containers confirmed that postlarvae continued to grow very rapidly when *C. closterium* was provided in excess (average of 68  $\mu\text{m}/\text{day}$  over 19 days from 2.2 mm initial SL, SE = 4.4,  $n = 6$ ).

Specific growth rates were highest from days 3 to 10, then declined during days 10 to 16 (Fig. 1F). On the three best diets, specific growth rate then climbed for 1 to 3 weeks and declined thereafter.

#### Postlarval Survival

Survival to day 68 correlated positively with growth rate ( $r = 0.88$ ,  $p = .05$ ,  $n = 5$ ). Survival was very low (<10% at day 37)

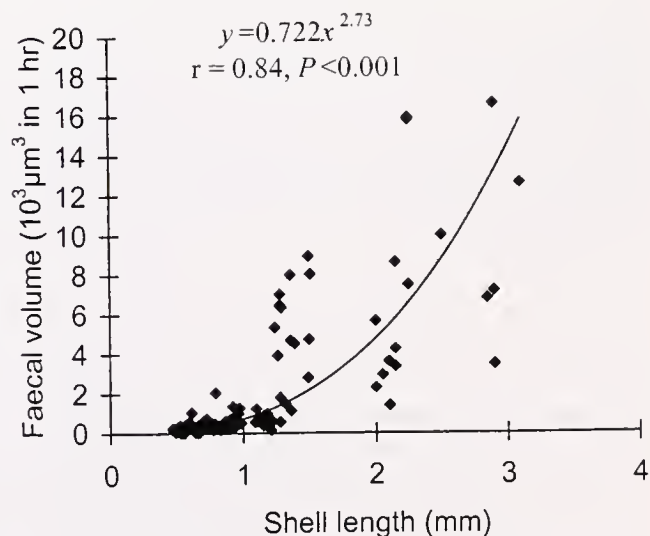


Figure 3. Relationship between postlarval shell length and faecal volume. Data are from all diets, except *Pleurosigma* sp. that produced abnormal feeding activity. The regression line and statistics assume a power relationship between  $x$  and  $y$ .

TABLE 3.  
Statistical comparison of growth rates (see Fig. 1E) of postlarvae fed five diatom diets.

Days Since Settlement	Diatom Diet				
	<i>Cocconeis scutellum</i>	<i>Cylindrotheca closterium</i>	<i>Navicula ramosissima</i>	<i>Navicula britannica</i>	<i>Pleurosigma</i> sp.
10	B	A	A	A	A
16	A	A	A	A	B
23	A	A	A	B	C
30	A	A	A	B	B
37	A	AB	B	C	

Tukey's groupings from ANOVA are presented for five times in the experiment. For each time, those diets in the same Tukey's group are given the same letter. Tukey's group "A" represents significantly ( $p < .05$ ) higher growth rate than "B," which is higher than "C." Low survival precluded the inclusion of *Pleurosigma* sp. beyond day 30.

on *Pleurosigma* sp., intermediate (48% at day 68) on *N. britannica*, and high (>70% at day 68) on the three best diets, *C. scutellum*, *C. closterium*, *N. ramosissima* (Fig. 1C). Survival to day 37 was near 100% on all diets other than *Pleurosigma* sp. (Fig. 1C). Mortality in the *Pleurosigma* sp., *C. closterium*, and *N. britannica* diets occurred steadily; whereas, the mortalities in the *C. scutellum* diet all occurred in a single week (Fig. 1C).

## DISCUSSION

Diatom strain was a major determinant of postlarval survival and growth, which were positively correlated. Both the ingestibility and digestion efficiency of diets were important, and their influence varied over time. This temporal variation was attributable to postlarval development and changes in diatom cultures.

### Dietary Benefits vs. Postlarval Size

Diatom diet made little difference to growth rates in the first 2 to 3 weeks after settlement, but older postlarvae grew more rapidly on the efficiently digested diatoms. Growth rates on the four ingestible diets (excludes *Pleurosigma* sp.) were similar for the weeks ending day 10 and day 16 (Fig. 1E), despite widely varying digestion efficiencies (Fig. 1A). Postlarvae were ~0.8–1.0 mm SL when divergence of growth rates among diets became significant (Fig. 1D, E, Table 3). Previous studies have suggested that dietary benefits were size dependent in postlarval abalone (Kawamura and Takami 1995, Kawamura et al. 1995, Kawamura et al. 1998a) but this is the first time that this relationship has been quantified with a continuous dataset.

There are several possible reasons for this relationship. The first relates to development of the digestive system. By day 2 postsettlement, postlarvae ingested diatoms, rotated them in the stomach, and excreted ruptured diatom frustules. It is possible, however, that small postlarvae lack the digestive capabilities to fully utilize diatom cell contents. We observed progressive development of the digestive gland from day 3, but we do not know when it became a functional organ. Takami et al. (1998) detected polysaccharases in *H. discus hamai* at ~1 mm SL, but not at ~0.5 mm SL, suggesting that digestive enzymes may develop at about the same time that postlarvae begin responding to better diets.

A second possible explanation relates to nutrition from residual yolk. As essential micronutrients from the yolk become depleted, postlarvae may require access to diatom cell contents to

obtain these compounds and maintain rapid growth. Diatoms with low digestion efficiency may not provide a nutritionally complete diet.

### Importance of Ingestibility

*Pleurosigma* sp. was a poor diet primarily because it was not efficiently ingested. The cells were very large, (115 × 16 × 8.5 μm) and loosely attached and were not ingested by the small postlarvae in the growth experiment. Even large postlarvae of 1.5–2 mm SL were inefficient at ingesting *Pleurosigma* sp. The importance of diatom cell size has previously been discussed in relation to size of the postlarval mouth (Seki and Kan-no 1981, Fleming et al. 1996). The mouth is 30 μm across just 2 days after metamorphosis begins (350 μm SL) (Seki and Kan-no 1981). Given that most diatom cells are much less than 30-μm wide, mouth size is unlikely to limit ingestion. Our observations suggest that a more important factor is the superior efficiency with which the radula handles small cells. Large and loosely attached *Pleurosigma* sp. cells were swept aside, or lost, from the radula during feeding. The efficiency of feeding on various diatoms may be determined by the morphology of the developing radula (Roberts et al. submitted).

The growth pattern seen on *Pleurosigma* sp. was typical of inadequate diets. The postlarvae fed *Pleurosigma* sp. grew rapidly to 540 μm SL by day 10, then very slowly to ~600 μm SL by day 30. Similar "tailing-off" of growth at 500 to 700 μm SL has been reported previously for poor diets, including trail mucus (Takami et al. 1997a), coralline algae without diatoms (Takami et al. 1997b), and noningestible *Cocconeis* spp. (Kawamura and Takami 1995, Takami et al. 1997a). The relatively rapid growth observed in the first week may be sustained by residual yolk and uptake of dissolved organic matter (DOM).

Previous research found that postlarval abalone only began ingesting *C. scutellum* once they reached about 800 μm SL (Daume et al. 1997, Takami et al. 1997a). Our experiment quantified that observation and documented a steady increase in ingestion efficiency rather than a sudden transition. The abruptness of the change may be influenced by the attachment strength of the diatoms and the number of loose cells in the culture. The reason for increasing ingestion efficiency on *Cocconeis* diets is not obvious. Increasing postlarval strength is unlikely to explain the change, because the radula of young postlarvae grazed smoothly over *C. scutellum* cells, rather than gripping cells but failing to remove/rupture them. The radula undergoes various morphological devel-

opments during this part of the postlarval period, but the link between these changes and the ingestion of *C. scutellum* is equivocal (Roberts et al. submitted).

#### Temporal Change in Diatom Digestion Efficiency

The digestion efficiency of *N. britannica* changed from low (0–30%) to high (80–90%) within 2 weeks. These are the first data documenting major changes in the digestion efficiency of a diatom strain. A similar increase was shown with *N. ramosissima*, but was not quantified at the time because of difficulty in accurately counting the small cells in cohesive fecal pellets.

The change in digestion efficiency was apparently caused by a change in the diatom cultures rather than postlarval development. After the digestion efficiency increased, we retested the digestion efficiency of small (600–800  $\mu\text{m}$  SL) postlarvae and found it to be high. The increase in diatom digestion efficiency persisted for at least several weeks (R. Roberts, unpubl. data). Two factors that can affect digestion efficiency are diatom attachment strength and structural strength (Kawamura et al. 1998a). There was no indication that the attachment strength of *N. britannica* and *N. ramosissima* changed during our experiment. Reduction in structural strength is a possibility but was not measured. The ability to control diatom digestion efficiency would be useful for abalone hatcheries.

#### Fecal Volume vs. Postlarval Size

Regression analysis showed that fecal production increased roughly cubically in relation to shell length (Fig. 3), as expected for a length-to-volume relationship. However, fecal volume varied greatly among replicates, and shell length explained only 70% of variation in fecal volume across four diets. Thus, the relationship was not precise, and the regression equation should not be interpreted accordingly. However, the data do confirm the rapidly increasing food consumption of growing postlarvae, and, with refinement, such a regression may be useful in predicting food requirements in a hatchery.

#### Overlap Between Yolk and Particulate Food

Our data suggest that nutrition sources other than particulate food are important in early postlarval life. Growth rates were relatively high between days 3 and 10 postsettlement, and dropped significantly for days 10 to 17. Our indirect evidence is consistent with the analyses of Shilling et al. (1996), who demonstrated that yolk and uptake of DOM can contribute substantially to early postlarval development in *Haliotis rufescens*. The lower growth rates in the second week of our experiment may reflect exhaustion of yolk reserves, reduction of DOM uptake, or a reduction in the

relative contribution from these energy sources. The metabolic requirements increase 3 to 5-fold from larva to early postlarva (Shilling et al. 1996) and would continue to increase rapidly as postlarvae grow. Presumably, there must be a point at which yolk and/or DOM uptake become insignificant as supplementary energy sources for feeding postlarvae.

#### Factors Controlling Postlarval Survival

Abalone in hatcheries often suffer 90 to 100% mortality by 2 months postsettlement (Searcy-Bernal 1992) and survival of 20 to 40% is regarded as excellent (e.g., Leighton 1989). Our experiments recorded >70% survival to 68 days on three out of five diets. Our experiments were conducted in small containers without antibiotics, with water exchanged each 3 to 4 days and postlarvae transferred to a new diatom culture weekly.

The species of diatom dominating the biofilm was very important, with both the ingestibility and the digestibility of the diatom affecting growth and survival (discussed above). Our data also suggest that constancy of food supply is important. The few instances of food limitation we encountered resulted in rapid slowing of postlarval growth (e.g., day 53 data in Fig. 1E). In our experiment, food limitation did not result in any immediate mortalities, but longer-term food limitation could be a major source of mortality. Although adult abalone can survive protracted periods without food (e.g., Carefoot et al. 1993), the tolerance of postlarval abalone has not been determined. Constant food supply in hatcheries is complicated by rapidly increasing food demand. This study suggests that fecal production rates (and presumably food consumption) increase approximately cubically in relation to shell length (Fig. 3).

This study emphasizes the importance of diatom diet in controlling the growth and survival of postlarval abalone. The ingestibility and digestibility of diatoms affects growth rates. We find that ingestibility of some diatom strains changes as postlarvae grow, and the digestibility of a diatom strain can vary markedly over time. Improved understanding and control of the diatoms used in abalone hatcheries should improve the efficiency and consistency of juvenile production.

#### ACKNOWLEDGMENTS

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## HISTOLOGICAL ANALYSIS OF MANTLE-CAVITY CILIATES IN *DREISSENA POLYMORPHA*: THEIR LOCATION, SYMBIOTIC RELATIONSHIP, AND DISTINGUISHING MORPHOLOGICAL CHARACTERISTICS

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**ABSTRACT** Dissection has traditionally been the sole method used in investigations of the parasites and other endosymbionts of zebra mussels, *Dreissena polymorpha*. This study demonstrates the value of histological analysis as a complementary technique capable of precisely determining the location of ciliates within zebra mussels and characterizing their symbiotic relationships at the cellular level. The photomicrographs included herein represent the first published histological images of mantle-cavity ciliates of zebra mussels, and we have highlighted morphological characteristics useful in distinguishing individual ciliate species in histological sections. Although zebra mussels from both North America and Europe were sampled for this study, only European populations were found to harbor mantle-cavity ciliates, and five species were observed. The host-specific species *Conchophthirus acuminatus* (Scuticociliatida: Conchophthiridae) was frequently recorded from epithelium covering the outer gill surfaces and occasionally from visceral mass epithelium, but also found in four previously unreported regions: frequently within gill water tubes and occasionally on labial palps, mantle epithelium, and within suprabranchial cavities. Although we sometimes observed zebra mussel sperm in food vacuoles of *C. acuminatus*, epithelial tissues in contact with high densities of these ciliates showed no evidence of pathology, thus confirming this species' commensal nature. The host-specific species *Sphenophrya dreissenae* (Rhynchodida: Sphenophryidae) was frequently recorded attached to mantle cavity epithelium and outer gill surfaces, but also found in three previously unreported regions: frequently within the gill water tubes, occasionally on the visceral mass, and rarely within the suprabranchial cavities. High-intensity infections with this parasitic ciliate did induce hyperplasia, cell hypertrophy, and vacuolization of the epithelia. The host-specific species *Hypocomagalma dreissenae* (Rhynchodida: Ancistrocomidae) was most frequently observed attached to epithelial cells lining outer gill surfaces, but also in five previously unreported regions: occasionally on the visceral mass, the mantle cavity epithelium, and in gill water tubes, and rarely on labial palps and within the suprabranchial cavities. This parasitic ciliate feeds on the contents of epithelial cells using a suctorial tentacle. The intensity of *H. dreissenae* infection, however, was usually very low, and no adverse effects on parasitized cells or nearby tissues were evident. The ciliate *Ancistrumina linnica* (Scuticociliatida: Ancistridae), a nonhost-specific commensal of mollusks, was recorded frequently within gill water tubes, occasionally on outer gill epithelia, and rarely within suprabranchial cavities. This species was also observed to have ingested *D. polymorpha* sperm cells. Commensal Peritrichia ciliates were also occasionally observed within the mantle cavity, but were likely carried there passively by water currents from their typical location on shell surfaces. The presence of "mantle cavity" ciliate species in the gill water tubes and the suprabranchial cavities of zebra mussels suggests that these ciliates probably can exit into surrounding waters to infect other zebra mussels via the exhalant siphon.

**KEY WORDS:** zebra mussels, ciliophora, *Conchophthirus*, *Sphenophrya*, *Hypocomagalma*, *Ancistrumina*, Peritrichia

### INTRODUCTION

Zebra mussels, *Dreissena polymorpha*, were likely transported from Europe to North America in the ballast water of transoceanic vessels (Carlton 1993). Within a few years after their discovery in Lake St. Clair in 1988 (Hebert et al. 1989), these freshwater, macrofouling, bivalves were found in high densities throughout the Great Lakes Basin. Populations have thus far been reported as far south as Louisiana and as far west as Oklahoma (New York Sea Grant 1998). Besides the economic impact of their fouling of

raw-water conduits within infrastructures (O'Neill 1996, O'Neill 1997), they have also caused significant environmental impacts (MacIsaac 1996). Although considerable research has been carried out to understand the ecological interrelationships of these bivalves with other aquatic organisms, relatively little effort has been made to investigate the diversity, distribution, and significance of endosymbiotic organisms present within these mussels. We conducted this investigation of *Dreissena*'s mantle cavity ciliates to address this information gap.

Interest in the prevalence and types of endosymbionts led to the

establishment of the International Research Consortium on Molluscan Symbionts (IRCOMS)—a project currently focusing on the endosymbionts of *Dreissena* spp. both in Europe and North America. Although comprehensive data on the prevalence and intensity of mantle-cavity infections in zebra mussels are currently being prepared for publication by IRCOMS members, we thought it best to issue this current report so that the scientific community might have access to the photographic images of the histological appearance of the mantle-cavity ciliates that we have observed to date, as well as the latest information on the nature of their symbiotic relationships and their precise locations within zebra mussels. The presented photomicrographs represent the first published histological images of *D. polymorpha*'s mantle-cavity ciliates. Previous investigations have relied almost exclusively on observations made during dissections of live zebra mussels to determine where endosymbiotic ciliates were present within *D. polymorpha* and whether these ciliates had any adverse effect. The present study demonstrates the value of using histological analysis as a complimentary technique. The information and images contained herein will, it is hoped, prove to be useful to parasitologists, as well as to other researchers (e.g., toxicologists, physiologists, etc.) who need to identify ciliates they encounter within zebra mussel tissue sections.

Of all symbiotic endofauna in zebra mussels, *Dreissena polymorpha*, mantle-cavity ciliates have been the most frequently reported (Molloy et al. 1997). In Europe, the following five species of mantle-cavity ciliates are believed to have an obligate and host-specific association with *D. polymorpha*: *Conchophthirus acuminatus*, *Conchophthirus klementinus*, *Hypocomagalma dreissenae*, *Sphenophrya dreissenae*, and *Sphenophrya naumiana*. *Ancistrumina limnica*, a nonhost-specific commensal of European freshwater mollusks, has been recorded only once from *D. polymorpha* (Raabe 1956). Among the five obligate European species, *C. klementinus* and *S. naumiana* have been reported from only one location (Lake Ohrid in Macedonia; Raabe 1966). The three other obligate species seem to have a much broader geographic distribution throughout Europe (Molloy et al. 1997). There is growing evidence that none of these European ciliates successfully accompanied zebra mussels in their trans-Atlantic crossing since these ciliates have not yet been observed from North American *D. polymorpha* populations (Molloy et al., 1997; authors, unpublished data).

#### MATERIALS AND METHODS

During 1992 to 1997, *D. polymorpha* populations were sampled both within North America (authors, unpublished data) and Europe (Table 1) and examined for endosymbionts by dissection. At the same time, subsamples were fixed for histology in 10% neutral buffered (sodium phosphate) formalin. After dehydration in increasing alcohol concentrations, the mussels were embedded in paraffin, sectioned at 5  $\mu\text{m}$ , and stained with hematoxylin and eosin. Photomicrographs of mantle-cavity ciliates were produced, and data on the precise location of ciliates within their hosts and their effect on host tissues were recorded. Species identifications of ciliates in the histological sections were facilitated by records of which ciliates were observed from the same sample during dissections, as well as from knowledge of which mantle-cavity ciliate species had previously been documented from *D. polymorpha* (reviewed in Molloy et al. 1997).

TABLE 1.

Source of *Dreissena polymorpha* used for photomicrographs.

Figure	Country	Population	Collection Date
1A, 1C	Russia	Ivankovskoye Reservoir, Dubnya	93/07/10 and 94/08/01
1E, 2A, 2B	Russia	Moscow River, Rublevo	93/07/11
1F	France	Moselle River, Mirgenbach	93/09/15
2D	Russia	Volkhof River, Novaya Ladoga	93/07/16
2E, H	Russia	Volkhof River, Novgorod	93/07/21
2F	Belarus	Dnieper-Bug canal, Pinsk	97/06/30
3A, B	Netherlands	Volkerak Lake, Ooltgensplaat	92/04/19
3C	Greece	Lake Volvi, Thessaloniki	95/10/27

#### RESULTS AND DISCUSSION

In this study, the following five species of ciliates were encountered within the mantle cavity of European *D. polymorpha* populations: *C. acuminatus*, *S. dreissenae*, *H. dreissenae*, *A. limnica*, and an unidentified species belonging to the subclass Peritrichia. It is rarely possible to accurately identify ciliates at the species level solely from histological slides, because key taxonomic characters, such as the number and pattern of kineties on the body, are not visible. Except for the peritrichs, however, these species have all been previously reported from European zebra mussels and are sufficiently different morphologically. When a ciliate's full length was visible in the section (e.g., approximately medial longitudinal or saggital cut through the ciliate), they could often be distinguished from each other using key morphological characteristics as discussed below and further detailed in Table 2.

##### *Conchophthirus acuminatus* (Scuticociliatida: Conchophthiridae)

*C. acuminatus* has never been reported in any other species of freshwater bivalve and is thus considered an obligate, host-specific endosymbiont of *D. polymorpha*. Key characteristics that distinguish it from other endosymbionts include the cytopharynx, which appears as a groove directed toward the anterior end of the body (Fig. 1A,B), the small micronuclei, the ovoid-oblong 15 to 20  $\mu\text{m}$  macronucleus located mainly in the anterior half of the body (Fig. 1B). Their body shape is also distinctive, because they are extremely laterally compressed. When creeping on or attached to a surface, they lie on their side; that is, not on their ventral surface (Fig. 1C).

*C. acuminatus* has been previously reported only from the epithelial outer surface of the gills and visceral mass. We frequently recorded this species at the point of attachment of the gills to the visceral mass. In addition, we also observed this species commonly throughout the entire length of gill water tubes (Fig. 1A,C) and occasionally on labial palps, mantle epithelium, and in the suprabranchial cavities. Ciliates were observed either free in the cavities (Fig. 1A,B,C) or adhering to epithelium (Fig. 1A) through use of their anterior thigmotactic cilia. Our observations supported the concept that *C. acuminatus* is a commensal, because no pathological effects on adjacent host tissues were observed, even when ciliate densities were high.

In studies of *Conchophthirus* and other scuticociliatids, several authors have reported food vacuoles in these ciliates to contain

TABLE 2.

Distinguishing morphological characteristics of the common mantle-cavity ciliates in European *Dreissena polymorpha* samples.

Species	Macronucleus Shape	Cytoplasm	Cell Size ( $\mu\text{m}$ )	Other Features
<i>Conchophthirus acuminatus</i>	Ovoid <sup>a</sup> to oblong	Relatively colorless <sup>b</sup> Heterogeneous with prominent inclusions and vacuoles	50–120 <sup>a</sup>	Prominent grooved cytopharynx Micronuclei often visible Presence of cilia
<i>Sphenophrya dreissenae</i>	Highly irregular <sup>c</sup> (often globular to banana-shaped)	Almost always pink-purple <sup>b</sup> Homogeneous without prominent inclusions and vacuoles	27–37 <sup>d</sup>	Lack of cilia Helmet shape Presence of protruding processes Highly basophilic macronucleus High macronucleus/cytoplasm ratio
<i>Hypocamagalma dreissenae</i>	Ovoid to spindle-shaped <sup>e</sup>	Often pink <sup>b</sup> with lightly stained anterior tip Heterogeneous with prominent inclusions and vacuoles	32–50 <sup>f</sup>	Presence of an attachment knob with clear tube Presence of cilia
<i>Ancistrumina limnica</i>	Ellipsoidal <sup>g</sup> to globular	Relatively colorless <sup>b</sup> Heterogeneous with prominent inclusions and vacuoles.	18–43 <sup>g</sup>	Presence of cilia Lateral elongate cilia on only one side of the body

<sup>a</sup> Raabe 1971.<sup>b</sup> Using standard hematoxylin and eosin staining procedures (see Materials and Methods section).<sup>c</sup> Dobrzanska 1961.<sup>d</sup> Dobrzanska 1958.<sup>e</sup> Jarocki and Raabe 1932.<sup>f</sup> Raabe 1970.<sup>g</sup> Measurements from present study, Raabe (1947) indicated a length of 35  $\mu\text{m}$  in species description, but did not include a range.

host tissues (Kidder 1934, Kirby, 1941, Antipa and Small 1971) and a mix of algae or bacteria (Kidder 1933, Kirby 1941). Because no study has been conducted on the food vacuoles of *C. acuminatus*, the diet of this species has remained unclear. Fenchel (1965) considered that *Conchophthirus* spp. in bivalves were incapable of ingesting suspended particles, because their adoral ciliature was very much reduced. If this is true of *C. acuminatus*, then this species likely feeds on particles present on epithelial surfaces. We sometimes observed *C. acuminatus* containing sperm cells within food vacuoles (Fig. 1C). A similar phenomenon was observed in a scuticociliatid ciliate species in *Mytilus edulis* (Kidder 1933). Because some of the zebra mussels containing these sperm-ingesting *C. acuminatus* were female, the ingested sperm had to be produced by another zebra mussel in the vicinity and taken into the mantle cavity through the female's inhalant siphon.

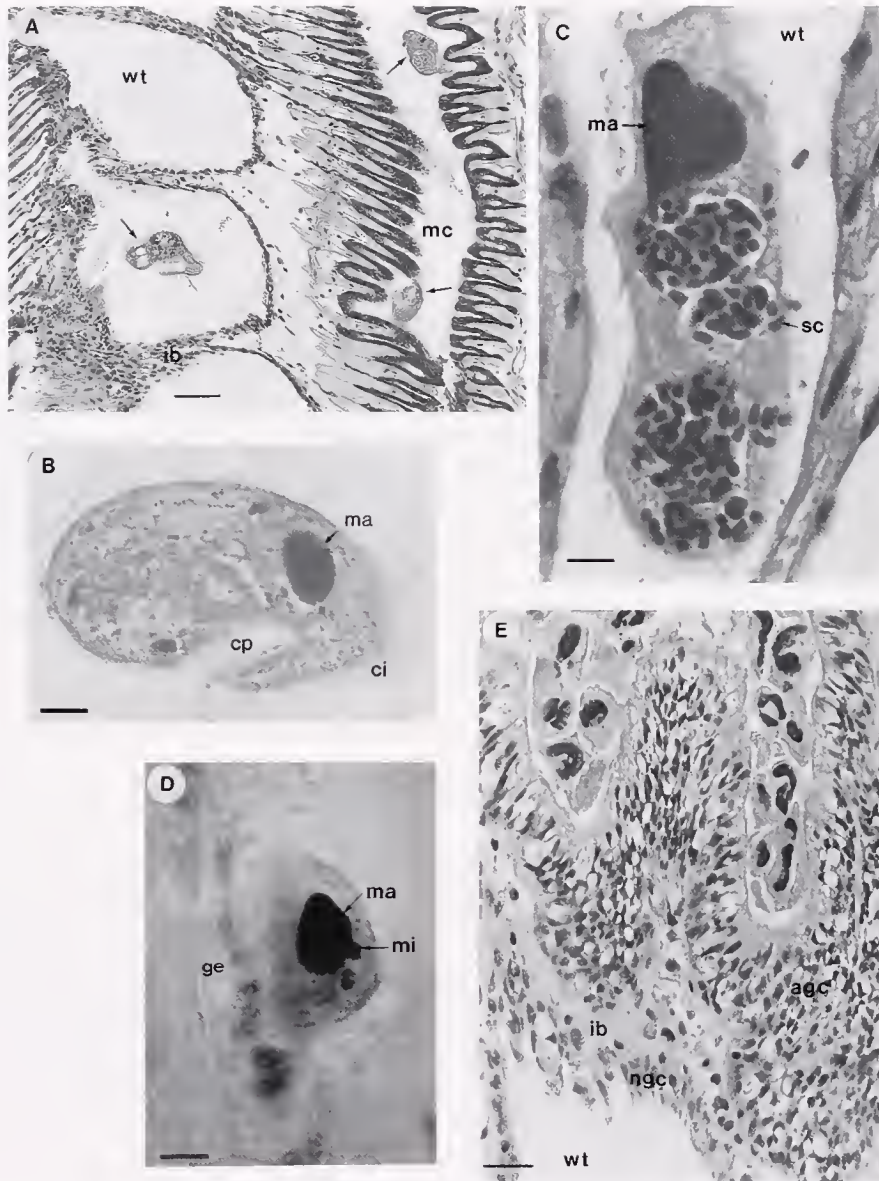
The only other conchophthirid species from *D. polymorpha*, *C. klimentinus*, is apparently very rare and has been reported only from one waterbody in Macedonia (Raabe 1966). Because it is similar in size (morphology reviewed in Molloy et al. 1997), it could be difficult to distinguish from *C. acuminatus* in histological preparations. Its overall shape, however, is different, because *C. klimentinus* has an ovoid outline and is slightly rounded at both ends (Raabe 1966, Raabe 1971).

#### *Sphenophrya dreissenae* (Rhynchodida: Sphenophryidae)

Ciliates in the family Sphenophryidae live on the gills of bivalves and are highly specialized morphologically. Adults lack cilia and have lost the streamline body form typical of mantle-cavity ciliates (Fenchel 1965). Relative to other mantle-cavity ciliates, *S. dreissenae* has a high macronucleus/cytoplasm ratio (Fig. 1D). The macronucleus is distinctive (Fig. 1E), because it is relatively large, densely basophilic, and highly irregular in shape (al-

though often globular to banana-shaped). The cytoplasm is particularly unusual, because it is rather homogeneous and does not contain prominent food vacuoles or cytoplasmic inclusions—a condition probably related to the absence of a mouth and absorption of dissolved nutrients directly from epithelial cells. In fact, all Sphenophryidae have no mouth opening, and in ciliate evolution this is considered a secondary phenomenon relating to parasitism (Fenchel 1965). Although the feeding process of sphenophryid ciliates has not yet been precisely determined, two hypotheses have been offered. Chatton and Lwoff (1921) suggested that *Sphenophrya* spp. used the entire surface in contact with the host epithelial cells as a "sucking disk", whereas, Kirby (1941) theorized that they fed osmotically.

*S. dreissenae* are typically attached to the gill epithelium using their flat clinging sole, and thus may appear flat or even concave (Dobrzanska 1958). Previously reported only from the epithelium on outer gill surfaces or lining the mantle cavity, we also observed these ciliates frequently within the gill water tubes, occasionally on the visceral mass, and rarely within the suprabranchial cavities (Fig. 1E, 2A,B). *S. dreissenae* reproduces by budding, and one to several layers of individuals were often observed coating the visceral mass epithelium or the gill inner epithelium (Fig. 1E, 2A,B). Foci of high ciliate intensity frequently resulted in tissue damage, including epithelial hyperplasia, cell hypertrophy, and extensive vacuolization (Fig. 1E). Epithelial cell hypertrophy and hyperplasia resulted in papillary protrusion of the epithelium into the water tubes thus increasing the surface area in contact with the ciliates (Fig. 2A,B). Secretory material, likely mucous, was frequently observed at the apical ends of the epithelial cells; in most of our photomicrographs, this secretory material had a homogeneous texture (see arrows, Fig. 2B) somewhat similar to the cytoplasm of the *S. dreissenae* cells themselves. Although *S. dreissenae* has always



**Figure 1.** (A) Oblique section through a gill showing three *Conchophthirus acuminatus* (arrows); note that one of them is in a water tube. (B) Lateral view of *C. acuminatus* free in the mantle cavity; note the cytopharynx, dense cilia, and macronucleus in anterior of cell. (C) Dorsoventral view of *C. acuminatus* in a water tube; note *Dreissena* sperm cells within its vacuoles, dense cilia, macronucleus in anterior of cell, and relatively long, lateral compressed body. (D) Isolated *Sphenophrya dreissenae* clinging on gill tissue; note the high macronucleus/cytoplasm ratio, lack of cilia, relatively homogeneous cytoplasm, and the helmet shape. (E) Details of an interlamellar bridge infected by *S. dreissenae*; note the protrusion of the epithelium caused by epithelial hyperplasia and the vacuolization of the cells on the infected side; compare to normal interlamellar bridges in Fig. 1A. Scale bar = 5  $\mu\text{m}$  (C,D); 10  $\mu\text{m}$  (B); 20  $\mu\text{m}$  (E); 50  $\mu\text{m}$  (A). age: abnormal gill cells, ci: cilia, cp: cytopharynx, ge: gill epithelium, ib: interlamellar bridge, ma: macronucleus, mc: mantle cavity, mi: micronucleus, ngc: normal gill cells, sc: sperm cell, wt: water tube.

been considered as being parasitic (Dobrzanska 1958, Dobrzanska 1961), our histological observations of adverse tissue reaction leading to gill deformities is the first conclusive evidence of pathology.

The relatively small size (27  $\times$  37  $\mu\text{m}$ ), helmet shape, lack of cilia, occasional presence of protruding processes, large nucleus/cytoplasm ratio, homogeneous cytoplasm, and dense irregularly shaped, highly basophilic macronucleus are characteristics that distinguish *S. dreissenae* from other recorded mantle-cavity ciliates. The only other *Sphenophrya* documented from *D. polymorpha*, *S. naumiana*, is different in shape (elongate to canoe-shaped),

has an elongate nucleus, and is generally larger in size (Raabe 1966).

#### *Hypocomagalma dreissenae* (*Rhynchodida: Ancistrocomidae*)

*H. dreissenae*, as with other *Hypocomagalma* spp., have at their anterior tip a suctorial tentacle that acts in part as an attachment knob (Fig. 2E,F). The presence of this structure readily separates this species from all other ciliates reported from *D. polymorpha*. Within the anterior of this species, we often observed a clear tube leading to the attachment knob—similar to the “hollow tube” re-



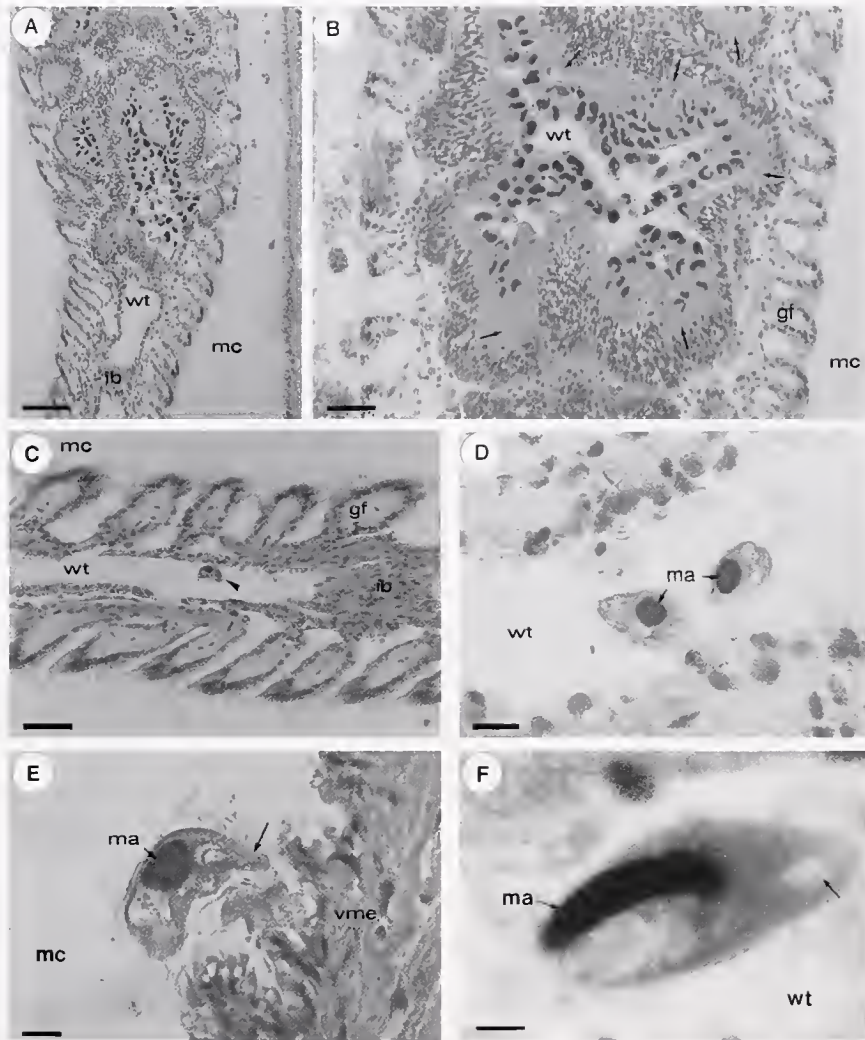


Figure 2. (A) and (B) Oblique sections through gill demibranch showing proliferations of *Sphenophrya dreissenae* in water tubes; note secretory material, likely mucous, at the apical ends of the epithelial cells (arrows) and the swelling of the epithelial lining in infected water tubes. (C) Oblique section through gill with a pyriform *Hypocomagalma dreissenae* (arrow) present within a water tube. (D) Two pyriform *H. dreissenae* within a gill water tube. (E) Banana-shaped *H. dreissenae* on the visceral mass epithelium; note globular macronucleus, cilia, and thin clear tube (arrow) in anterior tip. (F) *H. dreissenae* in a water tube; note spindle-shaped macronucleus partially obscured by vacuoles and the clear tube (arrow) at the anterior end. Scale bar = 5  $\mu\text{m}$  (F); 10  $\mu\text{m}$  (D,E); 50  $\mu\text{m}$  (B,C); 80  $\mu\text{m}$  (A). gf: gill filament, ib: interlamellar bridge, ma: macronucleus, mc: mantle cavity, vme: visceral mass epithelium, wt: water tube.

ported in other rhynchodid species (Lom and Kozloff 1968). Although there is some overlap in size with *S. dreissenae*, the presence of cilia can be used to distinguish *H. dreissenae* (Fig. 2E) from *S. dreissenae* (Fig. 1D). Other characteristics that help to distinguish *H. dreissenae* from the other mantle-cavity ciliates are the ovoid to spindle shape of its macronucleus and the heterogeneity of its cytoplasm (e.g., relatively prominent granules and vacuoles) (Fig. 2D,E,F).

*H. dreissenae* has previously been reported only from the outer gill surfaces. We observed this frequently, but also noted their presence in five other epithelial regions: occasionally on the visceral mass (Fig. 2E), on the mantle cavity epithelium, and in gill water tubes (Fig. 2C,D), and rarely on labial palps and in suprabranchial cavities. This species is considered as a true parasite of *D. polymorpha*, because it inserts its tentacle into the cytoplasm of an epithelial cell, and by some as yet unexplained means, nutrients from the host cell pass into the ciliate. Bradbury (1994)

indicated that *Hypocomagalma* spp. probably damage the cell to which they are attached, but because they are few in number per host, infection usually has little pathological effect. Our observations of *H. dreissenae* infection of *D. polymorpha* were similar.

#### *Ancistrumina limnica* (Scuticociliatida: Ancistridae)

*A. limnica* is a nonhost-specific invader of freshwater lamelli-branchs and gastropods (Raabe 1956). Ciliates such as this species typically feed on bacteria, diatoms, and other material extracted from water currents (Kirby 1941) and are, thus, not considered parasitic. As in the commensal *C. acuminatus*, however, we did sometimes observe zebra mussel sperm in food vacuoles. In the only previous report of *A. limnica* in zebra mussels, its precise location within the mantle cavity was not indicated (Raabe 1956). We observed *A. limnica* frequently within the gill water tubes (especially near the food groove) (Fig. 3A), occasionally on the

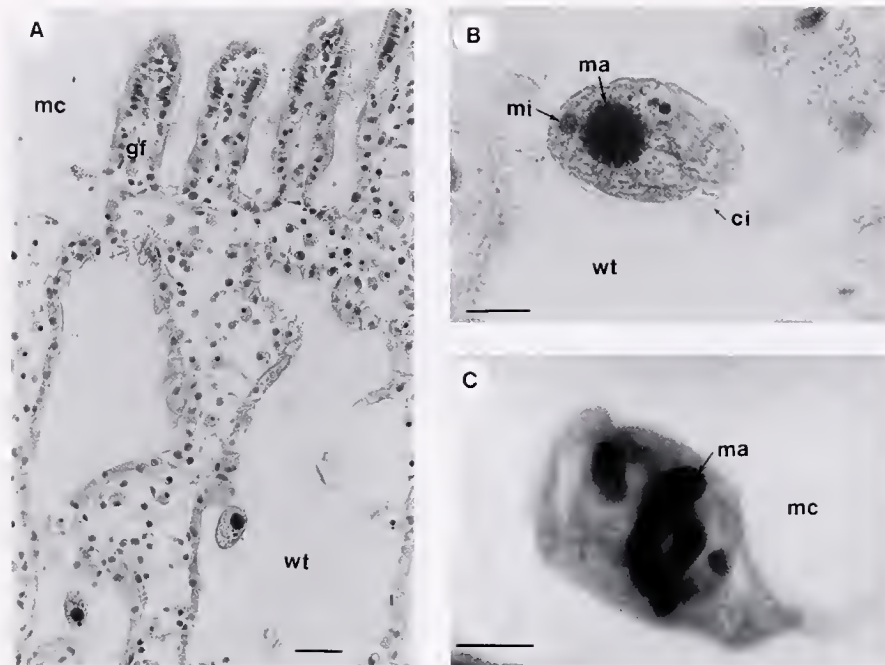


Figure 3. (A) Oblique section through gill demibranch showing two *Ancistrumina limnica* in adjacent water tubes. (B) *A. limnica* in a water tube; note the distinctive, elongate cilia (ci) on only one side of its body, as illustrated in Raabe (1947). (C) Peritrich ciliate attached to the visceral mass epithelium in the mantle cavity; note elongate, coiled macronucleus. Scale bar = 10  $\mu\text{m}$  (B, C); 35  $\mu\text{m}$  (A). ci: cilia, gf: gill filament, ma: macronucleus, mc: mantle cavity, mi: micronucleus, wt: water tube.

outer gill epithelium, and rarely in the suprabranchial cavities. No apparent signs of pathology were evident. Identifying characteristics of this species (Fig. 3B) are: (1) the presence of an ellipsoidal to globular macronucleus; (2) relatively colorless, highly vacuolated cytoplasm (similar in appearance to the much larger ciliate *C. acuminatus*); (3) the lack of an appearance of any firm attachment to epithelial surfaces (because they are free swimming and have, in contrast to *H. dreissenae* and *S. dreissenae*, no highly specialized attachment structures); and (4) the presence of rows of lateral elongate cilia on only one side of their body (rarely visible in most histological sections).

#### Peritrichia

Ciliates in the subclass Peritrichia with elongate coiled macronuclei were occasionally observed in the mantle cavity (Fig. 3C). Although peritrichs have been previously reported from bivalve mantle cavities (Fenchel 1965), this is the first report of these commensal ciliates within zebra mussels. It is likely that these ciliates were attached to visceral mass epithelium, and not simply free floating. In any case, no host reaction was evident in the adjacent epithelia. Because peritrich populations were observed externally on *D. polymorpha* shells, these ciliates were likely carried passively by water currents into the mantle cavity where they reattached.

#### CONCLUSIONS

Histological analysis proved to be a very useful technique to complement information gained through dissections of living material. Tissue sections revealed some symbionts to be more abundant than realized from dissections (authors, unpublished data), and the smaller in size and the more sessile the symbiont, the more this was true. Our observations of host tissue condition further strengthened current hypotheses regarding the symbiotic relation-

ships of these mantle-cavity ciliates; that is, *C. acuminatus*, *A. limnica*, and peritrich ciliates are commensals, and both *S. dreissenae* and *H. dreissenae* are parasites. Histological analysis was particularly valuable, because it revealed the precise location of each ciliate species on or within zebra mussel organs. It was previously unknown, for example, that the "mantle-cavity" ciliates of zebra mussels also inhabited their gill water tubes and suprabranchial cavities. It would seem, then, that these ciliates normally migrate with water currents into the water tubes through the gill ostia and then are carried with the water flow up into the suprabranchial cavities. The presence of these ciliates in these latter water cavities suggests a pathway by which these ciliates may exit their hosts to infect other zebra mussels. For example, laboratory transmission of *C. acuminatus* directly between infected and uninfected groups of zebra mussels has been achieved (Burlakova et al. 1998), but it has remained unknown from which orifice(s) these ciliates departed from their infected hosts. If such ciliates as *C. acuminatus* were only present in the mantle cavity *per se*, they could only leave by the inhalant siphon. This could certainly occur during pseudofeces ejection, when valves are quickly closed, rapidly forcing water, pseudofeces, and possibly, some ciliates out of the mantle cavity. The presence of "mantle-cavity" ciliates in gill water tubes and suprabranchial cavities, however, also gives these ciliates the ability to exit via their host's exhalant siphon, because it receives water from the suprabranchial cavities.

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**ABSTRACTS OF TECHNICAL PAPERS**

*Presented at the 19<sup>th</sup> Annual Meeting*

**MILFORD AQUACULTURE SEMINAR**

Milford, Connecticut

February 27–March 1, 1999



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**OVERVIEW, 19th MILFORD AQUACULTURE SEMINAR.**

**Walter J. Blogoslawski**, U.S. Department of Commerce, National Oceanic & Atmospheric Administration, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, 212 Rogers Avenue, Milford, CT 06460.

The 19<sup>th</sup> Milford Aquaculture Seminar attracted 41 speakers whose topics included shellfish diseases and their effects on the local industry, new techniques for bay and sea scallop culture, the culture of blackfish (*Tautog*) and summer flounder, biofiltration in recirculating systems, some Canadian experiences in aquaculture, the use of triploidy to enlarge crop animals, crop insurance availability, new regulations for permitting, and societal aspects of aquafarms. The 150 attendees from the US and Canada met in formal and informal sessions to discuss the recent problems in the aquaculture industry and to share potential solutions. The cooperation exhibited at the meeting highlighted the ability of persons sharing the same concerns to work together for acceptable resolutions of their common problems.

Attendees representing 42 public and private shellfish and finfish-aquaculture industry ventures were joined by persons from 15 educational institutions and 10 state and federal government agencies to exchange ideas and experiences in developing aquaculture technology.

The participation of our speakers and exhibitors is greatly appreciated as is the financial support from our sponsors, the U.S. Department of Commerce's National Marine Fisheries Service, Milford Laboratory, Milford, CT and the U.S. Department of Agriculture, Northeastern Regional Aquaculture Center in North Dartmouth, MA.

**GROWTH AND SURVIVAL OF JUVENILE BAY SCALLOPS FROM GENETIC LINES AT DIFFERENT DENSITIES AND DEPTHS: COLLABORATIVE STUDY BETWEEN THE NATIONAL MARINE FISHERIES SERVICE AND THE BRIDGEPORT AQUACULTURE SCHOOL.** **Joseph Choromanski, Sheila Stiles, Christopher Cooper, and Eric Bedan**, USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460; **Sherry W. Lonergan** and **Paul J. Trupp**, Bridgeport Regional Vocational Aquaculture School, 60 St. Stephens Road, Bridgeport, CT 06605.

Hatchery-reared juvenile scallops were field-tested to evaluate growth and survival of genetic lines of bay scallops (*Argopecten irradians*) in western Long Island Sound. Students and staff from the Bridgeport Regional Vocational Aquaculture School cooperated with scientists at the Milford National Marine Fisheries Service Laboratory in these collaborative grow-out studies. In May 1998, Milford scientists provided a total of five thousand scallops from different genetic lines for the students to use in their project. The lines used were large- and small-selected. The scallops were counted, measured, and their total volume determined. They then were divided into groups containing varying numbers of scallops

to determine effects of density. Chinese lantern nets and Japanese lantern and pearl nets were deployed off Bridgeport/Fairfield near Penfield Reef in Long Island Sound. Nets were suspended from a floating longline to a depth of approximately three meters. A second set of scallops was counted and measured, then placed in lantern nets anchored to the bottom (ten meters at high tide) and buoyed up into the water column to a height of two meters, to serve as comparisons for nets suspended from longlines.

Counts and measurements were made approximately midway through the experiment. Nets were heavily fouled with seaweed and tunicates. However, overall survival was high. Growth was not exceptional, which was presumed attributable to rather heavy current and wave action. A severe storm, which occurred in the area in July, loosened an anchor mooring and resulted in the loss of some of the nets. A videotape and visual inspection was made by NMFS divers to assess conditions and damage which had been caused by the storm. The final phase of this particular experiment ended in November when the nets were retrieved and evaluated. One longline net from the comparative depth study was lost, while both bottom nets were intact. Initial observations showed slightly better growth from the bottom nets, while survival was comparable.

This longline project was a unique opportunity for several reasons. While the final results of this first year may be preliminary, invaluable logistical experience in longline aquaculture was attained for the staff of the lab and the school; this will be used in planning future projects. The mutually beneficial experiment provided both valuable assistance for the NMFS scientists, and a learning experience for the students to become familiar with tools and protocols used in measuring the scallops in particular, as well as "hands-on" exposure to aquaculture in general.

**THE EFFECT OF DENSITY ON GROWTH OF ARGOPECTEN IRRADIANS IN LONG ISLAND SOUND: IN PARTNERSHIP WITH NATIONAL MARINE FISHERIES SERVICE SCIENTISTS.** **John J. Curtis, Sherry W. Lonergan, Thomas McGann, and Paul J. Trupp**, Bridgeport Regional Vocational Aquaculture School, 60 Saint Stephens Road, Bridgeport CT 06605.

Being consistent with its philosophy of infusing meaningful activities into the instruction at the Bridgeport Regional Vocational Aquaculture School, an invitation was accepted to have students work with National Marine Fisheries Service scientists of the Milford, Connecticut laboratory on a project to study "the effect of density on growth of *Argopecten irradians* in Long Island Sound." The initial study was recently concluded in part, in December 1998 with the harvest of the targeted crop.

The school's role was clearly defined with educational goals and objectives established at the onset of the project for the involved students. The species, *Argopecten irradians*, is one familiar to the students at the Aquaculture School since its introduction in a 1994–96 international collaboration with the People's Republic

of China. In that project the students and staff of our school learned not only the biology of the bay scallop but also the methods of spawning, grow-out and harvesting. In addition, procedures for statistical analysis of the collected scientific data were included for follow-up studies.

Students from the school's Intensive program were introduced to the project by NMFS scientists in the spring of 1998. They began work on design, construction and deployment of a long-line at the school's farm in Long Island Sound. The project began with NMFS scientists, students and staff transporting juvenile bay scallops, reared at the Milford Laboratory, to our test location. Scallops were sorted into test groups, measured and transferred to various style culture nets and attached to the long line. In the fall, students and scientists collected data on the growth rate (shell height and width) of the scallops which were then transferred to nets of a larger mesh size and returned to the water. The final phase of this project had two objectives. The first was to gather growth data on one group of test scallops and the second was to implement a separate long-line for another group of scallops for research on the effects of over-wintering.

The educational objectives of this project were many and varied. By immersing students in real-life scientific study, they were presented the procedures necessary to assist in the design and implementation of a high-level research project from beginning to end. Discussion of scientific methods, proper research techniques, data collection and analysis augmented the standard curriculum of science and technology. This project has offered our students opportunities to develop the skills of problem-solving in a meaningful activity that has already translated into higher academic performance and a better scientific understanding.

From an educational perspective, much has been learned to date and much more can be extracted from this project through continuation. The difficulties encountered in the initial attempt will be addressed through earlier phases of conditioning, spawning and placement at the farm site. The problems to gear presented by natural conditions are being addressed in the CAD classroom with students redesigning lantern nets and researching better methods of deployment. The students and staff of the Bridgeport Aquaculture School look optimistically to our continued involvement and the accomplishment of the prime goal of the project: to develop better methods to grow bay scallops which can be competitively sold in the market place.

**HISTOPATHOLOGICAL SURVEY OF THE QUAHOG, *MERCENARIA MERCENARIA*, ALONG THE CONNECTICUT COASTLINE.** Joseph DeCrescenzo, Inke Sunila, John Karolus and John Volk. State of Connecticut, Department of Agriculture, Bureau of Aquaculture, P.O. Box 97, Milford, CT 06460.

A histopathological survey was conducted along the Connecticut coast line on the hard clam, *Mercenaria mercenaria*. Quahog

Parasite Unknown (QPX), an economically important parasite, phylum *Labyrinthomorpha*, has been found off the coast of Massachusetts. The purpose of this survey was to detect QPX or other conditions which might possess a threat to Connecticut's hard clam harvest.

Eleven different locations were sampled along the Connecticut coast line. Samples of 30 clams each were harvested from either commercial or wild clam beds. A gross pathologic examination was then conducted before they were processed for histopathologic examination. The clams were shucked and placed into Davidson's fixative. Sections were then stained in hematoxylin-eosin.

Samples were diagnosed for infectious agents such as viruses, Chlamydia, bacteria, fungi or any protozoan or metazoan parasite. Histopathological lesions were classified as inflammations, degenerative process, cell or tissue death, or proliferative responses. The results showed no signs of the commercially important parasite QPX. However, some infectious and non-infectious agents were found in the examination. The following conditions appeared at low prevalences: *Chlamydiales*, ceroidosis, ciliates in the gill region, sloughing of the epithelia in the digestive diverticula, hemorrhage in the intestine and stomach, and mucus around the foot.

In conclusion, no economically important parasites were present in the samples. Recent mortalities in oyster beds due to infection with MSX have shifted more harvesting pressure toward hard clams. This study, based on the low prevalences of histopathological conditions and active gametogenesis in the gonads, predicts a positive future for Connecticut's clamming industry.

**THE INVERTED PROPELLER-BEANIE...A NEW WAY TO MIX LARGE MICROALGAL TANKS.** Mark S. Dixon, Barry C. Smith, and Gary H. Wikfors, USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460.

Large-scale open microalgal cultures can be viewed as bioreactors; they are defined as "stirred reactors" by chemical engineers. Energy must be supplied to the system, nutrients added at a rate equal to their use, wastes removed as they are generated, and cells must be exposed to the reactive surface (the culture/air interface). The Greenhouse for Research on Algal Mass Production Systems (GRAMPS) at the National Marine Fisheries Service Laboratory in Milford, Connecticut houses two 20,000 liter oval (5.5m x 3m x 1.2m) fiberglass tanks used for the production of large volumes of microalgae to feed post-set shellfish in a land-based nursery. Three stirring methods were considered for GRAMPS production tanks: 1) air mixing, 2) manual stirring with paddles, 3) mechanical stirring with a motorized device. Air mixing was rejected based upon previous experience showing that bubbles in dense, open microalgal suspensions encourage bacterial growth. The benefit of constant mixing by mechanical means needed to be established before investment in equipment

and its operation could be justified. Paddle-wheel mixers have been used in other large-scale microalgal production systems, but all are custom made and too expensive for routine application. In many industrial processes, tanks of reactants are stirred by a foil on a shaft, driven by a motor—essentially an inverted propeller beanie.

The recent addition of a propeller style mechanical mixer to one of the large tanks provided an opportunity for comparison with previous tank cultures which required regular manual mixing via a muscle-powered paddle. The reality of occasional hand mixing is a poorly-mixed culture which can only be maintained at relatively shallow depths. Reduced culture volumes, settling cells, and unequal exposure of cells to light all reduce the productivity of a culture. By contrast, a tank culture which is mixed continually allows all cells to be on the lighted, reactive surface on a calculated regular interval controlled by varying the speed of the propeller, thereby providing all cells access to light for photosynthesis and the culture/air interface for gas exchange many times during the day.

A culture of *Tetraselmis chui* (PLY429) has been maintained in the mechanically-mixed tank for the past 6 months using semi-continuous management. The mechanically-mixed tank was operated at a maximum depth of 1.1 meters or approximately 18,000 liters. Maximum operation depths for the hand-mixed tanks were less than 0.4 meters or approximately 6,000 liters. Cell densities in the mechanically-mixed tank often exceed  $1 \times 10^6$  cells/ml, while hand-mixed tanks average approximately  $1 \times 10^5$  cells/ml. Mechanically-mixed tanks are much longer lived than hand-mixed tanks under a semi-continuous harvest strategy; 6 months versus 6 weeks.

Higher productivity, greater culture volumes, a superior algal product, long lived cultures, and reduced maintenance are all benefits of mechanically-mixed, large-scale microalgal cultures. Economic analysis and evaluation of performance of the mixer as it is integrated into the overall automation of GRAMPS are planned for later this year.

**AQUATIC ANIMAL HEALTH AND UCONN AQUACULTURE PROGRAM: NEW FACULTY AND OPPORTUNITIES.** Richard A. French, Salvatore Frasca, Jr., Sylvain De Guise, and Herbert J. Van Kruiningen, University of Connecticut, Department of Pathobiology, Northeastern Research Center for Wildlife Diseases, 61 North Eagleville Road, Storrs, CT 06269.

The State of Connecticut has made a substantial investment in aquaculture industries and teaching aquaculture science. In April 1989, the Connecticut State Board of Education approved a request by the Bridgeport Board of Education to create a Regional Vocational Aquaculture School. The state appropriated 7.5 million dollars for the construction of the first of these schools, the Bridgeport Regional Vocational Aquaculture School. In 1994, the State Board of Education established the Sound School Regional Vocational

Aquaculture Center in New Haven. Both institutions provide high school students with specialized laboratories and classrooms that complement a marine science-related curriculum. Facilities at these centers include pathology laboratories, aquaculture tanks to grow finfish and shellfish, indoor boat shops, marine engine laboratories, and computer-assisted drafting laboratories.

The University of Connecticut has made a commitment to develop an undergraduate teaching program in aquaculture science, which is embodied in the formulation of a multidisciplinary aquaculture minor, including courses from the College of Agriculture and Natural Resources and the College of Liberal Arts and Science. The contribution of the Department of Pathobiology to this multidisciplinary aquaculture minor will be didactic and active teaching in the field of aquatic animal health (e.g., preventive medicine, disease recognition and treatment, mechanisms of disease, health surveillance). To this end, the University has hired three new veterinary research faculty in the Department of Pathobiology; Dr. Sylvain De Guise, Dr. Salvatore Frasca, and Dr. Richard French, and added an undergraduate course entitled, "Systemic Finfish and Shellfish Pathology and Microbiology," to be offered in Spring Semester 2000.

The aquaculture science program is affiliated with regional aquaria, fisheries, and professionals (Mystic Aquarium, The Maritime Aquarium, The Connecticut Department of Agriculture, Bureau of Aquaculture and Laboratory, and private industry), providing active instruction and cooperative training opportunities to students. In addition, a state-of-the-art Marine Science and Technology Center facility is under construction at the University of Connecticut Avery Point Campus, which will offer unique educational prospects to undergraduate and graduate students. The Connecticut Veterinary Diagnostic Laboratory, which provides autopsy service for state and private concerns, will expand and support the accession of numerous aquatic animal cases directed toward a primary teaching, diagnostics and research initiative. The Department of Pathobiology offers a Bachelor of Science undergraduate degree, and Master of Science and Doctor of Philosophy graduate degrees. Graduate students in Pathobiology may specialize in Pathology, Microbiology, Virology, Immunology, Clinical Chemistry, Avian and Aquatic Animal Pathology, and Wildlife Diseases. Regarding aquatic animal health, ongoing research includes studies of marine mammals, marine and freshwater finfish, and shellfish.

**VIBRIO PARAHAEMOLYTICUS AND OTHER SHELLFISH DISEASES OF PUBLIC HEALTH SIGNIFICANCE: A REVIEW.** Richard A. French, University of Connecticut, Department of Pathobiology, Northeastern Research Center for Wildlife Diseases, 61 North Eagleville Road, Storrs, CT 06269.

The incidence of foodborne illness associated with consumption of contaminated seafood products has recently triggered media attention that has helped to increase public awareness of issues

related to food safety. This media coverage has also generated a number of misconceptions regarding the safety of eating seafood. Microorganisms and other toxic substances commonly ingested by shellfish may accumulate within animal tissues and be passively transmitted to humans when they consume the tainted seafood products. Though generally relatively harmless to the affected shellfish, these microorganisms and chemicals are often pathogenic or toxic to humans. Pathogens of public health significance associated with contaminated seafood include causative agents of hepatitis and gastroenteritis, biotoxins (paralytic shellfish poisoning) and toxic industrial chemicals (heavy metals, polycyclic aromatic hydrocarbons, and chlorinated hydrocarbons). One important microbial pathogen of marine species, including crabs, shrimp, lobster, and oysters is *Vibrio parahaemolyticus*. Recent foodborne disease outbreaks associated with consumption of oysters in the Pacific Northwest (1997), Galveston Bay, Texas (1998) and Oyster Bay, New York (1998), have heightened awareness of *V. parahaemolyticus*. This *Vibrio* species is a halophilic bacterium that is part of the normal flora of estuarine and other coastal areas worldwide. Human illness associated with *V. parahaemolyticus* is characterized by a self-limiting, mild to moderate gastroenteritis occurring within 4–96 hours after consumption of raw or improperly cooked, and/or stored fish and shellfish. Several halophilic *Vibrio* species associated with mollusks are reported to cause gastroenteritis in humans. Disease is strain-specific within *Vibrio* species and correlated with production of various toxins, including enterotoxins, cytotoxins, and hemolysins. In addition to surveillance efforts designed to identify the pathogenic strains of *V. parahaemolyticus*, epidemiologic and pathogenesis studies are currently underway. Such research will help determine the geographic distribution of *V. parahaemolyticus* and provide a better understanding of the mechanisms of the disease process. Diagnostic methods for the detection of *V. parahaemolyticus* and species typing continue to improve. A review of shellfish-associated foodborne diseases and current efforts to improve food safety in the United States will be addressed.

**WAMPANOAG SHELLFISH AQUACULTURE.** David W. Grunden, Wampanoag Aquaculture Director, Island Aquaculture, Oak Bluffs, MA 02557.

The Wampanoag Tribe of Gay Head Aquinnah is a Federally Acknowledged Native American Tribe located on Martha's Vineyard Island. Their tribal offices are in the town of Aquinnah, MA. They have observed the decline of the local shellfishery in the town and have two goals for their aquaculture enterprise. The first, of course, is to make a profit. The second is to return a percentage of the yield from the hatchery back to the wild and to protect their cultural heritage of depending on the local shellfish as a food. They also hope that it will allow the fishery to recover so that many of their tribal members can continue to fish commercially within the local ponds. In working to achieve this second goal the tribe has a

Memorandum of Understanding with the Town to assist their shellfish department with any of its propagation and predator control programs. This has evolved into assisting the Town's shellfish department in developing a comprehensive plan to manage the shellstock.

A report on the early development of the Native American Wampanoag Tribe of Gay Head Aquinnah's commercial shellfish aquaculture enterprise will be presented. It will include an introduction to where the project is located, what has been done to date and the expected developments for 1999.

A hatchery is planned as well as grow-out of the seed to both field plant and market sizes. Additionally, the Tribe is working with the local Town to develop a comprehensive shellfish management plan.

**SUPERIOR GROWTH AS A GENERAL FEATURE OF TRIPLOID SHELLFISH: EVIDENCE AND POSSIBLE CAUSES.** Ximing Guo, Rutgers University, Haskin Shellfish Research Laboratory, 6959 Miller Avenue, Port Norris, NJ 08349, USA.

Triploids are organisms with three sets of chromosomes instead of the two sets found in normal diploids. Aquacultural interest in triploid shellfish so far has primarily focused on their sterility. The presence of an extra set of chromosomes poses a problem for meiosis and leads to complete or partial sterility in triploids. Because excessive gonadal development negatively affects meat quality of diploid molluscs, sterile triploids provide a high quality product that can be marketed year round. Triploid Pacific oyster is now widely used for aquaculture production. However, another important benefit of triploid molluscs, superior growth, has been largely overlooked by early studies and aquaculturists. During the past decade, triploids have been studied in over 20 species of molluscs. A review of recent data indicates that superior growth may be a general feature of triploid molluscs. Triploids exhibit significantly higher growth rate than diploids in almost all species studied so far. Triploids grow faster than diploids by 12–30% in *Crassostrea virginica*, 25–51% in *Crassostrea gigas*, 42–52% in *Crassostrea dalienwhanensis*, 72% in *Mulinia lateralis*, 27–58% in *Pinctada martensii*, 36% in *Argopecten irradians*, 32–59% in *Chlamys nobilis*, and 81% in *Chlamys farreri*. The adductor muscle of triploid scallops is larger than that of diploids, by 73% in *A. irradians*, 96% in *C. farreri*, and 167% in *Argopecten ventricosus*. The expression of the triploid advantage in growth may be influenced by genetic and environmental factors. Triploids may not show superior growth in food-limiting environments. Several hypotheses have been proposed to account for the superior growth in triploids. One hypothesis attributes the superior growth to increased heterozygosity in triploids. A positive correlation between heterozygosity and growth rate has been found in diploid molluscs. Triploids are theoretically more heterozygous than diploids. The heterozygosity hypothesis is supported by the observation that trip-

loids produced from blocking polar body I and diploid  $\times$  tetraploid mating, which are more heterozygous, grow faster than triploids produced from blocking polar body II. Another hypothesis views that sterility in triploids distributes more energy to growth rather than sexual reproduction. The energy relocation hypothesis cannot explain growth difference expressed before sexual maturation. Finally, it has also been suggested that triploid cells are larger than diploid cells and may contribute to an overall increase in body size. All these factors may contribute somewhat to the overall growth of triploids. Regardless of causes, triploid molluscs may benefit aquaculture by offering greatly improved growth. The challenge is that commercial production of triploids is technically difficult in most species. Commercial use of triploids may ultimately depend on the development of tetraploids, which can produce 100% pure triploids simply by mating with normal diploids. Tetraploids have been successfully developed for triploid production in the Pacific oyster, and success in other species may soon follow.

**VIBRIO PARAHAEMOLYTICUS—A NEW CHALLENGE FOR STATE SHELLFISH CONTROL AGENCIES.** William Hastback, New York State Department of Environmental Conservation, 203 North Belle Mead Road, Suite 1, East Setauket, New York 11733.

In late August 1998, the New York State Department of Environmental Conservation (NYSDEC) shellfish sanitation program was advised by the New York State Department of Health (NYSDOH) of a series of five (5) individual illnesses in shellfish consumers. Laboratory analyses of patient samples indicated that the illnesses were caused by the naturally occurring marine bacterium—*Vibrio parahaemolyticus* (VP).

The initial information available indicated that the shellfish implicated in the illnesses had been harvested from the area designated as NS-2, including Oyster Bay and Cold Spring Harbors, in northwestern Nassau County. On September 8, we learned that two individuals in New Jersey had become ill after consuming oysters from the same area. On September 9, the NYSDOH advised NYSDDEC of their determination of a statistical association between the consumption of shellfish and the illnesses. On September 10, the NYSDDEC Bureau of Marine Resources designated Oyster Bay and Cold Spring Harbors as uncertified for the harvest of shellfish on an emergency basis. The closure was in effect through October 22, a period of six weeks. The decision to reopen was based on declining water temperatures and the results of DNA probe examinations of oyster samples conducted by two FDA laboratories.

In the interim, the federal Centers for Disease Control identified the 03:K6 strain of VP isolated from patient samples. That strain of VP had been identified as the cause of an oyster-related illness outbreak that affected approximately 450 people in several states during June. Galveston Bay, Texas was the source of the oysters in that outbreak. The 03:K6 strain has also been respon-

sible for large seafood related illness outbreaks in southeast Asia, from India to Japan.

**THE ECONOMICS OF SEA SCALLOP GROW-OUT: AQUACULTURE AT AN OFFSHORE SITE.** Porter Hoagland, Hauke L. Kite-Powell, and Di Jin, Marine Policy Center, Woods Hole Oceanographic Institution, Woods Hole, MA 02543.

The extent to which offshore sea scallop aquaculture is a commercially viable business depends upon both the costs of growing scallops relative to wild harvest operations and conditions in the relevant product market.

Here we report on the development of a discounted cash flow model of the grow-out of sea scallops at an offshore farm, such as that represented by the Westport Fishing Corporation's sea scallop experiment off the coast of Martha's Vineyard, Massachusetts. We examine the economic viability of four alternative approaches to scallop farming: seabed seeding and three variations on cage culture: lantern cages; bottom cage trawls; and bottom cage clusters. For each alternative, we estimate capital and operating costs and revenues over a 20 year period. We assume a two-year cycle from collection of juveniles to harvest, and scale the farming operation in every case to produce 100 thousand pounds of scallop meat per two-year cycle (that is, every other year).

Under baseline assumptions, the only alternative that is profitable is seabed seeding. A 100 thousand lbs/cycle seabed seeding operation requires less than \$400 thousand in start-up capital and pays back the initial investment in four years. It requires a lease area of about 150 acres and requires the use of a large scallop vessel about 3 months out of the year, on average. The cage operations are not profitable because the higher survival rate and growth are not enough to justify the added cost of buying, maintaining, deploying, and harvesting the cages and associated moorings. Although they require smaller lease areas, the cage operations demand between \$1-2 million in startup funding. Of the three alternatives, bottom cage trawls come closest to break even because gear costs are relatively modest.

There are several sources of uncertainty in the model, including the ex-vessel price for sea scallops. In order to help manage this uncertainty, we have estimated a model of supply and demand for New England sea scallops using monthly data during the period 1985–93. The model is a linear representation of both supply and demand for "average size" sea scallops, implying a market equilibrium over the 1985–93 period of \$5.42 per pound.

It is useful to think of the production of scallops from an offshore farm as an inventory problem. At an offshore site, seed scallops grow over a period of about two years to a size that may command a premium over the average size scallop. We have developed a simple algorithm to help the farmer take advantage of historical monthly variability in sea scallop demand. If this variability persists, we find that when farm output is small relative to the market, the farmer should act as a price taker, harvesting and

marketing his product only in January. As potential output increases, however, the time profile of output shifts. Output of up to 150 thousand pounds should be produced in January and November. When output reaches 200 thousand pounds, there should be some level of production in every month except July.

It is costly to monitor sea scallop mortality at an offshore site. Because of uncertainty about mortality, the time profile of production is suggestive of a strategy for harvesting the aquaculture product. It may be sensible to sample the product through partial harvesting, say, in October. This sample will give the farmer an estimate of mortality. If mortality is low, then a production profile that places product on the market in every month might be followed. If mortality is high, then production should be adjusted accordingly, and product would be placed on the market in November or January. Note also that the production profile can be readjusted during the year as market conditions become revealed and as uncertainty about the quality of the farmed product is reduced. This research has been supported with funds from the Westport Fishing Corporation and the National Sea Grant College Program.

**THE POTENTIAL FOR BIVALVE AQUACULTURE IN MARYLAND'S COASTAL BAYS.** Mark L. Homer, Mitchell Tarnowski, and Robert Bussell, Maryland Department of Natural Resources, Tawes State Office Building, B-2, Annapolis, Maryland 21401.

It has been over a century since the coastal bays of Maryland supported a substantial public oyster fishery, nearly 30 years since hard clam catches peaked and then collapsed, and some 70 years since bay scallops even inhabited this region. Although a successful relay industry on private grounds was established for oysters after natural populations almost disappeared, it essentially ended about 50 years ago. Hard clams currently support only a remnant fishery, while, until this year, bay scallops had not been seen in the wild since the early 1930's.

There have been sporadic, and ultimately unsuccessful, attempts to culture oysters, hard clams, and bay scallops in the Maryland portion of Chincoteague Bay during the past seven decades. Oysters are particularly subjected to a hostile environment, related to environmental changes caused by the stabilization of the Ocean City Inlet in 1933. Three oyster parasites, Dermo, MSX, and SSO are active in the coastal bays, as are two species of highly abundant oyster drills. Any hard substrate placed into Chincoteague Bay is rapidly colonized by a variety of fouling organisms, including serpulid worms, colonial tunicates, hydrozoans, bryozoans, and barnacles. These factors tend to diminish the possibility of successful oyster aquaculture ventures in this region, with the possible exception of a rapid turnaround relay fishery.

Bay scallop culture has only recently been attempted in Maryland, although initial results are not encouraging. Through a Mary-

land DNR re-introduction project, growth and survivorship data are now available. Preliminary results indicate that growth rates may not be sufficient to produce marketable scallops before their second winter. Given the short life-span of this species and the labor involved in battling fouling organisms, bay scallop culture in Maryland has some serious problems to overcome.

Although Virginia has established a significant hard clam aquaculture industry, including production in Chincoteague Bay, few attempts have been made within Maryland's boundaries. The main impediment to hard clam culture appears to be associated with the permitting process, which includes three state agencies, five federal agencies, public hearings, and, on occasion, an appeals board. This daunting array of agencies, associated regulations, and opposition from waterfront property owners has attracted few individuals to the process. Environmental conditions in the Maryland coastal bays, however, appear to be sufficient to establish at least a modest hard clam aquaculture industry. There are areas outside Federal jurisdiction that provide clean, hard bottom for either planting bags or netting small beds of seed clams. Hatchery-reared clams are readily available from Virginia and there is a suitable, nearby market for hard clams.

**EFFECTS OF PHOTOPERIOD ON SURVIVAL, GROWTH AND PIGMENTATION OF SUMMER FLOUNDER (*PARALICHTHYS DENTATUS*) LARVAE IN LABORATORY CULTURE.** Marina Huber, Eric Moore, Neil Marcaccio, Robin Katersky, and David Bengtson, Department of Fisheries, Animal and Veterinary Science, University of Rhode Island, Kingston, RI 02881.

Summer flounder represents a promising species for commercial aquaculture in the northeastern United States. In order to optimize production, the effects of various environmental parameters on biological production parameters must be studied. We investigated the effects of photoperiod on three parameters important to hatchery production: survival, growth and abnormal pigmentation. The last parameter involves incomplete pigmentation of the eyed side, including minor non-pigmented blotches to complete albinism. Flounder larvae were reared in replicate 75-L aquaria under three light regimes, 24L:0D (constant light), 16L:8D (summer conditions), 8L:4D:8L:4D (abnormal conditions to trick the fish into physiologically living two "days" in one). No significant differences in survival or growth were detected in the larvae through metamorphosis; however, after metamorphosis, fish reared in constant light had significantly lower levels of abnormal pigmentation. The experiment was repeated with an additional treatment, 8L:16D (winter conditions); no significant differences in pigmentation were observed among treatments, but fish in the 8:4:8:4 treatment grew significantly more.

**A COMPARISON OF CHROMAGAR E. COLI, MILLIPORE COLI-COUNT SAMPLERS, AND THE MPN PROCEDURE FOR ENUMERATION OF COLIFORMS IN BAY SCALLOPS.** Diane Kapareiko and Richard A. Robohm, USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460.

Little information exists on whether bay scallops are capable of harboring microorganisms of human health significance. To assure a safe product resulting from our bay scallop aquaculture development activities, we are interested in surveying bay scallops for the presence of fecal coliforms (human health indicator organisms). The recommended procedure for coliform detection, the MPN procedure, requires a relatively large expenditure of time, labor and materials. Two recently-developed products, CHROMagar E. coli,<sup>TM</sup> and Millipore Coli-Count Samplers,<sup>TM</sup> appear to be simpler to apply for detection of coliform bacteria.

CHROMagar E. coli, a chromogenic plate medium, expedites the identification of *Escherichia coli* on the basis of contrasting colony colors. Millipore Coli-Count Samplers combine a grid-marked, 0.45  $\mu\text{m}$  Millipore membrane filter over a nutrient pad, which facilitates growth. Microorganisms present in the sample being tested affix to the filter pad and are cultured within its plastic case. Both products contain a chromogenic substance which reacts with genus or species-specific enzymes to produce a blue colony color for fecal coliforms. Other gram-negative bacteria remain colorless.

We tested both of these products for their accuracy in enumerating *E. coli* in comparison with the traditional MPN method. Aliquots of bay scallop homogenate were "seeded" in blender jars with three individual doses of a pure culture of *E. coli*; this consisted of a low dose (mean colony count =  $1.8667 \times 10^2/\text{ml}$ ), a medium dose (mean colony count =  $1.8667 \times 10^4/\text{ml}$ ), and a high dose (mean colony count =  $1.8667 \times 10^5/\text{ml}$ ). A fourth aliquot of scallop homogenate was not seeded in order to provide colony count information on possible pre-existing levels of *E. coli*. The number of organisms recovered from each dose was enumerated using the MPN procedure, CHROMagar E. coli and Millipore Coli-Count Samplers simultaneously; the experiment was repeated for a total of three trials. Individual counts were expressed as the ratio of the number of *E. coli* recovered (minus the background count of *E. coli*) divided by the dose administered (colonies/ml).

Ratios resulting from CHROMagar E. coli and Millipore Coli-Count Sampler counts for each dose were normally distributed. Analysis by two-sample t-test indicated no significant differences between the means of these ratios for each technique at the  $p = 0.05$  level of significance ( $p = 0.7564$  for low dose,  $p = 0.9542$  for medium dose,  $p = 0.2540$  for high dose). Overall ratios (regardless of dose) for the MPN, CHROMagar and Millipore procedures were also normally distributed and no significant differences existed when MPN ratios were compared either to CHROMagar E. coli ratios ( $p = 0.0957$ ) or to Millipore ratios ( $p = 0.0959$ ). A practical evaluation indicated that CHROMagar

*E. coli* had a longer refrigerated shelf-life for the powder concentrate, was more cost-effective than the Millipore Coli-Count Samplers (\$0.80/test for CHROMagar, \$2.50/test for Millipore), and always produced consistent results. <sup>TM</sup>Trademarks are used to identify products only and are not indicative of endorsement by the USDOC, NMFS.

**DEVELOPMENTS IN THE PRIVATE AQUACULTURE INDUSTRY ON MARTHA'S VINEYARD.** Richard C. Karney, Martha's Vineyard Shellfish Group, Inc., Box 1552, Oak Bluffs, MA 02557; and John C. Blake, Sweet Neck Farm, Box 1468, Edgartown, MA 02539.

The progress of private aquaculture ventures spawned by two National Marine Fisheries Service (NMFS) Fishing Industry Grants (FIG) is reported. Five growers have successfully taken thousands of 2 mm oyster seed to a 3 inch legal size in about three years. The single oysters grown in cages off-the-bottom are well shaped, deeply cupped and as good or better quality than most cocktail oysters seen in raw bars. The growers consistently received fifty to sixty-five cents apiece for their oysters. Despite the loss of up to 25 percent of the three year old oysters due to Seaside Organism (SSO, *Haplosporidium costale*) in the spring of 1998, production of market size oysters per grower for the past year ranged between 800-11,000 oysters with an average of about 3,000 oysters per grower.

Over a half million new seed oysters were cultured by twelve growers in 1998. Growth and survival have been excellent. Growers who have been conscientious about thinning and cleaning the seed, report that 2 mm seed set out in July was averaging 2 inches in September. Seed cultured in a tidal upwelling nursery grew to 2 inches (from 2 mm) in eight weeks! Some of these oysters reached 3 inches by December.

Island cultured shellfish were promoted under a \$4,000 grant from the Southeastern Massachusetts Aquaculture Center (SEMAC). The grant provided for the development of a logo, printing of promotional materials, and introducing the new cultured seafood products to Island chefs, retailers, and the general public.

Under a grant from the Massachusetts Department of Food and Agriculture, Jack Blake, an Edgartown grower, constructed and operated a floating hatchery/nursery prototype. The first two attempts at larval culture of quahogs failed. During the first attempt, fertilized eggs introduced to the flow-through larval culture system escaped when a drain screen dislodged. A second attempt was made to culture quahog larvae. This time the larvae were cultured in a closed system tank where water was changed every other day and cultured phytoplankton was fed daily. This culture succumbed to a *Vibrio* infection traced to source water which was drawn from a prefilter reservoir contaminated with oyster feces from an adja-

cent nursery culture system. In a third attempt, two million two-week-old oyster larvae introduced into the system were successfully grown in a closed system mode. Within a week, the veligers progressed to eyed larvae and were set on microcultch in the system. This culture attempt has resulted in over 110,000 of 2–5 mm oyster seed. Results from these early trials are promising and the innovative hatchery is scheduled to be run again next year.

**THE PRESENCE OF *HAPLOSPORIDIUM NELSONI* (MSX) AND *PERKINSUS MARINUS* (DERMO) IN *CRASSOSTREA VIRGINICA* ALONG THE CONNECTICUT AND NORTHERN LONG ISLAND SHORELINE IN 1998—AN EXTENSIVE SURVEY.** John Karolus, Inke Sunila, Stacey Spear, Joseph DeCrescenzo, and John Volk, Connecticut Department of Agriculture, Bureau of Aquaculture, P. O. Box 97, Milford, CT 06460.

Previous data generated by this laboratory determined a widespread prevalence of *Perkinsus marinus* (Dermo) starting in 1996 and *Haplosporidium nelsoni* (MSX) starting in August, 1997 in the *Crassostrea virginica* (eastern oyster) population along the Connecticut coast. An extensive survey was conducted in 1998 to include the entire coast line of Connecticut and the northern shore of Long Island.

Samples of 30 oysters each were collected at selected sites representing both leased oyster growing areas, seed areas and locations of special interest. For the diagnosis of MSX, oyster tissue was preserved in Davidson's fixative with 20% artificial seawater. Paraffin-sections were stained with hematoxylin—eosin and Ziehl's acid fast stain for detecting spores. For the diagnosis of Dermo, anal rectal tissue were cultured in Ray's Fluid Thioglycollate Medium.

*Haplosporidium nelsoni* was found at epizootic levels at most sites along the Connecticut shoreline in 1998. Ninety one percent of the Connecticut samples were found positive for MSX. Prevalence varied from 3 to 77%. Adult oysters at three locations were found to contain sporulating MSX during the autumn. Forty-four percent of the New York samples were positive. The prevalence range was from 3 to 37%.

For Connecticut sampling sites, the results for *Perkinsus marinus* in 1998 indicated no significant difference from the intensity of infection between the shallow waters (eight feet or less) and the deeper water samples. *Perkinsus marinus* was found in 100% of the Connecticut samples. In addition, there was no significant difference between the intensity of infection for 1997 versus 1998. In New York, 69% of the samples were positive for Dermo. However, the Dermo intensity of infection was not significantly different from that found in Connecticut during 1998.

MSX results for 1998 indicated most of the CT shoreline was experiencing a post-epizootic period. It appeared that Dermo had established an enzootic prevalence in Long Island Sound.

**EARLY INDUCTION OF SPAWNING OF A CAPTIVE TAUTOG BROODSTOCK BY LIGHT AND PHOTOPERIOD MANIPULATION.** Grace Klein-MacPhee and Aimee Keller, University of Rhode Island, Graduate School of Oceanography, Narragansett Bay Campus, Narragansett, RI 02882.

Broodstock collected by hook and line from the east passage of Narragansett Bay, RI was maintained in the laboratory in an 8 foot, black fiberglass tank with running seawater, an airstone, and several PVC tubes for shelter. The tank was tented with black plastic, and a fluorescent light set on a timer maintained a photoperiod of 10 hours light and 14 hours dark (approximate ambient photoperiod for winter). Seawater was at ambient temperature and salinity from November 25, 1997 to March 17, 1998. The fish were fed chopped quahogs, live crabs and whole mussels daily throughout December then every other day through March 17. It was a mild winter and water temperatures averaged 7.4°C (range 5.5–9) for November–December and 6°C (range 3.8–7.8) for January–March. During this time the fish were relatively inactive, spending most of the time in their shelters with an occasional sortie around the tanks to feed.

On March 17, the water temperature was raised to 14°C and maintained at an average temperature of 13.4°C (range 10.7–16.6) through March 31. The photoperiod was changed over a period of a week from 10L / 14D to 13L / 11D. On March 31 the fish began to spawn. The first large batch of eggs was collected on April 2 and the progeny from this spawning were raised through juvenile stage. The juveniles are now 10 months old and are healthy and active fish. The broodstock continued to spawn with periodic resting states through November 1998.

Tautog spawn in Narragansett Bay from May through August with a peak in June and July. Ichthyoplankton samples collected in the bay in 1998 contained tautog larvae in June–August.

We successfully succeeded in advancing the spawning date to late March using temperature and photoperiod manipulation, and obtained viable eggs and larvae. We intend to repeat the experiment this year with the addition of a control tank of fish which will be maintained at ambient temperature and photoperiod, and we will begin inducing spawning in February.

**GROWTH CHARACTERISTICS IN TRIPLOID PACIFIC OYSTERS—A NEW DIMENSION.** Brenda Landau and Ximing Guo, Rutgers University, Haskin Shellfish Research Laboratory, 6959 Miller Avenue, Port Norris, NJ 08349.

Standard practice has been to measure length and whole weight of randomly sampled oysters as a means of documenting performance. During routine random sampling earlier, the observation was made that the height or thickness of triploid oysters compared to their diploid controls was noticeably larger. To test this hypothesis, an allometric study was done to compare four measurements, length, width, thickness, and whole weight of triploid oysters to those of their diploid controls for two different year classes, 1994



and 1995. The triploids were produced from a previous study by diploid  $\times$  tetraploid matings. It has been previously documented that triploid oysters grow faster than diploids and that polyploid gigantism may, in part, account for the larger overall size, though thickness measurements were not used. A comparison of means shows triploids to be 17.0%, 19.7%, 42.8%, and 93.2% larger than their diploid counterparts for length, width, thickness, and whole weight, respectively, for the '94 year class; and, 5.1%, 12.2%, 25.2%, and 46.3% larger for the '95 year class. An analysis of variance for the general linear model in which group (triploid vs. diploid) and replicate ( $k=2$ ) are main factors shows the group effect to be significant ( $p=0.000$  to  $0.002$ ) in both year classes for each of the four measurements. Results of this study show that thickness, rather than length and width, is the primary dimension for the increased growth in triploid oysters. Consequently, triploid oysters are more deeply cupped than diploids. This study used only two replicates, so a follow-up study with more replicates is needed to confirm these results.

**A SOCIAL AND ECONOMIC EVALUATION OF AN OYSTER MARICULTURE TRAINING PROGRAM FOR LONG ISLAND COMMERCIAL FISHERMEN.** Steven Lang, York College, The City University of New York, 94-20 Guy Brewer Blvd., Jamaica, N.Y. 11451.

Despite a long history of shellfish mariculture, numerous public shellfish enhancement programs, and large tracts of available and potentially productive underwater land, New York's mariculture industry remains stagnant. For the most part, the constraints on shellfish mariculture are social, political and economic rather than environmental or technological. By far, one of the major obstacles hindering the development of mariculture has been the antagonistic attitude of commercial fishermen who have a long history of being opposed to the private use of public underwater land. The history of shellfishing in Long Island has been filled with struggles and conflicts between small-scale commercial fishermen who harvest wild shellfish from the public resources and shellfish farmers who cultivate shellfish and are dependent upon a system of exclusive property rights.

At the present time, amidst a steady decline in the natural stocks and shrinking opportunities caused by several factors, some fishermen are beginning to reconsider their negative attitude towards mariculture. While a few fishermen place great hope in mariculture's potential to create new opportunities, most are skeptical. For the potential of mariculture to be realized, attitudes will have to change so that it is viewed as a legitimate marine activity by members of the commercial fishing community.

In 1995, through the East End Institute, funds were made available from New York State to establish an oyster mariculture training program for Long Island commercial fishermen to learn simple off-bottom culture techniques. In 1996, additional funds from the Fishing Industry Grants Program of the National Marine Fisheries

Service were made available. Approximately 40 fishermen were given seed, culture gear, and informal training in oyster culture with the hope of them starting mariculture "cottage industries" that would supplement their incomes.

A major objective of the oyster mariculture training program was to encourage small-scale mariculture by changing attitudes on the part of fishermen who have traditionally been opposed to it. The operating logic of the training program was to create opportunities for a few fishermen to become successful so that other fishermen would become interested and pursue mariculture on their own. At the heart of the oyster mariculture training program is the notion that top-down development and management schemes initiated from distant external authorities are counterproductive and will not change negative attitudes on the part of fishermen. Change has to emerge from within the fishing community and must be facilitated in non-threatening ways which encourage active participation on the part of fishermen. The long and difficult process of institutionalizing mariculture as a way of life has to be based on some type of co-management arrangement between fishermen and government agencies. Development schemes have to incorporate attitudes and concerns of fishermen and include them in project designs and management arrangements. If developed rationally and in ways that are socially acceptable, mariculture could help to preserve traditional fishermen and enable them to follow the water in their customary ways.

For the most part, the oyster mariculture training program has been successful. This paper will explore some of the reasons for that success as well as implications for the future of small-scale mariculture in the region.

**THE TRANSITION FROM COMMERCIAL FISHING TO OYSTER CULTURE: RESULTS OF A NMFS FISHING INDUSTRY GRANTS PROJECT.** Richard Langan, Jackson Estuarine Laboratory, University of New Hampshire, 85 Adams Point Rd., Durham, NH 03824.

With support from the NOAA National Marine Fisheries Service Fishing Industry Grants Program, three New Hampshire commercial fishermen participated in a comprehensive oyster culture training program designed to give them an opportunity to evaluate shellfish culture as a part-time alternative to wild harvest fisheries. The fishermen were provided with guidance and assistance with site selection, permitting, evaluation of oyster culture methodologies, three year classes of oyster seed, and the supplies and equipment needed to continue in aquaculture after project completion.

Culture methodologies included remote setting of hatchery-reared eyed larvae on natural and artificial cultch, suspension nursery culture, and bottom grow-out. Permitting woes, shortages of larvae, extreme weather events, an oil spill, the specter of MSX, and predation of oyster drills and green crabs were balanced by some excellent sets, good growth, and a positive production outlook and provided the fishermen with the opportunity to experi-

ence first hand the risks and opportunities of shellfish culture. Of the three fishermen that participated in the project, two will very likely continue with oyster culture.

**EXPERIMENTAL TESTING OF FIELD TECHNIQUES FOR FARMING THE SOFT-SHELL CLAM (*MYA ARENARIA*).** Kenneth J. La Valley, Thomas L. Howell, and Riley Y. Morse, Spinney Creek Shellfish, Inc., Eliot, ME 03903; Brian Beal, and Bertrand Dubois, University of Maine, Machias, ME 04654.

The purpose of this USDA/SBIR Phase I research project was to determine the feasibility of commercially farming the soft-shell clam, *Mya arenaria*. To accomplish this objective the research proposed to: 1) Produce a high volume of high quality hatchery-reared seed; 2) Optimize Floating Up-weller techniques for soft-shell clam culture; 3) Investigate the added benefits of conditioning seed beds by harrowing; and 4) Investigate several seed planting/grow-out techniques.

To optimize hatchery production, larval stocking densities of 10K, 20K, 40K, and 100K larvae/gallon were evaluated for survival and maturity to settlement. Pediveliger metamorphosis occurred predictably from days 18 to 21 at 22°C. Highest larval survival was observed at stocking densities between 20K and 30K larvae/gallon.

The Floating Upweller System (FLUPSY) design exceeded our expectations, delivering 189 l/min. of upwelled water flow through each silo. The FLUPSY produced 20–22 mm animals in 2.5 months with near 100% survival. This reduced the grow-out time by up to three seasons compared to natural stocks. Soft-shell clams were stocked at high densities in upweller silos without a compromise in growth or survival, which reinforced the commercial application of the FLUPSY design. A design capacity of 100K (10 mm) seed clams per silo was determined, for a total capacity of 1 million (10 mm) seed clams per ten silo Floating Upweller System.

August field experiments were conducted at two sites in Kennebunkport, and one site in Portland, Maine to determine the added benefits of conditioning seed beds by harrowing. Soft bags similar to those used in the Florida quahog fishery were considered as a potential grow-out technique. Survival was a problem at all three sites. Netting, especially in the harrowed treatments at the Portland site, provided the best survival, and soft bags were found to be inappropriate, except, possibly in the softest muds.

Recognizing the potential and regional importance of soft shell clam farming, Spinney Creek Shellfish (SCS) has begun to address the administrative framework necessary for fostering this new commercial activity. SCS has drafted proposals for enhancing the existing framework along with the Maine Department of Marine Resources so that the best possible revised structure is in place at the point in time that this activity comes to full commercial potential.

In conclusion, the specific objectives were met, establishing this species as a candidate for commercial farming. The FLUPSY rapidly produced plantable seed clams with minimal maintenance, favoring scaling to commercial capacity. The bottleneck for this species was grow-out. Future research will include determining optimal planting size and sediment type, tidal height, tidal stage, and time of year for enhanced survival and growth.

**A TOUR OF UPWELLERS ON CAPE COD.** Dale F. Leavitt, Patricia L. Gohring, and William P. Burt, Southeastern Massachusetts Aquaculture Center, c/o Hurley Library—Mass. Maritime Academy, 101 Academy Drive, Buzzards Bay, MA 02532.

The use of upwelling culture systems for nursery grow-out of commercially important bivalve mollusks has become an important component of community and private shellfish aquaculture on Cape Cod. The nursery phase of bivalve culture is frequently a limiting step for bivalve seed production due to limitations in space and food availability in commercial hatcheries. A concerted effort has been made on Cape Cod to increase shellfish seed production by expanding the region's capability to raise seed through the nursery phase. During September, the Southeastern Massachusetts Aquaculture Center (SEMAC) conducted a tour of ten different upweller systems to investigate the design and operation of a variety of approaches to upwelling. The technical information compiled from these systems will be presented along with a pictorial display of various approaches to upweller design.

**THE NEED FOR AQUACULTURE IN THE WORLD TODAY.** Robert Link, Liquid Life Technologies, Inc., 1727 Veterans Memorial Highway, Islandia, NY 11722.

This paper will describe components of the finfish and shellfish aquaculture industries while explaining the challenges and opportunities that exist in those industries. Although the aquaculture industry is growing at a rapid pace, there are some impediments that affect all parts which include: regulations, marketing, and financing. All of these components are necessary for healthy growth and will be discussed in detail.

**RECENT STREAMLINING OF THE AQUACULTURE REGULATORY PROCESS.** Michael Ludwig, USDOC, NOAA, National Marine Fisheries Service, Habitat Conservation Division, Milford, CT 06460.

While aquaculture operations have been permitted and presently occur in the Gulf of Mexico, Atlantic and Pacific waters, initial permitting efforts for a given geographical area, a new culture species or innovative technology can be hampered by a lack of

understanding among the applicant, regulators and other involved parties. In addition, the public is often confused by what they perceive as conflicting positions taken by the regulatory agencies. Their confusion often arises from a perception that the agencies are a single entity rather than consortiums of representatives from a variety of programs, each with different (and occasionally conflicting) mandates and responsibilities. The differing expectations of all involved parties about the amount or type of information needed to describe an aquaculture proposal before it is deemed ready for evaluation can result in costly and protracted reviews.

The National Marine Fisheries Service (NMFS) Northeast Region's Habitat Conservation and Protected Resources Divisions has consolidated much of the guidance offered to aquaculturists across the US and Canada. The document was created to establish a standard level of information quality for use in seeking federal authorization of aquaculture projects. The document is intended to be all encompassing and not the minimum required for permit consideration. The all-species package contains guidance from which parties in a regulatory action can select elements for use in application forums. It is our expectation that the document will become the standard for submissions of environmental compatibility and regulatory acceptability in the Northeast and provide a semblance of uniformity for regional evaluation processes. It is our intention that compliance with the entire guidance package would be required only on occasions when the ecological sensitivity of a proposed culturing site or other project details are so complex as to warrant that degree of thoroughness. By seeking out those involved in the regulatory process as early as possible in the development of a proposal and using tools such as pre-applications meetings, an applicant will be able to identify the project assessment requirements to which one will be held. This will greatly facilitate and even expedite a project's evaluation.

However, we recommend that before undertaking any data gathering or committing to any physical site evaluation efforts, the applicant seeks guidance and thoroughly coordinate planning and site evaluation efforts with the appropriate regulatory agencies.

The Guidance package will be described and made available at the meeting.

#### **THE EFFECTS OF STOCKING DENSITY ON GROWTH OF LARVAL TAUTOG.** Lindsay Lydon and Grace Klein-MacPhee, University of Rhode Island, Graduate School of Oceanography, Narragansett Bay Campus, Narragansett RI 02882.

A preliminary study was conducted on larval tautog to determine if two different 15 gallon tank shapes, conical and rectangular, affected tautog survival. Although larval survival did not appear to be related to tank form, the stocking density influenced tautog growth.

Tautog eggs were collected from broodstock that spawned 7/29/98 and 7/30/98. After 8 hours of incubation, 10 mls of eggs were added to the four tanks with a static system. Green alga,

*Tetraselmis suecica* was added daily to each tank for the first 19 days. Larvae were fed a combination of dry and live food, rotifers, artemia, and a Kyowa™ diet. The tanks were transferred to flow-through systems after larvae were 19 days old. Survival was good in all tanks except for one conical which had 100% mortality by day 5. Although additional tautog were supplemented from a rectangular tank with a high density (848) of fish, these larvae in the conical tank developed abnormally. The majority of fish (92%) in this tank exhibited an unusual jaw development which prevented mouth closure. This jaw anomaly most likely interfered with their ability to eat dry food and to make a complete transition from live food, *Artemia nauplii*, to dry food. As a result, poor growth occurred in this tank.

After 14 weeks there was a statistically significant difference in size between fish groups. Fish growth was best in the tank with an initial stocking density of 2.8 fish/liter with a total of about 148 tautog. These fish had a mean length of 36 mm and weight of .90 grams compared to 29.28 mm and .50 grams for the tank with the highest larval density of 848 tautog at a stocking density of 15 fish/liter. The results of this study indicated that 15 gallon experimental tanks are suitable for rearing tautog larvae at low stocking densities. Larger systems would be required to raise tautog at higher stocking densities. Further studies should be conducted on larval tautog to determine stocking density in relation to optimum growth and survival. ™Trademarks are used to identify products only and are not indicative of endorsement by URI.

#### **PROGRESS IN BIOECONOMIC EVALUATION OF THE MILFORD LABORATORY SCALLOP NURSERY RECIRCULATING SYSTEM.** Gisele Magnusson and James Anderson, Environmental and Natural Resource Economics, University of Rhode Island, Kingston, RI 02881.

Costs and returns for a land-based recirculation nursery system for bay scallops (*Argopecten irradians*) were calculated and key economic factors affecting the financial viability of the system identified. The system under consideration was developed by the NMFS Milford Laboratory and incorporated a greenhouse for algae production with a re-circulating nursery to test several different filtration systems. A bioeconomic simulation incorporating the stochastic nature of key variables and the dynamic nature of an integrated production system was developed. Rudimentary hatchery and grow-out systems were incorporated to track costs and revenue impacts of various changes to the nursery system. Preliminary results of the model suggest that the average cost of algae from the greenhouse was within the range of published results. However, both the average cost of algae and the growth rate of juvenile scallops will have to change significantly to allow such a system to be financially viable. For the greenhouse system, labor, capital costs and nutrient costs are significant, while to the nursery, algae costs, labor and capital costs were most important. A value

for automation systems can be calculated based on the potential savings in labor and nutrient costs, over the base cost.

**AUSTRALIAN/TASMANIAN OYSTER CULTURE.** Harriette L. Phelps, University of the District of Columbia, Biology Department, 4200 Connecticut Ave., N.W., Washington, DC 20008.

In Australia, the two main cultured oyster species are the Sydney Rock Oyster (*Saccostrea commercialis*) and the Japanese Oyster (*Crassostrea gigas*). The Sydney Rock Oyster is presently cultured mostly in a few estuaries near Sydney and although the Japanese Oyster is found in some of those estuaries, the oyster farmers consider *C. gigas* highly detrimental because of competition for space from its earlier settlement pattern and faster growth.

The majority of commercial oysters are *C. gigas* spawned and grown in Southern Australia and the island of Tasmania. Tasmania is lightly settled and has numerous shallow estuaries with clean water mostly on the east coast and used for aquaculture. Presently, the oyster farmers send seed oysters for spawning to the oyster hatchery at Bicheno. The seed oysters are conditioned, spawned together, and the larvae raised in large tanks with some cultured algae addition. The oyster larvae set and transform on finely ground scallop shell added to the tanks. The young cultchless spat are returned to the farmers and reared in upwellers until transferred to bags set on trays in the shallow estuaries. The oysters are transferred to larger mesh bags until ready for sale at two years, at which time they are marketed mostly in coastal Australian cities as the live-shell product.

I saw several varieties of *C. gigas* being raised at one facility I toured: black, golden, striped-shell, etc. What I found interesting was that the central Bicheno hatchery made no attempt (unless requested) to separate the spawning oyster stocks, yet said they could tell which estuary where an oyster was raised in by its shape or other physical characteristics. Sometimes it was impossible to tell live *S. commercialis* from *C. gigas* except by the inner shell teeth of the Rock Oyster. However, the flavor was quite different.

**A COMPARISON OF ANTICLUMPING SOLUTIONS USED FOR INITIAL RECOVERY OF HEMOCYTES FROM THE BAY SCALLOP (*ARGOPECTEN IRRADIANS*).** Steven Pitchford and Richard A. Robohm, USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460.

Efforts to study the immune capabilities of bay scallop hemocytes *in vitro* were hampered by excessive clumping of cells upon withdrawal of hemolymph from adductor muscles. To resolve this problem, seven solutions used by others to prevent clumping of blood cells *in vitro* (five used in other invertebrate species and two in human medicine) were modified by adjusting

osmolality and pH to match that of scallop blood and examined for their ability to prevent scallop cell aggregation.

In brief, the protocol consisted of withdrawing hemolymph from scallop adductor muscles, dispensing it into each of the anticoagulant solutions in multi-chambered, glass, microscope slides and allowing the hemocytes to attach. This was followed by cell fixation. The number of single attached cells, small clumps (2–4 cells), and large clumps (> 4 cells) were counted in at least 15 fields for each of the anticoagulants. In addition, observations were made on the appearance and relative degree of attachment of the hemocytes to the substrate.

Results were collected using weighted values from three experiments; each experiment used hemolymph from three scallops—each exposed to all seven solutions. Dunnett's test for pairwise multiple comparisons showed that a modified solution, Adema's solution, previously used to prevent clumping in cells of a freshwater snail, was statistically superior to all but one of the other solutions. Three solutions developed by others for use with various molluscs were very poor in their ability to prevent clumping of scallop cells. Use of the best solution will be essential in many of our subsequent studies of bay scallop immunity.

**EFFECT OF DIETARY PH ON THE UTILIZATION OF SEMIPURIFIED DIETS BY TAUTOG, *TAUTOGA ONITIS*.** Laurel J. Ramseyer, National Academy of Sciences, USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460.

Experiments were conducted to determine whether semipurified diets would support growth in the tautog, *Tautoga onitis*, a stomachless fish. Semipurified diets contained 50% protein and either 7% or 12% lipid on a dry matter basis. A commercial salmonid feed containing 54% protein and 15% lipid on a dry matter basis was used as a reference feed. Semipurified diets were either as-is, with intrinsic pHs of 5.5–5.8, or with pH adjusted during preparation to 7.2–7.6. Experiments were conducted with 6–12 g tautog at 20°C for 28–31 d.

Tautog were fed the reference and the 7% lipid semipurified diets at rates providing 10, 12, or 14 g protein · kg<sup>-1</sup> body weight · d<sup>-1</sup>. Fish fed the semipurified diets required at least 14 g protein · kg<sup>-1</sup> body weight · d<sup>-1</sup> for weight gain, whereas fish fed the reference feed gained weight at all three feeding rates. Fish fed the pH 7.2 diet gained more weight than fish fed the pH 5.5 diet. However, when dietary lipid was increased to 12%, weight gain of fish fed the lower pH diet was not significantly different from weight gain of fish fed the pH 7.6 diet or the reference feed. The pH of digesta was 8.7–9.1 throughout the gut regardless of dietary treatment. The results indicated that the alkalization of digesta in tautog is an energy-dependent process. Semipurified diets supported growth in tautog, but should be alkalized to a standard pH before use in tautog nutrition studies.

**VIBRIO PARAHAEMOLYTICUS—A NEW PROBLEM FOR THE SHELLFISH INDUSTRY IN THE NORTHEAST.** David R. Relyea, Frank M. Flower and Sons Inc., P.O. Box 88, Oyster Bay, NY 11771.

During the time period between 8/10/98 and 8/29/98, eight cases of gastroenteritis occurred and were eventually reported to the New York State Department of Environmental Conservation (NYSDEC). The illnesses occurred in Nassau and Suffolk counties in New York (6) and (2) cases were from New Jersey. Stool samples from patients indicated that the illnesses were caused by a naturally occurring marine bacterium, *Vibrio parahaemolyticus*. Tagging information seemed to indicate that the source of the bacteria was oysters and clams from area NS2 which includes Oyster Bay and Cold Spring Harbor. However, most patients had also eaten other seafoods (crabs, shrimp, etc.) that are known to be sources of *Vibrio parahaemolyticus*. Health Department officials claimed that the only food common to all patients was shellfish from NS2. New York State Department of Health (NYSDOH) and USFDA notified NYSDEC that NS2 had to be closed and NS2 was closed to shellfishing on 9/10/98.

Due to confusion and insufficient Federal guidelines the area was not able to be reopened until 10/22/98. During that time Frank M. Flower and Sons with 40 employees and about 50 individual baymen had no source of income. This presentation gives the industry perspective of this perplexing problem.

**NOAA FISHERIES AND AQUACULTURE.** Edwin Rhodes, USDOC, NOAA, National Marine Fisheries Service, 1315 East-West Highway, Silver Spring, MD 20910.

Aquaculture has played a significant role in NOAA Fisheries and its predecessor agencies since their origins in the 19th century. The continuing efforts by the agency in its 127 year history contributed some of the key science in the field of aquaculture, including research that contributed to the commercial development of salmon, shrimp and shellfish culture.

Since the 1980's, agency priorities in the area of fisheries management, coupled with budget limitations, have restricted the participation of NOAA Fisheries in aquaculture. Recently, aquaculture has reemerged as an important consideration as NOAA Fisheries plans for the new century. This planning and policy development stage is critical because it is through this process that agency priorities are set and budgets are driven. The NOAA Fisheries strategic plan has as one of its objectives to "promote the development of robust and environmentally sound aquaculture" and outlines specific goals in the areas of technology development, siting, permitting and financial assistance. Partly based on this plan, the Northeast and Northwest Centers of NOAA Fisheries have reorganized to include aquaculture divisions, and new aquaculture industry financing programs are being developed.

At the NOAA level, NOAA Fisheries, the National Ocean Ser-

vice and the Office of Oceanic and Atmospheric Research have collaborated to put a new aquaculture policy in place that recognizes the significant role that environmentally sound aquaculture will play in meeting future demand for seafood, as well as the potential to contribute to wild stocks through enhancement. The NOAA policy also foresees a major aquaculture effort for the production of non-food products such as bait, aquaria species, chemicals and pharmaceuticals.

Finally, an active task force is developing a Commerce-wide policy for aquaculture, and its formulating plans to facilitate aquaculture permitting in the U.S. exclusive economic zone. This policy and planning activity has helped to generate a new interest in aquaculture in the Department and the recognition of the potential for aquaculture speaks to an optimistic future.

**CONTROL OF EUTROPHICATION BY BIVALVES: FILTRATION OF PARTICULATES AND REMOVAL OF NITROGEN THROUGH HARVEST OF RAPIDLY GROWING STOCKS.** Michael A. Rice, Department of Fisheries, Animal and Veterinary Science, University of Rhode Island, Kingston, RI 02881.

Filter feeding by populations of bivalves has been suggested as a means of reducing eutrophication in coastal estuaries by exerting control of phytoplankton populations in the water column. In some estuaries, programs have been established for the purpose of improving water quality and, frequently, large populations of mature shellfish that reside behind pollution closure lines in estuaries represent a large filter feeding biomass. The rate of filter feeding by bivalves is size dependent and allometrically related to shell dimensions, so the largest and oldest individuals filter the greatest volumes of water. In most areas closed to shellfishing, bivalve populations are composed of mostly older adults. These large animals are slow growing, have a low rate of new tissue production in relation to standing crop biomass, and have a neutral nitrogen balance (organic-N assimilated =  $\text{NH}_3\text{-N}$  excreted). These large adults increase sedimentation through filter feeding, but since they are neither harvested nor growing rapidly, they do not directly remove much nitrogen from the estuary. Although it is possible that increased sedimentation can lead to greater denitrification rates in the sediments. The only way filter feeding can directly remove nitrogen from the environment is through tissue growth. The dry weight of the soft tissues of most bivalves is typically around 30% protein, so for each kilogram of shucked shellfish meats harvested there are 16.8 grams of organic nitrogen removed from the estuary. Nutrient removal from estuaries can be maximized through management of shellfisheries for maximum biomass production and harvest, and the development of aquaculture projects in which rapidly growing shellfish are harvested regularly. This is publication 3681 of the College of the Environment and Life Sciences, University of Rhode Island.

**SUMMER FLOUNDER CULTURE IN THE NORTHEAST: UPDATE ON RECENT RESEARCH AND INDUSTRY STATUS.** **Gregg Rivara**, Cornell Cooperative Extension-Suffolk County, 3690 Cedar Beach Road, Southold, NY 11971; **David A. Bengtson**, Department of Fisheries, Animal and Veterinary Science, University of Rhode Island, Kingston, RI 02881.

The Northeastern Regional Aquaculture Center and Sea Grant have been sponsoring research on summer flounder as an emerging aquaculture species in the Northeast. As part of outreach and extension efforts, a workshop is being held for industry just prior to the 19th Milford Aquaculture Seminar. Researchers are presenting their results to industry at that workshop and also describing the future research for which they have received funding. A roundtable discussion involving researchers and industry is also part of the workshop so that industry can describe their research needs. The industry is still small, but is making progress and starting to sell product. A summary of the workshop activities, major research findings and industry status will be provided to the participants at the 19th Milford Aquaculture Seminar.

**AN OVERVIEW OF AQUACULTURE RESEARCH IN ATLANTIC CANADA.** **Shawn M.C. Robinson**, Dept. Fisheries and Oceans, Biological Station, St. Andrews, New Brunswick, Canada, E0G 2X0.

The aquaculture industry in Canada is in a growth phase. Since 1986, shellfish culture has grown in production volume at an annual rate of 10% and finfish culture has grown at an annual rate of 28%. This rapid growth in the industry has fueled a push in research to support the development of existing species in culture as well as to bring new species on-line. There is also active research on factors that affect the industry such as disease and environmental interactions.

In Atlantic Canada, the lead federal agency for aquaculture research is the Dept. of Fisheries and Oceans. Its role is to provide scientific knowledge for the sustainable development of aquaculture including the development of an economically competitive and environmentally sustainable industry. The research laboratories are located at the Biological Station in St. Andrews, New Brunswick, the Gulf Fisheries Centre in Moncton, New Brunswick, the Bedford Institute of Oceanography in Dartmouth, Nova Scotia and the Northwest Atlantic Fisheries Centre in St. John's, Newfoundland. However, there are other major research organizations in Atlantic Canada as well such as: 1) provincial aquaculture agencies in Newfoundland, Nova Scotia, Prince Edward Island, New Brunswick and Quebec 2) the National Research Council-Institute for Marine Biosciences in Halifax Nova Scotia 3) the universities (UNB, Moncton, UPEI-AVC, NSAC, Acadia, Dalhousie, Laval, Quebec and Memorial) 4) the Canadian Centre for Fisheries Innovation and 5) the industry itself including the provincial aquaculture associations. The majority of the research is done in collaboration with industry partners.

Research on finfish has been strongly emphasized to date. Atlantic salmon is the most commercialized species so far and much of the early developmental work has been done. Research on this species is ongoing in the Bay of Fundy and Newfoundland and is concentrating on broodstock (new strains, transgenics), fish health (record of performance, husbandry practices, therapeutants), nutrition (area and species specific diets), grow-out technology and environmental linkages (freshwater discharge, waste management). There are also a number of new finfish species that are being studied such as: halibut, haddock, winter flounder, striped bass, steelhead and American eels. These studies are going on primarily in Newfoundland, Nova Scotia and New Brunswick. The scope of research on these new species falls into four categories: 1) Broodstock/seedstock (environmental influences on maturation, influence of diets, prediction of maturation, capture techniques) 2) Fish health (identification and life histories of diseases and parasites, baseline data on normal fish) 3) Nutrition (nutritional requirements, culture techniques for native plankton, micro-encapsulated larval diets, larval feeding behaviour) and 4) Grow-out (early rearing techniques, tank and grow-out designs, refinement of automatic feeding techniques, *in situ* estimation of fish size in cages).

Shellfish research is active in all provinces. The industry is presently mostly located in Newfoundland, the Atlantic coast of Nova Scotia and the Gulf of St. Lawrence although it is starting to grow in the Bay of Fundy. In general there are four research areas being targeted: 1) Broodstock/seedstock (hatchery development, natural spat collection) 2) Shellfish health (identification of diseases and parasites, diagnostic tools and treatments) 3) Grow-out (optimize rearing of juveniles, seeding densities, predator control, roe enhancement) 4) Environment (environmental effects on growth and survival, site selection, carrying capacity, effects of winter ice). Species being studied are: blue mussels, sea scallops, American oysters, European oysters, hard-shell clams, soft-shell clams and sea urchins. There is also some work being done on bio-fouling species such as tunicates.

There is a small program on algal research. Programs are underway on the dynamics of phytoplankton blooms and some shell fish sites are being monitored for toxic algal effects. Grow-out trials are being done on some macro-algal species such as dulse (*Palmaria palmata*), nori (*Porphyra* spp.) and kelp (*Laminaria longicruris*).

As the marine culture industry develops, there is an increasing research effort being directed toward the linkages between the commercial culture of various species and the environment. Oceanographic modeling techniques are being developed for area management strategies, site assessment and remediation techniques are being studied, and practical methods for monitoring by the industry are being developed.

Past research has contributed substantially to the development of the Atlantic Provinces aquaculture industry and there is strong support from industry for work in the future. The major research impediment to-date has been securing reliable long-term research funds.

**POST-METAMORPHIC GROWTH OF SUMMER FLOUNDER IN LABORATORY CULTURE: DO EARLY-SETTLING LARVAE GROW FASTER THAN LATE SETTLERS?** Tessa L. Simlick, Robin S. Katersky, Neil Marcaccio, and David A. Bengtson, Department of Fisheries, Animal and Veterinary Science, University of Rhode Island, Kingston, RI 02881.

Laboratory-reared summer flounder larvae begin to settle to a benthic existence 30 to 35 days after hatching but settlement can continue for about a 30-day period, because completion of metamorphosis among individuals does not occur simultaneously. We perform weekly gradings (i.e., removal of settled flounder) until all fish have settled in order to prevent cannibalism and stress, because newly settled juveniles tend to be larger than swimming larvae. Although we know there is a strong correlation between larval growth and time of settlement (fastest growers settle first), no data exist on post-settlement growth variability. We wanted to know whether fast-growing larvae become fast-growing juveniles or whether slow-growing larvae can 'catch up' in growth rate. Experiments were designed and conducted at the Narragansett Bay Campus Research Facility to explore these inquiries. Settled fish were graded from the larval tank at 32 days after hatch (DAH) (Grade 1), 39 DAH (Grade 2), and 46 DAH (Grade 3). Graded fish were randomly placed in three replicate 75-L aquaria per grade, at a density of 30 fish per aquarium. Flounder were fed *Artemia* for 30 days after removal from the larval tank and were then weaned onto a commercial diet. All fish were measured by Image Analysis at bi-weekly intervals until the fish were 95 DAH. No significant differences in post-settlement growth rate were seen among the three grades. In the final set of measurements, the fish exhibited an increase in size variation within replicates and cannibalistic attacks were again causing mortality. Future experiments will continue to investigate specific growth rate variation in all stages of juvenile growth.

**FERTILIZATION RATES AND PROCEDURES USING COMMERCIAL "F/2" NUTRIENT MIXES TO GROW T-ISO (*ISOCHRYSIS SP.*) AND PLY429 (*TETRASELMIS CHUI*).** Barry C. Smith, Sara Barcia, Jennifer H. Alix, and Gary H. Wikfors, USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460.

Two developments intended to make microalgal feed culture more convenient have collided. First, pre-mixed concentrated nutrient products, formulated according to Guillard's "f/2" recipe, are entering wide use; these products are sold in two solutions that are kept separate to avoid chemical complex formation until used to make culture media. The second development is the use of automated methods of adding nutrients to culture water, e.g., metering pumps and venturi eductors. The two-part nutrient mixes will require duplicate apparatus for their automated dispensing, increas-

ing technical complication, chances for malfunction, and cost. A simple solution (pun intended) to this dilemma would be a dilution with water of the combined two-part product that would remain stable. Several dilutions of combined f/2 concentrates were observed over two weeks for visible precipitates. Then, culture media were prepared with the concentrate dilutions, and their ability to support growth of T-ISO and PLY429 was compared to freshly-prepared f/2 of two brands. In a nested Analysis of Variance design, four concentrate dilutions (Part A:Part B:water, 50:50:0, 40:40:20, 30:30:40, 20:20:60) were used to prepare four final nutrient media (f/4, f/2, f, 2f) each. Algal division rates were calculated from optical density readings of triplicate test-tube cultures, and final population densities were determined by microscope cell counts.

All nutrient dilutions precipitated but were re-dissolved easily. For *Tetraselmis chui*, PLY429, nutrient pre-dilution and final concentration had significant effects upon division rate and final population. Maximal division rates were higher when nutrient solutions were pre-diluted, and significantly higher in the 2f media as compared with lower enrichments. Final cell densities tended to be higher when nutrients were not pre-diluted, especially at lower fertilization rates. Predictably, higher fertilization rates led to higher final cell yields, but a nitrogen budget analysis showed nearly half of the added nitrate was not taken up by PLY429 at the 2f enrichment. For *Isochrysis sp.*, T-ISO, division rate was highest in the most pre-diluted nutrients and lowest at the highest fertilization rate. Cell yield of T-ISO tended to be higher in nutrient mixes less pre-diluted and was significantly lower in the f/4 enrichment compared with higher fertilization rates. Nitrogen budget analysis of T-ISO cultures showed that nitrate remained unassimilated at all concentrations above the f/4 enrichment.

Indications from these experiments are: 1) enrichments above the "f" level for PLY429 and above "f/4" for T-ISO result in wasted nutrients, suggesting that some other nutrient (perhaps vitamins) limited T-ISO in these experiments; 2) pre-dilution of two-part commercial algal fertilizers increases maximal division rate, but not final cell yield, suggesting that chemical complexation is reversed during algal growth, making nutrients available to algae over time. Pre-dilution of combined two-part algal fertilizer products can affect performance of cultures; the decision to pre-dilute will depend upon whether cultures are being optimized for rate or yield.

**UPDATING THE PLANS FOR SEA SCALLOP AQUACULTURE IN MASSACHUSETTS.** Ron Smolowitz, Coonamessett Farm, 277 Hatchville Rd., East Falmouth, MA 02536; and Harlyn Halvorson, UMASS-Boston, ECOS, College of Arts and Sciences, 100 Morrissey Blvd., Boston, MA 02125.

The Sea Scallop Working Group (SSWG) was started five years ago to provide a forum for discussion of interests in aquaculture among various stakeholders. These discussions led to the

development of a Blueprint for Sea Scallop Aquaculture in 1995. A SSWG summit meeting was held February 8–9 at the Massachusetts Maritime Academy to evaluate our progress to date on various sea scallop projects and to review the problems encountered. Through the use of breakout groups, the recommendations of the 1995 Blueprint were reexamined and priorities set for SSWG activities in the coming year. The results of this summit meeting will be reviewed.

**METHODOLOGY FOR THE GENERATION OF POLYMORPHIC MOLECULAR TAGS IN THE BAY SCALLOP, *ARGOPECTEN IRRADIANS*.** Jeff Southworth, Maronda Brown, Department of Molecular & Cellular Biology, University of Connecticut, Storrs, CT 06269; Sheila Stiles, USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460; and Linda Strausbaugh, Department of Molecular & Cellular Biology, University of Connecticut, Storrs, CT 06269.

Several promising species for aquaculture lack genetic and morphological markers. Consequently, there is a critical need for the development of physical and genetic tags for monitoring and identifying brood stocks. We are examining methods to differentiate bay scallop populations (*Argopecten irradians*) at the genotypic level using Type I and Type II markers. We have chosen to investigate the potential of a Polymerase Chain Reaction (PCR), and Random Amplification of Polymorphic DNA (RAPD) [similar to DNA Fingerprinting (DAF)] or Arbitrarily Primed PCR (AP-PCR) techniques.

We have examined levels of polymorphism within and among 5 populations of bay scallops collected from the United States northeast coast. Approximately twenty PCR primers were analyzed for their effectiveness at revealing polymorphisms among individuals and populations of this marine organism. A significant amount of time was spent optimizing the primers and the PCR conditions to obtain clearer, more consistent results. We have determined that there is a high degree of polymorphism at the level of individual organism genotype. In addition, RAPD analysis is reliable and reproducible, as well as extremely sensitive. In a complementary approach we have selected coding regions that might provide species and/or strain-specific markers as well as promoters for genetic engineering applications. Additional investigations include the design of core histone gene primers from both *Drosophila melanogaster* and *Strongylocentrotus purpuratus* to screen a genomic library of both *Placopecten magellanicus* (sea scallop) and of the bay scallop *Argopecten irradians*. Development of molecular tags can provide a screen for genetic diversity, ultimately circumventing inbreeding depression.

**DISEASE-RESISTANT OYSTERS, *CRASSOSTREA VIRGINICA*, IN LONG ISLAND SOUND.** Inke Sunila, John Volk and John Karolus, State of Connecticut, Department of Agriculture, Bureau of Aquaculture, P.O. Box 97, Milford, CT 06460; Terry Backer, Long Island Soundkeeper Fund, Inc., P.O. Box 4058, East Norwalk, CT 06855; Stan Czyzyk, Bluepoints Co., Inc., Atlantic Avenue, P.O. Box 8, West Sayville, NY 11796; Ed Lang, P.O. Box 314, Clinton, CT 06413; Matt Mroczka, Cedar Island Marina Research Laboratory, P.O. Box 181, Clinton, CT 06413; Karen Rivara, AEROS Cultured Oyster Company, 41 Heathcote Court, Shirley, NY 11967.

Under heavy infection pressure, oysters develop resistance to parasitic diseases such as MSX (*Haplosporidium nelsoni*). Resistant oysters still get infected, but their mortality rate is lower than that of susceptible oysters. Genetic resistance can be developed against other economically important oyster diseases such as Dermo-disease (*Perkinsus marinus*) or JOD (Juvenile Oyster Disease).

A MSX-epizootic, associated with high mortalities in some areas, raged in Long Island Sound (LIS) starting in 1997. Hatchery-raised, highly susceptible seed experienced 99% mortality. Connecticut's commercial oyster companies were advised to increase the prevalence of resistant oysters in two different ways: 1. Establishing brood stock sanctuaries in heavily infected sites to permit survivors to produce resistant seed. This could be done by not harvesting part of the infected lot (10% area) for a period of three years, and 2. Selecting disease-resistant strains when using hatchery-raised seed.

The prevalence of potentially MSX-resistant oysters, based on histological characteristics, in the field increased eightfold from 1997 to 1998 on previously exposed sites. A cooperative program was initiated with production hatcheries to produce a commercially available, disease-resistant oyster seed especially bred for Long Island Sound conditions. Present management strategies respect traditional oyster culture methods, which include deployment of hatchery-raised seed concurrently with natural set. This gave rise to the need of developing a hatchery stock with a spawning cycle compatible with wild oysters. Spawning time is an inherited characteristic, which is maintained upon transplantation. The new oyster strain would have the characteristics for spawning time, growth, temperature and salinity tolerance and hardiness of the parent population in LIS. In addition, it would have been selected for disease resistance. Brood stock was created from survivors of 90% mortality (83% MSX prevalence, 100% Dermo prevalence) from Clinton, Connecticut. A commercially available, local seed was used as a control. "Clinton" strains have been tested since the spring 1998 in an infected location for growth, mortality and infection rates. During the first season, "Clinton" had a 16% higher growth rate than the control. Both strains acquired MSX and Dermo infection. "Clinton" experienced a 1% mortality, control 11%. The first generation



“Clinton” showed superior survival and growth characteristics. This seed has been deployed in commercial scale at several sites in LIS. Grow-out facilities have been established to grow hatchery-raised seed prior to dispersing it to the field. Brood stocks are exposed in different locations to other oyster diseases such as Dermo, JOD and SSO (Seaside Organism or related species).

**REFLECTIONS ON BIOFILTER SELECTION FOR SHELLFISH CULTURE.** James C. Widman Jr., USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460.

Selecting biofilters for shellfish recirculating systems can be a perplexing process. Many of the biofilters used in finfish recirculating systems have numerous disadvantages when the requirements of shellfish culture are considered. One of the main requirements is live phytoplankton as a food source. Many commercially available biofiltration systems are capable of trapping and removing phytoplankton-size particles. Finfish systems, on the other hand, are designed to remove fecal material and unused food from the water stream. Many of them also use high flow rates essential to many finfish species but usually detrimental to shellfish. These systems must be modified before being considered for shellfish recirculating systems.

Bead filters are effective at removing and trapping phytoplankton size particles and sand-bed filters have similar drawbacks. Protein skimmers/foam fractionators not only remove organics, but also phytoplankton-size particles. Numerous drum filters capable of removing fish wastes do not appear to be appropriate for the fragile waste products of shellfish; some mesh sizes also remove phytoplankton. Many of these systems are still undergoing modifications and may eventually evolve into effective shellfish biofilters in the future.

There are systems that appear to satisfy the requirements of shellfish culture today. Many of these systems act as contact filters, basically providing large surface areas for nitrifying bacteria to grow. Rotating biological contact filters (RBC's), Aquacube, moving bed biofilters, and submerged panels are examples of this technology.

Trickle filters and fluidized sand bed filters may be effective, but may be subject to fouling, or damage to the phytoplankton. Caution must be exercised when biofilter specifications mention the removal of solids. Pads, mats, foams and types of trickle filter media can become fouled with both waste material and phytoplankton, making them less efficient at nitrogen removal.

**FEEDING RATIONS AND REGIMES FOR POST-SET OYSTERS, *CRASSOSTREA VIRGINICA*, FED CULTURED MICROALGAE IN A LAND-BASED NURSERY.** Gary H. Wikfors, Jennifer H. Alix, Mark S. Dixon, and Barry C. Smith, USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460.

Filter-feeding bivalves, such as the eastern oyster, have evolved feeding behaviors that respond to changes in both quantity and quality of suspended particles. In near-shore waters, particle loadings are highly variable and beyond the control of the shellfish farmer relying upon natural primary production to feed his or her seed oysters. The risks associated with “raw water” nursery culture of oysters (poor nutrition; exposure to pollutants, disease, and predators; vandalism), and seasonal limitations, eventually will exceed the costs of controlled, land-based nursery culture. As this occurs, information needs about oyster feeding will shift from describing responses to varying environmental conditions to providing feeds in a way that optimizes their use by the spat. Feeding standards developed for animal agriculture that list daily nutritional input, in biochemical terms, and growth obtained on these specific diets, represent a useful model for aquacultured animals. For filter feeders, however, the “daily allowance” concept is complicated somewhat by the behavioral responses to particle loadings mentioned above.

To begin the process of identifying practical feeding rations and regimes for post-set oysters, we conducted an experiment in which daily rations were varied (1, 2, 5, or 10% of oyster live weight in dry matter of feed) and each ration was provided in 2, 4, or 16 feedings each day; a 50:50 mix of two high-lipid *Tetraselmis* strains, PLY429 and PLAT-P, was used for all experimental treatments. Oyster growth was determined weekly—in terms of live weight, volume displacement, and shell size—and at the end of the seven-week experiment in terms of dry weight. Feed conversion efficiency was calculated from change in oyster dry weights and the sum of algal dry weight feed provided during the experiment.

Oysters (65 mg live weight initially) grew progressively faster on 1, 2, and 5% rations, regardless of regime, but there was no significant increase in growth when the ration was increased to 10%. Oysters grew fastest when fed most often, but the statistical significance of this effect was dependent upon ration, e.g., at the highest ration, effect of regime was not significant. Feed conversion efficiency was inversely related to growth, and was in the range of 2–15% from high to low rations. Results of this experiment indicate that the optimal feeding ration for oysters on a qualitatively suitable diet will lie within the range of 2–5% of live weight in dry matter per day, and that providing the daily ration in multiple daily feedings becomes more important at lower rations. These findings confirm results obtained previously with bay scallops, *Argopecten irradians*, in that maximal growth is obtained on daily rations between 2 and 5% and multiple daily feedings improve growth. Oysters, however, with a maximal conversion effi-

6tyciency  
of less than 20% appear to be less efficient at converting  
feed to growth than scallops which can achieve a conversion ef-  
ficiency of 24% with identical nutritional input.

**FEEDING STUDIES ON JUVENILE TAUTOG, TWO EXPERIMENTS: WEANING JUVENILE TAUTOG TO AN ARTIFICIAL DIET AND EFFECTS OF FEEDING FREQUENCY ON GROWTH OF JUVENILE TAUTOG.** Steve Yankocy, Grace Klein-MacPhee, and Aimee Keller, University of Rhode Island, Graduate School of Oceanography, Narragansett Bay Campus, Narragansett RI 02882-1197.

Focus was placed on reducing costs and labor by enhancing growth through selection of a good commercial diet for juveniles and determining the best feeding schedule. Two feeding experiments were conducted on juvenile tautog with the goal of finding an optimum feeding regime. The first experiment dealt with the type of food which would be consumed by the fish. The experiment utilized two types of food: Kyowa™ brand dry food and live brine shrimp. Three different feeding regimes were used. One group was fed Kyowa, one live brine shrimp and the third a com-

bination of brine shrimp and Kyowa. There were three replicates of each treatment. The fish were weighed and measured prior to the experiment with each tank receiving an equal weight of fish. The experiment lasted two weeks. The fish fed the Kyowa diet were larger than those fed brine shrimp or a combination of both, however the results were not statistically significant. Based on the cost of brine shrimp cysts and the extra effort to hatch and enrich them, the kyowa diet was more economical.

The second experiment dealt with feeding frequencies. Feeding schedule has been shown to influence growth patterns or food conversion rates significantly in a number of species. Three groups were set up all of which would receive the same amount of Kyowa brand dry food on different feeding schedules. One group received two feedings a day, the second four feedings a day and the third six feedings a day. The fish were weighed and measured at the start, and there were three replicates of each treatment. Feedings were done by hand and with the help of mechanical feeders. Fish fed four and six times a day were significantly larger than fish fed twice a day, but were not significantly different from each other. ™Trademarks are used to identify products only and are not indicative of endorsement by URI.

**ABSTRACTS OF TECHNICAL PAPERS**

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## BIOLOGY AND MORPHOLOGY OF SHELLFISH

### THE PRESENCE OF THE TURBELLARIAN *URASTOMA CYPRINAE* FROM DIFFERENT AREAS OF THE GILLS OF THE EASTERN OYSTER *CRASSOSTREA VIRGINICA*.

**Nicole T. Brun** and **Andrew D. Bogen**, Department of Biology, Université de Moncton, Moncton, NB, Canada E1A 3E9; **Jacques Allard**, Department of Mathematics and Statistics, Université de Moncton, Moncton, NB, Canada E1A 3E9.

*Urastoma cyprinae* is a turbellarian that occurs on the gills of various bivalve species. Recent investigations indicate that *U. cyprinae* can induce pathology to the gill tissue of mussels. In Atlantic Canada, this worm has been reported from the gills of the Eastern Oyster *Crassostrea virginica*. Past work has demonstrated that *U. cyprinae* is attracted to oyster mucus on which it may be actively feeding. The present study focuses on the distribution of *U. cyprinae* in oysters in relation to certain properties of mucus associated with different areas of the gills. The worms occur in higher numbers along the basal food tract compared to the medial and ventral regions of the gills. *U. cyprinae*'s preference for this site may be explained by a number of factors, including the fluidity and accessibility of the mucus, as well as the protection provided compared to more exposed parts of the gills when the oysters are actively feeding.

**CILIA AND MUCOCYTES ON THE ABFRONTAL SURFACE OF BIVALVE GILLS.** **Suzanne C. Dufour**, Scripps Institution of Oceanography, University of California, San Diego, La Jolla, CA 92093-0202; **Peter G. Beninger**, Laboratoire de biologie marine, Université de Nantes, Nantes cédex 3, France.

The lack of fundamental data on the abfrontal surface of bivalve gills has prompted a comparative study of cilia and mucocytes on this surface. These features have been studied by scanning electron microscopy and histology on eight species of bivalves, representing seven families and the four major gill types (*Mytilus edulis*, *Modiolus modiolus*, *Arca zebra*, *Placopecten magellanicus*, *Crassostrea virginica*, *Spisula solidissima*, *Mya arenaria* and *Mercenaria mercenaria*). Inter-species variations were found; gradients in the numbers and diversity of cilia and mucocytes were observed for each gill type. These results seem to indicate that the abfrontal surface had a primitive role in mucociliary cleaning (prior to filament folding), and that the cilia and mucocytes here observed are vestigial. The loss of this primitive function brought forth two possibilities: 1. selective pressures led to the reduction in numbers and types of abfrontal mucocytes and cilia; 2. the abfrontal cilia and/or mucocytes were retained as they assumed new functions. In general, the degree of loss of abfrontal cilia and mucocytes follows the degree of evolution of the gill: eulamellibranchs have less abfrontal cilia and mucocytes than homorhabdic filibranchs.

**HABITAT AND PREY PREFERENCES OF VEINED RAPA WHELKS (*RAPANA VENOSA*) IN THE CHESAPEAKE BAY: DIRECT AND INDIRECT TROPHIC CONSEQUENCES.** **Juli M. Harding** and **Roger Mann**, Virginia Institute of Marine Science, Gloucester Point, VA 23062.

The recent discovery of Veined Rapa Whelks (*Rapana venosa*) in the lower Chesapeake Bay has ecological consequences beyond the obvious potential for predation on commercially valuable shellfish prey species (e.g., *Crassostrea virginica*, *Mercenaria mercenaria*). In the Black Sea and in their native Sea of Japan, *Rapana* have been reported primarily from hard bottom habitats. Adult Chesapeake Bay *Rapana* have been collected from both hard and soft bottom habitat. Laboratory observations indicate that adult *Rapana* prefer sand bottom and will burrow almost completely into the sand at water temperatures >20 C (i.e., not overwintering behavior). Burrowing behavior by these large apex predators expands the potential suite of vulnerable prey items to include infaunal shellfish (e.g., *Mya arenaria*, *Ensis directus*, *Cyrtopleura costata*). The presence of large (>100 mm) empty *Rapana* shells in Chesapeake Bay may enhance growth of the local hermit crab (*Clibanarius vittatus*). Recent collections of *Clibanarius vittatus* from the Hampton Roads area indicate they use empty *Rapana* shells as shelters and are reaching previously unrecorded sizes. The implications of abnormally large crustacean scavengers on Chesapeake Bay benthic epifauna (e.g., oyster spat) are discussed.

**DEAD SCALLOPS DO TELL TALES: ARCHAEOLOGICAL BAY SCALLOP MORPHOLOGIES AND COMPOSITION OF ANCIENT METAPOPOPULATIONS.** **Dan C. Marelli** and **William S. Arnold**, Florida Department of Environmental Protection, Florida Marine Research Institute, 100 8th Avenue SE, St. Petersburg, FL 33701-5095.

Shell middens are conspicuous features of some sites along the Florida Gulf coast, representing thousands of years of human exploitation of marine resources. The bay scallop (*Argopecten irradians*) was extensively harvested by prehistoric Floridians, but many populations of Floridan bay scallops have declined precipitously during the past 4 decades probably because of the effects of overharvest, habitat alteration, water quality degradation, and toxic algal blooms. Traditional metapopulations may lose stability in Florida because of the collapse of large local populations that traditionally acted as sources for less temporally consistent local populations. Unfortunately, because of our late start on the problem, much of the useful information from local populations that would allow us to reconstruct the traditional metapopulations may already be lost. Some backcasting of past populations structure is possible using biochemical techniques, but the only uncorrupted data we have are the scallop shells left behind by the original

Floridians. Examining morphologies of archaeological scallops, comparing these morphologies with those of modern scallops, and referencing allozyme data will allow us to speculate on what ancient metapopulations may have looked like and how we can use this information in attempting to enhance or restore local populations of bay scallops in Florida.

**BREAKING STRENGTH AND FAILURE ENERGY OF *M. EDULIS* SHELLS.** Laurie A. McDuffee and T. Jeffrey Davidson, Department of Health Management, and William P. Ireland, Department of Anatomy and Physiology, Atlantic Veterinary College, University of Prince Edward Island, Charlottetown, PEI C1A4P3.

Mussel processors on Prince Edward Island (PEI) have estimated that approximately 3% of marketable mussels entering the processing line have to be culled due to cracked or broken shells. The mussel processing sector also observed that mussels from certain estuaries have a greater percentage of broken/cracked shells during processing compared to other areas. With PEI's mussel industry worth \$24 million a year, this problem represents an annual loss to the industry of \$720,000.

We hypothesized that one reason for differences in shell cracking may be differences in mechanical properties of mussel shells between estuaries. Because mechanical properties are influenced by the geometry, we also hypothesized that measurements of shape and size would effect mussel breaking strength.

Mussels (sp. *M. edulis*) were obtained from PEI Department of Fisheries and Tourism who collected mussel samples from seven mussel growing areas of PEI. Mussels were cleaned, measured with an electronic caliper, and weighed. Measurements included length, width, height, weight, and thickness of the shells at the highest point. Shells were separated and meats removed. The half shells underwent biomechanical testing in a materials testing system under a single compressive load at a rate of 0.07 cm/sec. Mechanical properties and failure configurations were determined. Mechanical variables of interest included the maximum failure load, (also considered breaking strength), failure energy, and stiffness. These variables were obtained from the load deformation curves generated for each specimen. Maximum failure, or breaking strength, was defined as the highest load that the shell could withstand before breaking. Maximum failure energy was determined from the area under the load deformation curve to the maximum failure point. Stiffness was calculated as the slope of the linear region of the load deformation curve. Mechanical properties between estuaries were compared statistically. There were significant differences in breaking strength and failure energy between the various mussel growing areas of PEI. For instance, it required almost twice the force to break a shell from March Water than it did from the Brudenell River.

**EFFECTS OF TEMPERATURE ON THE HEART AND VENTILATION RATES OF THE AMERICAN LOBSTER (*HOMARUS AMERICANUS*).** S. M. C. Schreiber, S. Krull, S. H. Jury, and W. H. Watson III, Dept. of Zoology, University of New Hampshire, Durham, NH 03824.

The behavior of lobsters is strongly influenced by the temperature of their surroundings. However, the underlying physiological responses to temperature are not well understood. In this study, we examined the effects of a series of 5°C temperature increases on the heart and ventilation rates of intact animals and isolated hearts. Heart rates of intact animals increased continuously over the range of 5–25°C. However, this increase was non-linear, with  $Q_{10}$  values decreasing from near 2.5 at the low end to near 1.5 at the high end of the range. Ventilation rates were strongly correlated with heart rates. In contrast, the isolated hearts showed very little response to temperature.  $Q_{10}$  values for isolated hearts were markedly lower than those in intact animals for all temperature ranges. Their maximum rates were exhibited between 14–19°C. At higher temperatures, beat rate decreased dramatically and, in many instances, the heart failed. Basal heart rates of isolated hearts were significantly lower than those of intact animals at all but the lowest temperatures, suggesting that there is modulation occurring in the whole animal that is absent in the exposed preparation. Currently, we are carrying out experiments to determine if the effects of temperature on heart activity are mediated by the cardioregulatory nerves or by neurohormones.

**HAEMOLYMPH FREE AMINO ACIDS IN THE NORWAY LOBSTER (*NEPHIOPS NORVEGICUS* L.) AND CHANGES ASSOCIATED WITH INFECTION BY THE DINOFLAGELLATE PARASITE *HEMATODINIUM*.** Grant D. Stentiford and Douglas M. Neil, Division of Environmental & Evolutionary Biology, Institute of Biomedical & Life Sciences, University of Glasgow, G12 8QQ, Scotland, UK.; Graham H. Coombs, Division of Infection and Immunity, Institute of Biomedical & Life Sciences, University of Glasgow, G12 8QQ, Scotland, UK.

Changes in plasma free amino acid (FAA) concentrations have been used to assess various stresses, including disease, in a number of animal groups. However, with the exception of osmotic stress studies, this measure has received relatively little attention in Crustacea, despite their large tissue FAA pools. Norway Lobsters (*Nephiops norvegicus* L.) on the west coast of Scotland undergo a seasonal infection by a parasitic dinoflagellate (*Hematodinium* sp.) and this systemic disease causes gross histopathological changes in host tissue, eventually leading to death. We therefore set up a study to investigate the profile of FAA compounds in normal and *Hematodinium*-infected *Nephiops*. Results show an almost doubling of FAA compounds during severe infection, with most of this increase being due to the sulfonic amino acid taurine. In heavily infected animals, plasma taurine increased both absolutely (from 0.22  $\mu\text{mol} \cdot \text{ml}^{-1}$  to 2.56  $\mu\text{mol} \cdot \text{ml}^{-1}$ ) and relatively (from 5.8% to

40% of total identified FAA) when compared with healthy animals, and also made the dominant contribution to the observed rise in the non-essential/essential plasma FAA ratio. We also found that plasma taurine increased proportionally to infection stage, as estimated by parasite accumulation in the pleopods of *Nephtrops*. Increased plasma taurine may originate from host tissue or from the parasite and this is currently under investigation. Such elevated taurine levels may have implications in predator attraction to infected *Nephtrops* and on the general functioning of the host nervous system. We believe that plasma taurine concentration has the potential for use as an indicator of disease and possibly other stresses in captive and wild Crustacea.

## BIVALVE GENETICS AND MOLECULAR BIOLOGY

**GENOTYPE AND ENVIRONMENTAL VARIATION IN REVERSION OF TRIPLOID *CRASSOSTREA GIGAS* TO THE HETEROPLOID MOSAICS STATE.** Standish K. Allen, Jr., Aimee Howe, and Tom Gallivan, Aquaculture Genetics and Breeding Technology Center, Virginia Institute of Marine Science, Gloucester Point, VA 23062; Ximing Guo and Greg DeBrosse, Haskin Shellfish Research Laboratory, Rutgers University, Port Norris, NJ 08349.

In 1993, we were surprised by the occurrence of heteroploid mosaics among supposedly "certified triploid" individuals. At the time, their origin was not clear although reversion—loss of chromosomes from the triploid yielding heteroploid mosaicism—was suspected. In 1995 we began an experiment to unequivocally demonstrate reversion among triploid *Crassostrea gigas*. Triploids produced using cytochalasin (CB) treatments and from tetraploid × diploid matings were deployed in a quarantine system adjacent to Delaware Bay and in the Chesapeake Bay. Observations on labeled individuals were carried out for two years. The frequency of reversion in CB induced triploids was about 2–3 times higher than in tetraploid × diploid ones. The frequency of "reversion" also varies between grow out sites. Harsher environments may exacerbate the problem of reversion. Reversion is progressive with more and more diploid cells accumulating with time. The gametes produced in mosaics seldom have haploid cells. Cytogenetic data suggests that chromosome elimination may be caused by unusual chromosome clumping, as evidenced by mitotic metaphase spreads.

**MOSAICISM OF SOMATIC AND GAMETIC TISSUES IN *CRASSOSTREA GIGAS* AND *C. ARIAKENSIS*.** Whitney Chandler, Aimee Howe, and S. K. Allen, Jr., Aquaculture Genetics and Breeding Technology Center, Virginia Institute of Marine Science, Gloucester Point, VA 23062.

Putative two-year old mosaic *Crassostrea ariakensis* and *C. gigas* were conditioned for two months, sacrificed and biopsied. Percentage mosaicism among the tissue types (gill, gonad, heart,

adductor muscle, digestive gland and hemolymph) as well as the frequency of haploid gamete formation was examined by flow cytometry. Gametic tissue was examined via histology. Of the six tissue types examined, hemolymph contained the highest proportion of diploid cells and gill and gametic tissue contained the lowest, in both species. No haploid gametes were found. In *C. ariakensis*, 66% were male, 17% female, and 17% hermaphrodite. In *C. gigas* 25% were male, 62% females and 13% hermaphrodite. Gametogenesis in mosaic oysters was deemed nearly identical to that in triploids. Follicles were few and incompletely formed and gametes were in various stages of maturation within the same animal. We conclude that hemolymph is a more sensitive indicator of mosaicism than other tissue types and should be used for routine certification. Also, there is no evidence of normal (diploid) gametic activity in two-year-old male mosaics, although the eventual formation of haploid cells cannot be ruled out.

**OPTIMIZATION OF REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR) FOR USE WITH THE EASTERN OYSTER *CRASSOSTREA VIRGINICA*.** Tachih Cheng, Department of Veterinary Science, John T. Buchanan, Department of Oceanography and Coastal Sciences, Jerome F. La Peyre, Department of Veterinary Science, Terrence R. Tiersch, School of Forestry, Wildlife, and Fisheries, Richard K. Cooper, Department of Veterinary, Louisiana State University, Baton Rouge, LA 70803.

In order to monitor gene expression and library construction of complement DNA (cDNA) in oysters, RT-PCR condition was optimized to amplify actin cDNA. To determine the importance of DNase I digestion before reverse transcription, standard PCR and RT-PCR were used to amplify actin gene and products were sequenced to identify whether introns existed. A specific primer set was designed to amplify a 770 base pair segment of a published actin cDNA sequence. The genomic DNA (gDNA) and total RNA from hemocytes were isolated. The gDNA was used in PCR as templates; total RNA was used for RT-PCR after digestion with RNase-free DNase I and transcribed to single strand cDNA (sscDNA) by reverse transcriptase and a universal poly deoxythymidylate (pol dT) primer. Single strand cDNA was used as the template. In 2% agarose gel electrophoresis, an ~770 base pair band was found in PCR and RT-PCR amplifications. The bands were recovered and sequenced and showed >93% similarity with each other. These results indicate that RT-PCR with the specific primer set not only amplified the expected size actin cDNA, but also an actin-like gDNA of similar size and sequence. This data suggests that there is no large intron in the oyster actin gene and that it was necessary to digest total RNA with RNase-free DNase I to eliminate the gDNA before reverse transcription to avoid generating false positive RT-PCR.

**MOLECULAR EVOLUTION OF THE GPI LOCUS IN BAY SCALLOPS, *ARGOPECTEN IRRADIANS*.** Maureen K. Krause, Natural Science Division, Southampton College of Long Island University, Southampton, NY 11968.

Previous research demonstrated that allozyme variation at the polymorphic enzyme-coding locus, *glucose phosphate isomerase*, explains a large and significant proportion of variation in production-related traits in the bay scallop *Argopecten irradians*. Additionally, apparent *in vivo* biochemical functional differences among allozyme genotypes are correlated with higher-order phenotypic effects. The collective evidence suggests that natural selection may be maintaining genetic variation for *Gpi*, but whether the observed association between *Gpi* genotype and production-related traits is due to selection at the *Gpi* locus itself or to loci in linkage disequilibrium is unclear. The hypothesis for selective maintenance of genetic polymorphism can be tested using DNA sequence data and recently developed statistical methods. This paper presents the initial results from a molecular evolutionary study of nucleotide variation at *Gpi*. We have isolated and characterized a large portion of the coding region of bay scallop *Gpi*. The 917 bp cDNA shows approximately 66% nucleotide homology with *Calanus finmarchicus* *Gpi*, 73% homology to the human gene, and 69% homology with *Drosophila melanogaster*. Initial analyses of polymorphisms and correspondence to allozyme variation will be presented.

**IMPACT OF CULTURE PRACTICES ON THE HETEROZYGOSITY OF SUSPENSION-CULTURED BLUE MUSSELS.** Bruno Myrand, Station Technologique Maricole des Îles-de-la-Madeleine, Cap-aux-Meules, Qué., G0B 1B0; Réjean Tremblay, Groupe Interuniversitaire de Recherche en Océanographie du Québec, Qué., G1K 7P4; and Jean-Marie Sévigny, Institut Maurice-Lamontagne, Mont-Joli, Qué., G5H 3Z4.

The mean heterozygosity of suspension-cultured mussels (*Mytilus edulis* L.) from the Magdalen Islands, southern Gulf of St. Lawrence, is lower than that of the spat used for sleeving. This may have important impacts on the productivity of the local mussel culture industry because of the apparent relationship between heterozygosity and some fitness components such as resistance to stress and, probably, to summer mortality. To understand the causes of such decrease in heterozygosity, spat was sampled from collectors in October 1996 and then at various steps of the commercial sleeving operations. Graded spat was sleeved at usual (~850 per m) and high density (~1 950 per m) or kept in pearl-nets to impede fall-offs. The only significant decrease in heterozygosity (measured at 7 enzymatic loci) occurred once the sleeves were attached to longlines in the lagoon. The major decrease occurred before early June 1997. In September 1997, the mean heterozygosity of sleeved mussels was  $2.44 \pm 0.14$  (usual density) and  $2.14 \pm 0.21$  (high density) while that of the graded spat used for sleeving was  $3.13 \pm 0.20$ . Heterozygosity of the graded spat in pearl-

nets was  $2.83 \pm 0.14$  and did not decrease significantly. We hypothesize that more heterozygous spat goes out of the sleeves' meshes more rapidly and/or in greater numbers than do more homozygous individuals. If so, they could be more prone to fall-offs, mainly those resulting from turbulence created by heavy winds over the shallow lagoons in fall and spring.

**TETRAPLOID ZHIKONG SCALLOP (*CHILAMYS FARRERI*) PRODUCED BY INHIBITING POLAR BODY I.** Huiping Yang,<sup>1</sup> Ximing Guo,<sup>1,2</sup> and Fusui Zhang,<sup>1</sup> <sup>1</sup>Institute of Oceanology, Chinese Academy of Sciences, Qingdao, Shandong 266071, China; <sup>2</sup>Haskin Shellfish Research Laboratory, Institute of Marine and Coastal Sciences, Rutgers University, 6959 Miller Avenue, Port Norris, NJ 08349.

Triploid shellfish are useful for aquaculture because of their sterility, superior growth and improved meat quality. Triploid scallops have greatly enlarged adductor muscle (by 73–167% over normal diploids), making them extremely valuable for aquaculture. Although triploids can be produced by blocking polar body II, the ideal way for triploid production is through diploid × tetraploid mating. In this study, we tested tetraploid induction in the zhihong scallop, *Chlamys farreri*, by inhibiting the release of polar body I (PB1) in newly fertilized eggs. Cytochalasin B (0.75 mg/l) was applied at 7 ~ 10 min post-fertilization (PF) and terminated when PB1 was released in about 60% of the untreated eggs. The treatment and its control were repeated 12 times using the different pairs of parents. Blocking PB1 greatly altered chromosome segregation and the ploidy of resultant embryos. Variable proportions of aneuploid, triploid, tetraploid and pentaploid larvae were produced among the replicates. Most of the tetraploid, pentaploid and aneuploid larvae died with the first two weeks PF. At three months PF, five tetraploids (or 1.8%) were found in a sample of 271 spat (2–4 mm) from one of the replicates. Although the tetraploid percentage is low, this result suggests that tetraploid zhihong scallop can survive to at least the juvenile stage.

## COLD WATER AQUACULTURE HEALTH

**RESISTANCE TO BROWN RING DISEASE IN CLAMS: POTENTIAL CELLULAR MECHANISMS.** Bassem Allam, Kathryn A. Ashton-Alcox, and Susan E. Ford, Haskin Shellfish Research Laboratory, Rutgers University, Port Norris, NJ 08349.

Brown Ring Disease (BRD) is a temperature-controlled disease that has affected the clam *Ruditapes philippinarum* in Western Europe since the late 80s. The etiological agent, *Vibrio tapetis*, colonizes the periostracal lamina, disturbing the normal calcification process. In this study, flow cytometry was used to analyze hemocyte parameters in experimentally infected and control clams



from France (FR) and the US west coast (Oregon). Four weeks following challenge with *V. tapetis*, BRD prevalence was 52 to 57% in US *R. philippinarum* and 93 to 100% in FR specimens, suggesting some resistance in the US clam. Only 21–37% of the native European species, *R. decussatus*, developed BRD. In the hemolymph and the extrapallial fluid of animals that did not develop BRD, total hemocyte counts, the percentage of granulocytes, the percentage of viable cells, and the phagocytic activity against latex beads by the hemocytes were significantly higher in US than in FR *R. philippinarum*. In diseased animals, phagocytosis of *V. tapetis* was elevated in *R. decussatus*, remained unchanged in US *R. philippinarum*, and decreased significantly in FR *R. philippinarum*. Phagocytosis of *V. tapetis* by hemocytes in extrapallial fluid could be an important defense mechanism in BRD. Our results suggest that the relative resistance of *R. decussatus* and the US population of *R. philippinarum* to BRD could be related to differences in cellular parameters of the defense system and particularly the number and the phagocytic activity of hemocytes.

**QUAHOG PARASITE X (“QPX”) OF HARD-SHELL CLAMS, *MERCENARIA MERCENARIA* AND *M. MERCENARIA* VAR. *NOTATA* IN ATLANTIC CANADA—OBSERVATIONS FROM WILD AND CULTURED CLAMS.** Gregory S. Bacon and Sharon E. McGladdery, Gulf Fisheries Centre, Moncton, NB, E1C 9B6; Bruce A. MacDonald, Department of Biology, University of New Brunswick, Saint John, NB, E2L 4L5.

Quahog Parasite Unknown (QPX), has been associated with mortalities of hatchery broodstock in PEI, Atlantic Canada, since the early 1990's, however, no similar mortalities have been detected in wild populations since the 1960's. Investigations were, therefore, started in 1996, to determine if uninfected populations are present which could be used for hatchery broodstock. Seven hundred and twenty (720) adult clams (48–80 mm) were collected from four sites in three provinces (St. Andrews, NB (Bay of Fundy); Shediac Bridge, NB, Wallace, NS, and West River, PEI (Gulf of St. Lawrence)). Only Shediac Bridge clams were negative for QPX using histological screening. Quahogs from St. Andrews, Wallace and West River had prevalences ranging from 3.3–6.7%, 6.7–20.0% and 3.3%, respectively. The highest prevalence (20.0%) occurred in clams collected from St. Andrews in summer 1997, with a mean intensity of 86.8 and range of 1–465 organisms/tissue section. Culture results indicate that QPX infections in healthy clams and in smaller size-groups may be difficult to detect using histology. Smaller clams (13–18 mm) which have not yet been found to be infected in the wild, or in hatcheries, were infected experimentally with QPX from infected clams from St. Andrews. Investigations of culture vs. histology results are ongoing.

**GEOGRAPHIC DISTRIBUTION OF GONADAL NEOPLASMS IN SOFTSHELL CLAMS, *MYA ARENARIA*, FROM MAINE AND ATLANTIC CANADA.** Bruce J. Barber, School of Marine Sciences, University of Maine, Orono, ME 04469; Gregory S. Bacon, Gulf Fisheries Centre, Moncton, NB, E1C 9B6.

Gonadal neoplasms (germinomas) in softshell clams, *Mya arenaria*, have only been reported from locations in Maine, USA despite the fact that *M. arenaria* occurs from Labrador to North Carolina on the east coast of North America. To more accurately determine the geographic distribution of this disease, adult clams ( $n = 18$ –60 per sample) obtained between 1989 and 1997 from sites along the entire coast of Maine and from Atlantic Canada (New Brunswick, Nova Scotia, and Prince Edward Island) were examined histologically for the presence of neoplasms. Gonadal neoplasms were found at six of the 28 locations sampled, with prevalences ranging from 3.3% to 50%. All sites positive for neoplasms were located between Penobscot Bay, Maine and Passamaquoddy Bay, New Brunswick. Definition of the geographic distribution of this disease may provide insight into its presently undetermined etiology.

**A NOVEL SPECIES OF ALPHA-PROTEOBACTERIUM IS ASSOCIATED WITH SIGNS OF JUVENILE OYSTER DISEASE (JOD) IN *CRASSOSTREA VIRGINICA*.** K. J. Boettcher, J. T. Singer, and B. J. Barber, Univ. of Maine, Orono, ME.

In 1997, we deployed juvenile *C. virginica* on Maine's Damariscotta River as part of a study designed to elucidate the etiology of JOD. Cumulative losses in those oysters ranged from 55% in groups receiving weekly treatment with the antibacterial agent norfloxacin to over 84% in untreated groups. Bacteriological analysis of JOD-affected animals yielded counts  $\geq 10$ -fold higher than those obtained from healthy animals, and one specific colony type represented 40–95% of total recoverable colony-forming units (CFUs). These colonies achieved 1-mm diameter after 4–5 d and produced a non-diffusible pigment that resulted in a pinkish-beige appearance after 7 d. Over 200 representative isolates were phenotypically characterized; all were Gram-negative motile rods, oxidase positive, weakly catalase positive, and produced no reaction in oxidative/fermentative media. The 16S rRNA genes from two isolates were amplified, sequenced, and found to be identical. Alignments produced using the Ribosomal Database Project indicate that these bacteria are a previously undescribed species of the marine alpha-proteobacteria. In 1998, the impact of JOD on animals we deployed on the Damariscotta River was minimal (less than 4% cumulative mortality); however, bacteriological analysis of affected oysters revealed the presence of CFUs morphologically identical to those isolated from 1997 JOD-animals. These bacteria

were not detected in healthy oysters cultured nearby at that site, nor in *C. virginica* maintained simultaneously in a non-JOD enzootic area. These results provide further evidence that a bacterium, specifically a novel species of alpha-proteobacteria, is involved in JOD mortalities.

**THE ASSOCIATION BETWEEN THE TURBELLARIAN *URASTOMA CYPRINAE* AND THE EASTERN OYSTER *CRASSOSTREA VIRGINICA*.** Andrew D. Boghen, Nicole T. Brun, and Erick Bataller, Department of Biology, Université de Moncton, Moncton, NB, Canada, E1A 3E9.

Turbellarian flatworms are primarily free-living. Many are also facultative commensals and occasionally parasitic. Such is the case for *Urastoma cyprinae*, a common gill-worm of bivalves that induces serious damage to gill tissue in mussels. In Atlantic Canada the worm is widespread in the Eastern Oyster, *Crassostrea virginica*. Given the importance of the oyster industry on Canada's east coast, studies were initiated to shed light on the nature of the host-symbiont relationship between this worm and the oyster. While initial histological and biochemical studies provide inconclusive evidence of pathology, our work confirms that *U. cyprinae* is strongly attracted to the mucus secreted by the gills. The distribution of the worms in different areas of the gills, coupled with specific behavioral activities as revealed by in-vivo endoscopic observations, support the likelihood that the worms may be actively feeding on mucus. Such findings are further reinforced by studies employing zymographic techniques that demonstrate higher levels of protease activity in mucus for parasitized compared to non-parasitized oysters. Because of the visible presence of worms on the gills, investigations are currently underway to eliminate the worms prior to marketing using short-term depuration methods based on salinity tolerance.

**EFFECT OF COLD WATER ON LIMITING OR EXACERBATING SOME OYSTER DISEASES.** Susan M. Bower and Gary R. Meyer, Department of Fisheries and Oceans, Pacific Biological Station, Nanaimo, British Columbia, V9R 5K6, Canada.

Like most other pathogens, agents of oyster diseases are influenced by environmental conditions. Information accumulated to date indicates that some parasites are only pathogenic when water temperatures are cold for extended periods while others do not produce disease when temperatures are low. For example, controlled laboratory studies revealed that *Mikrocytos mackini*, the agent of Denman Island disease in *Crassostrea gigas*, did not develop in oysters held at  $>15^{\circ}\text{C}$  and temperatures  $<10^{\circ}\text{C}$  were required for at least 3 months for the development of disease and

associated mortalities. This temperature requirement explains the occurrence of the disease only in the spring of the year and its absence in oysters south of British Columbia despite the extensive historic relocation of potentially infected oysters to the Pacific United States. Conversely, other oyster diseases seem to be curtailed by cold temperatures. For example, *Nocardia* sp. has been detected in *C. gigas* in British Columbia throughout the year. However, nocardiosis occurs only towards the end of exceptionally warm summers. The literature also indicates that *Ostracoblabe implexa*, the agent of oyster shell disease, requires  $>22^{\circ}\text{C}$  for more than two weeks to proliferate and optimum growth occurs at  $30^{\circ}\text{C}$  (Alderman & Jones 1971, Trans. Br. Mycol. Soc. 57:213–225). This requirement for warm temperatures probably explains why this fungus is not a problem in British Columbia although we have detected it in a few oysters. Diseases caused by *Perkinsus marinus* and *Bonamia ostreae* have not been detected in Canada possibly because these parasites require warm temperatures to replicate. The question that research must now address is how long can these pathogens survive in hosts held at temperatures not suitable for their pathogenic expression before potential carriers can be certified as free of infection.

**IMPACT OF *BONAMIA OSTREAE* ON CULTURED *OSTREA EDULIS* AT TWO SITES ON THE DAMARISCOTTA RIVER, MAINE.** Ryan B. Carnegie and Bruce J. Barber, School of Marine Sciences, University of Maine, Orono, ME 04469.

The oyster parasite, *Bonamia ostreae*, persists in *Ostrea edulis* populations at several locations in Maine. However, *B. ostreae* prevalence is always low, and heavy infections are rare. To assess the potential role of temperature on growth and prevalence of *B. ostreae*, we deployed 2000 *O. edulis* seed in July 1997 among eight surface trays at each of two sites on the Damariscotta River. One location (Little Point) was in a warm ( $>21^{\circ}\text{C}$  in summer) area in the upper river, and the other (Lowes Cove) was a cooler ( $<18^{\circ}\text{C}$ ), mid-river site. Shell height, mortality, and prevalence of *B. ostreae* were measured bimonthly beginning in May 1998. Temperature was measured 4x daily and salinity weekly. Growth was significantly greater at the warmer Little Point site, where the oysters had reached  $70.1 \pm 2.8$  mm in shell height by September 1998. At Lowes Cove, average shell height was  $48.8 \pm 3.0$  mm. However, cumulative mortality was also greater at Little Point:  $41.4 \pm 11.6\%$  in September 1998, vs.  $21.8 \pm 6.8\%$  at Lowes Cove. Instantaneous mortality was 31.8% at Little Point in July 1998 following a significant mid-June rain event that reduced salinity below 22‰. *B. ostreae* was observed only in animals reared at Little Point. Prevalence of *B. ostreae* was 3.1% in May 1998, and 9.4% in July 1998, and infection intensity in all cases was low. Thus, it is more likely that the mortality was due to a sharp decrease in salinity than to *B. ostreae*.

**MYCOTIC PERIOSTRACAL SLOUGHING.** T. Jeffrey Davidson, Claude Morris, and David Groman, Atlantic Veterinary College, University of Prince Edward Island, 550 University Ave., Charlottetown, PEI, Canada C1A 4P3.

A brown discolouration has been observed on the shells of market-sized blue mussels (*Mytilus edulis*) for the past number of years. Histopathology and electron microscopy have identified the pathology as loss of the shell's periostracum due to a marine fungus, hence the name of the new disorder, Mycotic Periostracal Sloughing. This fungus appears to deposit on the shell in late summer/early fall in the first growing season in socks. It has not been observed in spat or mussels in their first growing season before October. The pathogenesis begins with single foci on the shell. The lesions extend horizontally causing coalescing of the foci and vertically, causing deterioration and sloughing of the periostracum and to a lesser degree the prismatic layer.

The brown discolouration seen on the shell could be due to the loss of the periostracal layer causing a decrease in refractivity. Studies are ongoing to determine if this disorder affects the health of the meat or growth parameters. All mussel growing areas on P.E.I. are affected. It has also been observed in *Mytilus trossulus* and *M. galloprovincialis* in Washington State.

**INVESTIGATION OF THE STRESS RESPONSE, SUMMER MORTALITY AND DISEASE RESISTANCE OF OYSTERS, CRASSOSTREA SPP.** C. S. Friedman, G. N. Cherr, J. S. Clegg, A. H. Hamdoun, J. L. Jacobsen, S. A. Jackson, and K. R. Uhlinger, Bodega Marine Laboratory, P.O. Box 247, Bodega Bay, C.A. 94923.

The ability to mount a stress response is often essential for an organism's survival, especially for oysters inhabiting dynamic and stressful environments. We have characterized the response of Pacific oysters, *Crassostrea gigas*, to heat shock (HS), hypo-osmotic acclimation and disease. Pacific oysters synthesized heat shock proteins (HSP) in the 70 kD family and exhibited prolonged induced thermotolerance (ITT, at least 3 wk) after exposure to heat shock. The temperature needed to induce a stress response appears to be related to the upper thermal limit of the oyster (43.5–44 C) not on the magnitude of thermal shock. Despite an increase in thermal tolerance after HS, we observed a significant reduction in chemotaxis and percent phagocytosis in oysters after HS relative to those without a heat treatment. Hypo-osmotic acclimation delayed HSP production and tolerance to lethal temperatures. Oysters challenged with *Nocardia* synthesized HSPs in a pattern similar to control animals. However, the degree of ITT was reduced in oysters with nocardiosis. Although reproductive stress does not alter the stress response of Pacific oysters, the chemotactic ability of hemocytes from gravid oysters was significantly reduced as compared with nonreproductive animals ( $p < 0.001$ ).

The Eastern oyster, *C. virginica*, produced HSPs after heat shock a similar manner as the Pacific oyster. However, the magnitude of ITT was reduced relative to that of Pacific oysters held under the same culture and experimental conditions.

**PROSORHYNCHUS SQUAMATUS (DIGENEA: PLATYHELMINTHES) INFECTION OF BLUE MUSSELS, MYTILUS EDULIS, IN ATLANTIC CANADA.** Sharon E. McGladery, Mary F. Stephenson, and Fiona McArthur, Gulf Fisheries Centre, Moncton, NB, E1C 9B6.

In May, 1997, a sample of mussels (*Mytilus edulis*) from the Atlantic coast of Nova Scotia, was found to contain the sporocysts of the digenean *Prosorhynchus squamatus* at a prevalence (%P) of 13.3% ( $n = 60$ ). A single infected specimen (%P = 3.3%) was found at a neighbouring site but, with the exception of a single mussel from the Magdalen Islands and another possible specimen from northern New Brunswick, no other infections had previously been recorded. Mussels at the affected site demonstrated reduced tolerance of processing handling and mortalities were observed in the samples collected. Mantles showed abnormal colouration (patchy yellow-white) in heavily infected individuals, however, early/light infections could not be detected by gross observation. Four species of fish (*Gadus morhua*, *Hippoglossoides platessoides*, *Melanogrammus aeglefinus* and *Myoxocephalus scorpius*) have been recorded as definitive hosts of *P. squamatus*, in Atlantic Canada, but this is the first record of the sporocyst stage in blue mussels. Metacercarial host(s) has/have yet to be identified. Due to concern about mussel health and possible spread of the parasite, along with routine mussel transfers, a survey was initiated in 1997/98 to determine what size-groups were infected, the life-cycle, seasonal dynamics, pathogenic effects and current distribution of infection. Results from this survey were compared with records from 2300 mussels, collected throughout Atlantic Canada between 1990–1997 and confirmed an apparent focal distribution. Work on the other hosts involved in the life-cycle is ongoing.

**MONITORING SHELLFISH HEALTH IN NEWFOUNDLAND: A PREVENTATIVE APPROACH.** Kelly Moret, Cyr Couturier, G. Jay Parsons, and Kate Williams, Fisheries and Marine Institute of Memorial University of Newfoundland, St. John's, NF, Canada, A1C 5R3.

The rapid expansion of the mussel aquaculture industry in Newfoundland prompted the University, Industry Association and funding agencies to initiate a research project aimed at establishing a health/disease profile of cultured mussels throughout the province. Prior to the establishment of this program, farmers wishing to assess the health status of their stock were required to submit samples to either the Department of Fisheries and Oceans, Mon-

ton, NB, or the Atlantic Veterinary College, UPEI, Charlottetown, PEI. The status of the local industry is ideal for compiling a health baseline survey as imports, exports, and transfers to the island have been limited, thereby allowing us to take a preventative approach to health care. In 1997, a total of 13 farms participated in the initial survey (792 mussels). Shell examination revealed that 2 of the 13 farms had a very low incidence of the fungus identified as Mycotic Periostracal Sloughing Disease ("MPS"). Histological examination revealed that 76.92% of the farms had mussels which contained parasites. The most prevalent parasites were the gill ciliates, *Ancistrum mytili* and *Sphenophrya*-like ciliates. The total parasite prevalence for the 792 mussels was low (average 7.8%). For 1998, sampling was expanded to 23 sites. In addition to routine histological examination, other research included studies on the fungus "MPS" and stress protein analysis. Conclusions to date include: parasites found in Newfoundland mussels are similar to those found in mussels throughout Atlantic Canada and the parasite load in Newfoundland cultured mussels is apparently lower than that in mussels in the rest of Atlantic Canada.

**TEMPERATURE EFFECTS ON BROWN RING DISEASE SUSCEPTIBILITY AND DEFENSE-RELATED ACTIVITIES IN THE MANILA CLAM, *Ruditapes philippinarum*.** Christine Paillard, Bassem Allam, and Radouane Oubella, UMR CNRS 6539, Institut Universitaire Européen de la Mer, UBO, Brest, France; Susan E. Ford, Haskin Shellfish Research Laboratory, Rutgers University, Port Norris, NJ 08349.

Brown Ring Disease (BRD) in the manila clam, *Ruditapes philippinarum* is a bacterial disease, caused by *Vibrio tapetis*, which perturbs the calcification process. Field and experimental studies confirm that BRD can be classified as a cold-water disease. It occurs in wild and cultured clam populations along the European Atlantic coasts, but it is practically absent in areas that have high summer temperatures. It has never been reported in manila clams in the USA or Japan. Epizootological surveys of BRD along the French Atlantic coast have shown inter-site prevalence variations that could be explained by temperature extremes. Experimental *Vibrio tapetis* infections carried out at different temperatures (8, 14 and 21°C) have documented variations in BRD prevalence as a function of temperature, i.e. 20% at 21°C versus 100% at 8 and 14°C, 4-weeks after inoculation. Bacterial growth rate is maximal between 14 and 21°C and declines at higher temperatures. Total hemocyte counts, phagocytosis, and leucine-aminopeptidase activities were significantly higher in control or inoculated clams at high than at low temperature. Higher lysozyme activity was measured at 21°C, compared to the lower temperatures, in clams inoculated with *V. tapetis*. Greater activity of potential cellular defense mechanisms at high temperature, as well as reduced bacterial growth, could be associated with the recovery process occurring at temperatures of 21°C or higher.

## FEEDING PHYSIOLOGY & ECOLOGY OF BIVALVES

**CTENIDIA AS THE SITE OF PARTICLE SELECTION IN BIVALVES: A COMPARISON BETWEEN SIMPLE AND COMPLEX CTENIDIAL SYSTEMS.** Shirley M. Baker and Jeffrey S. Levinton, Dept. of Ecology and Evolution, State Univ. of New York, Stony Brook, 11794; J. Evan Ward, Dept. of Marine Sciences, Univ. Connecticut, Groton, 06340.

Suspension feeding bivalves compensate for fluctuations in the quality and quantity of their food supply by changing pumping and ingestion rates, and by rejecting non-nutritive particles as pseudofeces. Sorting and rejection of particles have been thought to occur on the labial palps. Recently however, Ward *et al.* (1998) report that in oysters (*Crassostrea virginica* and *C. gigas*) particle selection takes place on the ctenidia, while in mussels (*Mytilus trossulus*) the ctenidia play little role in particle selection. They suggest that the complex structure of the oyster ctenidia (plicate, heterorhabdic) contributes to the ability to sort particles. Using video endoscopy, we have found that other bivalves are also able to sort particles on the ctenidia. These include the zebra mussel, *Dreissena polymorpha*, and the freshwater unionid mussels *Amblyema plicata*, *Pyganodon cataracta*, and *Elliptio complanata*, which have non-plicate, homorhabdic ctenidia. In *D. polymorpha*, material transported in the dorsal ciliated tract was primarily preferred particles and underwent little further processing by the labial palps. Particles were carried in two ways at the ventral food groove; ultimately rejected particles were transported in a mucus string above the food groove, while ultimately accepted particles were often transported deep within the groove. This type of two-layer transport in the ventral food groove was also observed in the unionids. At high particle concentrations, the unionid species appressed the ventral areas of the two inner demibranchs. While ultimately accepted particles continued to be transported in the ventral food grooves, rejected particles were carried in a mucus string in the "groove" formed by the two demibranchs. Where the two inner demibranchs parted, near the pedal gape, the mucus string was transferred to the mantle and expelled. These examples provide evidence for some degree of particle sorting on ctenidia that are less complex than those of oysters.

**THE END OF THE PARTICLE PROCESSING LINE: MANTLE PSEUDOFECES REJECTION MECHANISMS IN SUSPENSION-FEEDING BIVALVES.** Peter G. Beninger, Laboratoire de Biologie Marine, Université de Nantes, Nantes, 44322 France, and Anne Veniot, Département de Biologie, Université de Moncton, Moncton N.B. E1A 3E9 Canada.

Characteristics of pseudofeces voidance from the mantle were investigated in the four major bivalve particle processing systems using scanning electron microscopy, to determine what mecha-

nisms underlie the final step in the rejection route. The entire mantle surfaces of *Mytilus edulis* (homorhabdic filibranch), *Mya arenaria* and *Spisula solidissima* (eulamellibranch), *Crassostrea virginica* and *C. gigas* (pseudolamellibranch), and *Placopecten magellanicus* (heterorhabdic filibranch) were surveyed and photographed. In both the homorhabdic filibranchs and the eulamellibranchs, the mantle rejection tracts previously located using video endoscopy were characterized by cilia which were extraordinarily long compared to the cilia of the general pallial surface. These long cilia were grouped into closely-adhering tufts, herein termed composite cilia. In *Mytilus edulis* and *Mya arenaria*, the general pallial surface presented shorter simple cilia, whereas in *Spisula solidissima* the general pallial surface presented short simple cilia dorsally, long composite cilia ventrally, and an intermediate band of composite cilia. Effective mucociliary transport using long cilia can only be accomplished if they are grouped, as in all three species with mantle rejection tracts. In *C. virginica* and *C. gigas*, pseudofeces are carried atop short simple cilia, which are themselves elevated above the general pallial surface by prominent, specialized radial ridges. These four species all possess a gill ventral particle groove, hence the site of pseudofeces production is the labial palp, and a discrete mantle rejection tract is necessary to transport pseudofeces to the inhalent siphon for expulsion. The long composite cilia and the specialized rejection ridges within this tract provide vertical isolation of pseudofeces from the general pallial surface.

In the heterorhabdic filibranch *Placopecten magellanicus*, no specialized cilia were observed on the mantle; this corresponds to the absence of a mantle rejection tract in this system, which does not possess a gill ventral particle groove and relies on valve adduction to expel pseudofeces. These results suggest that elevation above the surrounding mantle cilia is the rule in species relying on mantle rejection tracts for the avoidance of pseudofeces. Cilia mapping is thus seen to be a valuable technique in the ongoing study of particle processing mechanisms in suspension-feeding bivalves.

**CHALLENGES OF EXAMINING POSTINGESTIVE SELECTION IN BIVALVES.** Martha G. S. Brilliant and Bruce A. MacDonald, Biology Department and Centre for Coastal Studies and Aquaculture, University of New Brunswick, Saint John, NB, Canada E2L 4L5.

Postingestive selection is known to occur in several species of bivalves, however the factors responsible for selection have not been established. Studies on postingestive selection have not usually attempted to isolate which particle variables selection is based on or test particles were not presented to the bivalves simultaneously so that no choice was given. We have shown that size and density of particles play a role in postingestive selection in the sea scallop (*Placopecten magellanicus*). Determining the role of particle chemistry or quality is more challenging. Factors which must

be considered when choosing test particles to study postingestive selection by chemical properties are 1) test particles should appear physically identical but chemically distinct to the bivalve, 2) test particles must be easily distinguishable from one another by the researcher, 3) test particles must be traceable and quantifiable after passage through the bivalve, 4) preferably the test particles should be natural food particles and, 5) the integrity of the test particles in the bivalve stomach should be similar. We have addressed most of these factors by developing a protocol using two colours of fluorescent carboxylate-modified microspheres, one coated with protein. Scallops will be fed coated and uncoated beads simultaneously with the feces being collected and analyzed by flow cytometry. Gut retention times of the two bead colours will be compared to determine if postingestive selection has occurred. This method should provide a means of determining whether scallops can distinguish particles within the stomach on the basis of chemical properties alone.

**SEASONAL VARIATION IN FOOD UTILIZATION BY SEA SCALLOPS AND BLUE MUSSELS.** Peter J. Cranford, Fisheries and Oceans Canada, Bedford Institute of Oceanography, P.O. Box 1006, Dartmouth, NS, B2Y 4A2.

Seston utilization by adult *Placopecten magellanicus* and *Mytilus edulis* cohorts was measured using an *in situ* method during the spring, summer and fall of 1995. Large seasonal changes in the rates and efficiencies of feeding and absorption were observed, but only 28% of the variance in daily ingestion rates of both species could be explained by empirical models developed using a wide range of potential environmental influences (temperature, seston abundance and composition and vertical particle flux). Ingestion and absorption rates of scallops and mussels showed similar seasonal patterns with highest rates observed during the spring, when diet quantity and quality were high, and during late-autumn, when quantity and quality were both low. These data indicate that seston utilization and related growth was not caused solely by seasonal food and temperature fluctuations, but imply physiological regulation of feeding and digestion. Both species displayed a large capacity for controlling clearance and absorption rates. Clearance rates during October and November were at least twice as high as observed at other times of the year, and absorption efficiency gradually decreased at high diet quality and increased when quality was low. The accuracy of available bivalve clearance (filtration) rate models was assessed by comparing predicted responses with average *in situ* clearance rates. Only those models based on natural seston rations provided adequate predictions of observed clearance rates. Clearance rate predictions based on algal cell rations greatly overestimated *in situ* clearance at all times of the year and do not appear to apply to animals in nature. Future directions in ecophysiology need to focus on the *in situ* responses of bivalve filter feeders.

### **PARTICLE AGGREGATES IN SESTON AND THEIR ROLE IN BIVALVE PARTICLE SELECTION.**

**Jon Grant** and **Mike Nickerson**, Department of Oceanography, Dalhousie University, Halifax, NS B3H 4J1.

Natural suspended particles consist of a complex mixture of phytoplankton, detritus, and inorganic material. A persistent feature of marine particles is that they exist as aggregates which make the characterization of food quality for suspension feeders particularly troublesome. Bulk measures of food including organic content do not contain information about particle size or aggregation state which determine aspects of suspension feeder handling such as selection efficiency. There are few options for measuring aggregation state, since particles are fragile and subject to sampling artifacts. Moreover, although there has been theoretical and empirical progress regarding particle aggregation (e.g., fractal dimension), the organic components of aggregates are poorly known. Two approaches to these problems include novel ways of sampling and analyzing particles from the field, and manipulation of aggregates in the laboratory for use in bivalve feeding experiments. Newly developed small volume particle samplers were used to examine the size and composition of natural aggregates collected in the field, with particular reference to organic components. In addition, feeding experiments were initiated using clay-protein aggregates to examine selection efficiency in blue mussels. Initial results from these experiments are discussed in terms of assessment of the food supplies available to suspension feeding bivalves and their implications for understanding the energetics of feeding.

### **A COMPARISON OF FEEDING PHYSIOLOGY IN DIFFERENT SIZES OF CULTURED AND WILD *MYTILUS EDULIS* AND *M. TROSSULUS*.**

**Melissa Mooney, G. Jay Parsons,** and **Cyr Couturier**, Fisheries and Marine Institute of Memorial University of Newfoundland, St. John's, NF, Canada A1C 5R3.

Seasonal patterns in food demand were examined in two species of cultured and wild blue mussels from a single site in Newfoundland to contribute towards an improved model of estimating production capacity. Small and large (shell length  $\leq 20$  or  $\geq 50$  mm, respectively) cultured and wild blue mussels were obtained monthly. Mussels were acclimated to laboratory conditions, maintained at ambient temperature, and fed low, medium or high food rations ( $< 3500$ ,  $5000\text{--}7500$  or  $> 9000$  cells/mL, respectively). Diets consisted of 50/50 mixtures of *Isochrysis galbana* and *Chaetoceros muelleri*. Weight specific rates of filtration (g/h/g), clearance (L/h/g) and metabolism ( $\text{mg O}_2/\text{h/g}$ ) were measured. Overall, clearance and filtration rates were significantly higher at higher temperatures (ANOVA,  $P < 0.05$ ), lower during spawning (ANOVA,  $P < 0.01$ ) and higher in smaller mussels compared to larger mussels (ANOVA,  $P < 0.01$ ). Metabolic intensity was significantly higher at higher temperatures (ANOVA,  $P < 0.01$ ). *Mytilus trossulus* demonstrated a significantly higher clearance rate and meta-

bolic intensity than *M. edulis* (ANOVA,  $P < 0.01$ ). No significant differences were observed between cultured and wild mussels for any of the factors tested. The proportion of different size mussels and species on a given site has direct implications for stocking density, and will impact the overall production capacity of a given culture site. The results validate the importance of grading, and suggest practices such as stocking smaller mussels near the outer boundary of a site (where food availability is maximal) may be more effective.

### **THE EFFECTS OF CURRENT SPEED AND PARTICLE CONCENTRATION ON MUSSEL (*MYTILUS EDULIS*) FILTRATION RATE: A RECIRCULATING FLUME STUDY.**

**Carter R. Newell**, Great Eastern Mussel Farms, Inc., P.O. Box 141, Tenants Harbor, ME 04860.

Bivalve filtration rates are one of the most important yet uncertain terms in models of shellfish carrying capacity. Previous workers (Newell et al., 1998) investigated mussel shell gape in the field using a time lapse video over two tidal cycles. They found that shell gape (distance between valves), an indication of pumping rate, was positively correlated with filtration rate in experimental chambers. Due to the difficulty of separating covarying environmental variables, namely current speed and particle concentration, in the field, a laboratory flume approach was chosen to calibrate the shell gape assay.

After acclimation to experimental diets, individual mussels were attached to plexiglass stands and subjected to various concentrations of food with current speed constant and various current speeds with food concentration constant while a time-lapse video system recorded shell gape and exhalant siphon area. Each run included a 15–20 minute acclimation period followed by data capture over 40–45 minutes. Video tapes were analyzed with an Optamus system. Mussel exhalant siphon area showed a significant decrease with an increase in current speed from 5 to 30  $\text{cm s}^{-1}$ . Below about 5 million particles  $\text{l}^{-1}$ , mussels significantly reduced their pumping rates. The negative effects of high current speeds on mussel pumping and filtration rates have important implications for the suspension culture of mussels.

### **ROLE OF OYSTERS IN MAINTAINING ESTUARINE WATER QUALITY.**

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Environmental changes in Chesapeake Bay, such as elevated phytoplankton biomass and loss of benthic plants, are often thought to be largely a function of nutrient-driven eutrophication. We propose, however, that populations of the eastern oyster, *Crassostrea virginica*, which have been reduced to  $< 1\%$  of their historic levels, may have exerted "top-down" control on phytoplankton

stocks and also reduced turbidity, thereby increasing light available to benthic plants. In laboratory incubations under oxic and anoxic conditions we measured changes in sediment geochemistry, nutrient fluxes, and denitrification in response to loading by different amounts of algal paste, an experimental analog of oyster biodeposits. Increased organic loading to the sediment under oxidized conditions resulted both in higher rates of coupled nitrification/denitrification and denitrification in the presence of water column nitrate. In contrast, coupled nitrification/denitrification was suppressed under anoxic conditions. Similar incubations in the presence of benthic microalgae showed negligible ammonium fluxes from sediments, with the algal/microbial community efficiently retaining ammonium and fixing nitrogen. Because no DIN was recycled to the water column under oxic conditions we conclude that rehabilitation of natural oyster stocks will have the beneficial effect of removing phytoplankton from the water column without stimulating further phytoplankton production. Furthermore, nitrogen will be removed from the Bay via increased denitrification. These data also suggest that private-sector oyster aquaculture should be encouraged not only for the obvious economic value but also for the broader ecological benefits to the Bay.

**COMPOSITE CILIA: DESCRIPTION OF A NEW TYPE OF CILIAM USED IN PARTICLE PROCESSING IN BIVALVES.** Anne Veniot, Département de Biologie, Université de Moncton, Moncton N.B., E1C 3E9, Canada; Peter G. Beninger, Laboratoire de Biologie Marine, Université de Nantes, Nantes, 44322 France.

Cilia are the elementary mechanism responsible for the creation of water currents in the pallial cavity, as well as for particle capture and subsequent processing. Recent scanning electron microscopy observations of several pallial surfaces involved in particle capture and processing in bivalves have shown the presence of unusually long, grouped cilia. These cilia were named composite cilia to distinguish them from the known compound cilia (cirri) and simple cilia. However, high-resolution ultrastructural observations and determination of the characteristics of composite cilia are necessary in order to accurately determine whether these cilia represent a new ciliary type. We used scanning electron microscopy, light microscopy and transmission electron microscopy to examine the structure and ultrastructure of composite cilia in four Bivalve species: *Mytilus edulis*, *Mya arenaria*, *Spisula solidissima* and *Crassostrea virginica*. Data show that composite cilia are made up of a group of simple cilia originating from a single cell. In contrast to compound cilia (cirri), the component simple cilia present no fusion at any point along their length. The component cilia are densely packed and uniformly spaced, with one microvillus between adjacent cilia, a striking contrast with both compound cilia, which have no space or microvilli between adjacent cilia, and simple cilia which are unevenly spaced with several intervening microvilli. These characteristics suggest that composite cilia are a

new category of cilium, with a distinct arrangement of non-fused component cilia. The coarse frontal cilia of frontal filaments of the gill of *C. virginica* are shown to belong to this category of cilium. We suggest that mechanisms of adhesion in compound cilia may consist of weak molecular interactions, such as hydrogen bonds or attraction between protein groups of opposing polarity. The structure of composite cilia impart properties which enable them to perform specialized tasks in particle processing.

#### **MEDIATION OF FEEDING AND SELECTION BY SECONDARY METABOLITES OF DETRITAL PARTICLES.**

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In environments dominated by kelp forests, a large amount of the suspended detrital material is derived from the seaweeds. These kelp-derived particles can support growth of suspension feeding bivalves. We employed flow cytometry and video endoscopy to study the effects of kelp detritus on feeding and particle selection in the oyster, *Crassostrea gigas*. Oysters were fed mixtures of phytoplankton and similar size detrital particles prepared from ground kelp. Fresh particles derived from the kelp *Agarum fimbriatum* had higher concentrations of phenolic compounds than those from *Costaria costata*. Aging for 4 days reduced phenolic concentrations of particles from both species. Clearance rates of oysters were significantly inhibited by high phenolic concentrations of the kelp detritus, and clearance increased as *Agarum* particles aged. Selection by the gill of the oyster was also affected by phenolic concentration of the kelp detritus. Particles with low (aged *Costaria*) and high (fresh *Agarum*) phenolic concentration produced low selection, whereas particles with intermediate phenolic concentration produced high selection. In all assays, kelp particles were significantly rejected in the pseudofeces. Our data suggest that phenolics are inhibitory to feeding and selection mechanisms in oysters.

#### **GROWTH AND CULTURE OF SHELLFISH**

**EFFECT OF FISHING PRESSURE ON SIZE OF ADULT MALE BLUE CRABS IN MARYLAND: CALVERT CLIFFS AND THE PATUXENT RIVER—1998.** George R. Abbe and Brian W. Albright, Academy of Natural Sciences Estuarine Research Center, St. Leonard, MD 20685.

The Maryland blue crab fishery has experienced increasing fishing pressure since the 1940s. Regulations enacted in 1994 to limit or reduce effort and improve the fishery have had little posi-

tive effect. Data from near Calvert Cliffs in Chesapeake Bay have shown a declining trend in mean size for total male crabs and legal size males since 1968. To determine if this decline was due to fishing pressure we compared results from Calvert Cliffs, where fishing pressure is intense, with an area in the Patuxent River where fishing pressure is low. Crab pots are the gear of choice on the Bay, but are restricted for commercial use in the river. The two areas examined were about 8 km apart by air and had similar temperatures and salinities. Peeler pots of 1-inch mesh baited with menhaden were fished at both areas at similar depths from June to November 1998. During this time 411 and 670 pots were fished on the river and Bay, respectively, yielding 974 and 1888 crabs. Total catches per unit effort were similar at 2.4 and 2.8, but river crabs were 89% male compared to 41% on the Bay. Legal males ( $\geq 5$ -inch carapace width) made up 69% of the river catch compared to 10% of the Bay catch, and males  $\geq 6$  inches accounted for 28% of the river catch compared to only 2% in the Bay. Mean size of all males was 5.5 and 4.6 inches in the river and Bay, respectively; legal males were 5.8 and 5.6 inches, respectively. Reduced fishing pressure in the river allows many male crabs the opportunity to molt again after reaching minimum legal size as evidenced by the percentage of 6-inch males, whereas most males in the Bay are caught shortly after reaching legal size. Further regulations to reduce effort in the Chesapeake or an increase in minimum legal size may be necessary to improve the quality of the fishery.

**TRIPLOID PRODUCTION OF MYTILUS EDULIS IN PRINCE EDWARD ISLAND—AN INDUSTRIAL INITIATIVE.** John W. Brake, Jeffrey Davidson, Atlantic Veterinary College, University of Prince Edward Island, and Dr. Jonathan Davis, Taylor Resources Inc., Quilcene, WA.

The mussel aquaculture industry in Prince Edward Island (PEI), Canada is a well established major contributor to the island economy. In 1997 farm gate value exceeded \$12 M (Can) and export value exceeded \$24 M (Can). The industry currently supports over 1250 full and part time jobs.

Harvesting and marketing during the spawning season is currently an area of concern for the industry. Mussels that have recently spawned have a low (less appealing) meat yield, while those close to spawning can spawn out en route to the market from processors. In both cases the potential exists to increase consumer dissatisfaction with the product. Triploids have very poorly developed gonads, thus more energy can be used for meat production instead of gonad production, allowing for the possibility of larger meat yields than normal diploids during the spawning season. The production of triploid mussels might therefore alleviate these problems, allowing the marketing of a high quality product year round. The production of triploid Pacific oysters (*Crassostrea gigas*) is currently extensively practiced in the Pacific Northwest. Triploid clams (*Ruditapes philippinarum*), scallops (*Placopecten magellanicus*), and mussels (*M. galloprovincialis*) have all been produced as

well as others. Identified methods of triploid induction (used at different levels or in combinations) in shellfish include temperature and/or pressure shocking and the use of chemicals such as caffeine, cytochalasin B, or 6-dimethylaminopurine.

The mussel industry has recognized the potential of harvesting triploid mussels during the spawning season. The objective of this study is to elucidate the best triploid induction methods for commercial use in PEI by the use of a matrix of previously identified triploidy induction methods. These combinations of methods will be ranked by % induction and % survivorship, as well as feasibility in order to determine the best method. Hatchery techniques will be fine tuned for the species, then the performance of triploids in the field will be evaluated to find when it might be more or less advantageous to utilize them.

**DIFFERENTIAL EFFECTS OF TWO ISOLATES OF AU-REOCOCCUS ANOPHAGEFFERENS, IN UNIALGAL AND MIXED SUSPENSIONS, ON FEEDING AND GROWTH OF BIVALVES.** V. Monica Bricej, Scott MacQuarrie, Institute for Marine Biosciences, National Research Council, 1411 Oxford Street, Halifax, NS B3H 3Z1, Canada; Roxanna M. Smolowitz, Laboratory for Aquatic Animal Medicine and Pathology, University of Pennsylvania, Marine Biological Laboratory, Woods Hole, MA 02543.

Previous work examined only short-term effects of *Aureococcus anophagefferens*, the causative agent of brown tides in mid-Atlantic estuaries, on feeding of bivalves. We conducted three-week laboratory trials to determine the effects of two cultured isolates of *A. anophagefferens* on survival, growth (in shell length, biovolume and total organics) and histopathology of juvenile (6 mm) quahogs, *Mercenaria mercenaria*. The algal strains were isolated in 1986 and 1995 from Long Island, New York, bays. Three unialgal treatments were used: *Isochrysis galbana* (t-Iso) at  $60 \times 10^6$  cells  $L^{-1}$ , and *A. anophagefferens* at a high cell density ( $1 \times 10^9$  cells  $L^{-1}$ ) simulating natural blooms, and moderate density ( $400 \times 10^6$  cells  $L^{-1}$ ); two were mixed suspensions of t-Iso ( $60 \times 10^6$  cells  $L^{-1}$ ) spiked with either 400 or  $20 \times 10^6$  *Aureococcus* cells  $L^{-1}$ . A non-fed control was included. Survival was not significantly affected in any of the treatments. No growth was observed at high or moderate densities of *Aureococcus*. Marked growth suppression relative to the t-Iso control occurred in the mixture spiked with  $400 \times 10^6$  *Aureococcus* cells  $L^{-1}$ , supporting our findings with adult mussels that *Aureococcus* inhibits capture of other non-toxic algae co-occurring in a mixed assemblage. Less severe growth inhibition of clams occurred in the mixture containing  $20 \times 10^6$  *Aureococcus* cells  $L^{-1}$ , and growth performance in this treatment increased gradually over the course of three weeks. In contrast, growth trials with the 1986 isolate showed positive growth of *M. mercenaria* juveniles in both high and moderate concentrations of this strain, and comparable growth in the t-Iso control and mixture spiked with  $400 \times 10^6$  *Aureococcus* cells  $L^{-1}$ .



These results indicate that cell densities ( $20 \times 10^6$  cells  $L^{-1}$ ) an order of magnitude lower than previously documented, can have deleterious effects on bivalve growth, but that acclimation to such low levels of *Aureococcus* can occur over time. They also support our bioassay results with adult mussels which show no evidence of toxicity with the 1986 brown tide isolate, whereas two 1995 isolates elicit strong feeding inhibition.

**EVALUATING THE PERFORMANCE OF NON-NATIVE OYSTER SPECIES IN VIRGINIA.** Gustavo W. Calvo, Mark W. Luckenbach, and Eugene M. Burreson, School of Marine Science, Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062.

The Pacific oyster, *Crassostrea gigas*, and the Suminoe oyster, *Crassostrea ariakensis*, have been suggested as potential substitutes for and/or enhance native oyster, *Crassostrea virginica* stocks which have been devastated by disease and other factors in Virginia. Field experiments with triploid progeny of quarantined broodstock have been established as a means to evaluate the performance of non-native oysters, while preventing undesired introduction of disease agents and/or uncontrolled proliferation of exotic species in Chesapeake Bay and the Atlantic Coast of Virginia.

During a one year experiment using juvenile oysters at 9 sites covering a wide range of salinity, *C. gigas* has demonstrated overall higher disease resistance and superior growth and survival at high salinity sites (>25 ppt), as compared to *C. virginica*. Conversely, at low salinity sites (<15 ppt), *C. virginica* has had higher survival and growth than *C. gigas*. Performance at intermediate salinity sites (15–25 ppt), was similar for both species. In an ongoing study using 2 year old oysters at a subset of 6 of the study sites described above, *C. ariakensis* is exhibiting overall higher disease resistance and equal or superior survival and growth than *C. virginica*, after six months of deployment.

The results of these field studies provide only a partial basis for evaluating potentials and pitfalls of using non-native oyster species to enhance the oyster fishery and aquaculture industry in Virginia. Other factors not addressed in these studies, including reproductive capabilities of the exotic species and its ecological interactions with other species in the new environment, must also be understood to more fully evaluate the environmental impact of potential introductions.

**JUVENILE GROWTH OF CAGE-REARED STIMPSON'S SURFLCLAMS (*MACTROMERIS POLYNYMA*) IN MAINE, USA.** Christopher V. Davis, Pemaquid Oyster Company, Inc., PO Box 302, Waldoboro, ME 04572.

Stimpson's surfclam (*Mactromeris polynyma*, Stimpson 1860) is a circumboreal species ranging throughout northwestern Atlantic and northeastern Pacific inshore waters. Distinguished from other Mactrids by its purple foot, siphon, and mantle edge which

turn brilliant orange-red when cooked, this species is highly sought after for the Japanese sushi market. A preliminary investigation of this species' mariculture potential in Gulf of Maine waters was conducted in 1997 from biological, technical, and commercial perspectives. Laboratory and field growout studies investigated the effects of substrate type and growing site on juvenile growth and survival during one growing season.

Cage-reared surfclams set out in the low intertidal zone exhibited both poor growth and survival. Surfclams grew an average of 1.6 mm in shell length (6.4% increase in SL) and had a 30% cumulative mortality over a 3.5 month period. In contrast, subtidally-reared juvenile surfclams grew from an initial size of 23 mm SL to 33, 35 and 39 mm SL at the Horseshoe Cove, Damariscotta River and Beals Island sites, respectively. Mean size among cohorts at the three growing sites were significantly different from one other after two months growth and continued to diverge in subsequent months. Mortality was nil at all three growing sites. Although the overall time to rear this species to the target size of 50 mm SL in Maine waters is still unknown, this growth information, when combined with data from other year/size classes, will assist in predicting the growing time required to reach market size.

**SURVIVAL AND GROWTH OF JUVENILE GREEN SEA URCHINS ON DIFFERENT MACROALGAL SETTLEMENT SUBSTRATES.** Nils T. Hagen, Department of Fisheries and Natural Science, Bodø College, N-8002 Bodø, Norway.

The effect of settlement substrate on the survival and growth of juvenile green sea urchins (*Strongylocentrotus droebachiensis*) was investigated in a laboratory experiment which was independently replicated 3 times. Five substrates were investigated: the articulate coralline alga *Corallina officinalis*; the crustose coralline alga *Lithothamnion glaciale*; the foliose red alga *Palmaria palmata*; the minute green alga *Ulvelva lens*; and the natural biofilm which developed in empty experimental containers. A total of 4750 competent urchin larvae gave rise to 2064 juveniles during Phase 1 of the experiment, which lasted between 6 and 11 weeks. During Phase 2 of the experiment, which lasted between 8 and 11 weeks, all surviving juveniles were fed *P. palmata*. The total number of surviving juveniles was reduced to 1153 by the end of Phase 2. In Phase 1 the control treatments with natural biofilm had the best survival rates ( $\approx 60$ – $70\%$ ), closely followed by *Corallina* and *Lithothamnion*, whereas survival on *Palmaria* was 25–50%, and survival on *Ulvelva* was  $\leq 10\%$ . In Phase 2 the pattern of survival changed substantially, and juveniles which had settled on the coralline algae clearly had the best overall survival. The growth of juveniles settled on the coralline algae was consistently better than controls during both phases of the experiment, although juveniles settled on *Palmaria* had similar growth in the third replicate of the experiment and in Phase 1 of the first replicate. In conclusion, the best overall growth and survival was obtained in treatments with coralline algae.

**INCREASING THE SOMATIC GROWTH RATE OF JUVENILE GREEN SEA URCHINS (*STRONGYLOCENTROTUS DROEBACHIENSIS*) USING PREPARED DIETS.** Eddy J. Kennedy and Shawn M. C. Robinson, DFO, Biological Station, St. Andrews, NB, Canada E0G 2X0; G. Jay Parsons, Marine Institute, Memorial University, St. John's, NF, Canada A1C 5R3.

As the world market demand for sea urchins continues to rise, the wild fishery is reaching a limit for providing good quality sea urchins thus other means, such as aquaculture, must supply world demand. In order to provide a constant seed supply for a sea urchin aquaculture industry, a hatchery must become a realization. Part of such an operation is maximizing juvenile somatic growth rates to shorten the overall production cycle, increase quality and increase production revenue. One approach is to use prepared diets as these have a positive effect on gonad growth rates of adult sea urchins. Components of the prepared diets must be investigated to determine the effects on juvenile growth so that a diet yielding maximal growth rates can be developed. This study investigated the effect of three factors: protein source, protein concentration and juvenile size on somatic growth rates. Protein sources used were (a) soybean protein, (b) 95% soybean protein and 5% fish protein, and (c) 50% soybean and 50% fish protein. For each set of diets, 4 different protein concentrations were used: 20%, 30%, 40%, and 50%, for a total of 12 diets. The two cohorts of sea urchins had a size range of 1–8 mm and 12–20 mm initial test diameter. Each treatment consisted of 4 replicates in a randomized block design. Twenty-four tanks, with 4 treatment baskets per tank, were subject to the prepared diets while 2 "control" tanks were fed a natural diet of seaweeds for comparison. Results of the study will be further discussed with regard to establishing diets for future hatchery/nursery production of the green sea urchin.

**SHELLFISH RESTORATION: HAVE WE BEEN SUCCESSFUL?** Dorothy L. Leonard, Habitat Conservation Office, National Marine Fisheries Service, Silver Spring, MD 20910.

The climate of diminished public funding and the desire to maintain and improve shellfish and its habitat has motivated many in the scientific, educational, public, nonprofit and private sectors to join forces and share resources to achieve shellfish restoration goals. Also concerned with declines in environmental quality and fisheries are numerous local volunteer organizations who have developed successful programs to identify problem areas, recommend improvements and monitor progress. As a result the last five years have seen an acceleration in the number of shellfish restoration activities undertaken by individuals and partnerships formed to facilitate funding and implementation. The question raised in this presentation is how successful these efforts have been. Is there an increase in shellfish waters approved for harvest? Have shellfish landings increased? Are the enhancement and aquaculture efforts improving the health of the ecosystem? And, what have the costs been? Examples of successful shellfish restoration projects are presented with an evaluation of the impacts.

**EFFECTS OF SEA LETTUCE, *ULVA LACTUCA*, MATS ON ABUNDANCES OF SOFTSHELL CLAMS, *MYA ARENARIA*, AND ASSOCIATED INVERTEBRATES IN NEW JERSEY.** Clyde L. MacKenzie, Jr., James J. Howard Marine Sciences Laboratory, Northeast Fisheries Science Center, 74 Magruder Road, Highlands, NJ 07732.

Eutrophication of estuarine waters throughout the world has resulted in a proliferation of *Ulva* sp. and other algae. In the Navesink River in northeastern New Jersey, sea lettuce, *Ulva lactuca*, mats grew over beds of softshells, *Mya arenaria*. The softshells initially extended their siphons several cm out of the substrates and within a few weeks their entire bodies emerged from the substrates and the softshells died. The mats also sharply reduced the abundances of associated invertebrates, such as polychaetes, other mollusks, and crustaceans. Few invertebrates lived on the surfaces of the mats. In southern New Jersey, other investigators showed that sea lettuce mats provide habitat for some small fishes. We need studies to determine effects of the presence of sea lettuce on a broad spectrum of animals before we can remove it to increase abundances of softshells for the benefit of commercial and recreational fishermen in this area.

**SPATIO-TEMPORAL VARIATION IN SESTON FLUX, GROWTH AND PRODUCTION OF THE BLUE MUSSEL, *MYTILUS EDULIS*, HELD IN SUSPENDED CULTURE, IN A SUB-ARCTIC ENVIRONMENT.** Gina McNeil and Cyr Couturier, Fisheries and Marine Institute of Memorial University of Newfoundland, St. John's, NF, Canada A1C 5R3.

The Newfoundland mussel culture industry has experienced significant growth over the last five years and growers are beginning to fully utilize the available space. This has raised questions as to the extent/level of maximum/optimum stocking levels. A reciprocal transplant experiment of three mussel populations was undertaken at three commercial aquaculture sites with different hydrographic and environmental regimes. Variations in growth, survival and production were assessed bimonthly in relation to seston flux, temperature and salinity which were measured every 2–3 weeks at several stations on each site. CaSO<sub>4</sub> cylinders (15 cm long × 3.75 cm in diameter) were calibrated with S4 current probes to assess relative current speeds. A strong positive relationship was established between cylinder dissolution and actual current speeds, providing a useful index for calculating seston flux. Mussel growth and production varied according to season, populations, site and location on the site (ANOVA,  $p < 0.05$ ). There were no observable differences in natural mortality amongst populations. Differences in mussel performance were related to the relative seston flux, generally showing higher growth and production in areas of higher flux. The importance of relative food flux measurements is discussed in relation to site evaluation and production capacity estimates.

**THE EFFECT OF LOCATION AND TIME OF YEAR ON MUSSEL PRODUCTIVITY IN AN AQUACULTURE ESTUARY.** Linda E. Waite, Thomas Landry,<sup>1</sup> and Jeff Davidson, Atlantic Veterinary College, PEI. <sup>1</sup>Department of Fisheries and Oceans, Gulf Fisheries Centre, Moncton, NB.

The PEI aquaculture shellfish industry, worth more than \$25 million annually, is beginning to see signs of production limitations. Production differences among bays and within bays have been questioned by the industry and this uncertainty is leading to concerns on the capacity of grow-out sites to withstand further development. A DFO (Department of Fisheries and Oceans) Strategic Science Fund initiative research program called COSAD (Coastal Oceanography and Sustainable Aquaculture Development) has been formed to address this issue. It recognizes the importance to understand the relationship between marine habitats and aquaculture production. As part of the COSAD initiative, studies have been conducted to determine if geographic location and time of year have an effect on mussel productivity. Mussel productivity, measured as the incremental increase in shell length and physiological condition factor over a specified time period, was sampled from five sites over two years from May to November and one year from January to March. These sites are in Tracadie Bay, a productive aquaculture estuary on the north side of Prince Edward Island. They were chosen for their diverse geographic locations and cover the entire bay where aquaculture is located. Analysis of mussel productivity depending on the location within the bay showed differences with higher productivity near the mouth of the bay. Analysis of mussel productivity depending on the time of year showed differences with the highest productivity in June with a second peak in early October. The effect of physical and biological interaction on mussel productivity will be discussed.

**MITIGATING PREDATION BY THE EUROPEAN GREEN CRAB, *CARCINUS MAENAS*, UPON PUBLICLY MARI-CULTURED QUAHOGS, *MERCENARIA MERCENARIA*.** William C. Walton and Gregory M. Ruiz, Smithsonian Environmental Research Center, PO Box 28, Edgewater, MD 21037; Bethany A. Starr, Martha's Vineyard Shellfish Group, PO Box 1552, Oak Bluffs, MA 02557.

To supplement local clam, *M. mercenaria*, fisheries, municipalities on Martha's Vineyard, MA (USA) seed local mud flats with juvenile clams. Despite the apparent modest success of this program, managers would like to improve seed survival. Observed seedings indicate a rapid (<72 hrs) decline in seed numbers coincident with evidence of predation (e.g., shell fragments). Field per capita feeding experiments and the overwhelming predominance of green crabs relative to other predators indicate that the European

green crab, *Carcinus maenas*, is the most damaging predator. To mitigate this predation, we tested several mechanistic seeding factors: timing, size and density. Monthly outplants of seed from April through October did not indicate any 'window of decreased predation'. In cage experiments, however, seed size significantly influenced predation rates of green crabs. Further laboratory experiments indicated that large seed (18+ mm shell length) were relatively safe from green crabs (by far the most common crab) and lady crabs *Ovalipes ocellatus*, but to a lesser degree from blue crabs, *Callinectes sapidus*. Lastly, per capita predation rates by green crabs enclosed in field cages increased with increasing seed density (8 to 144 per sq. m). Open field plots of different seed densities, however, exhibited no such increase; rather, predation was approximately 50% across the range tested. In conclusion, we present a preliminary model integrating these factors, as well as efforts to directly control green crabs, to provide managers with an optimum seeding strategy.

## LOBSTER ECOLOGY AND FISHERIES

**LOBSTER ( *HOMARUS AMERICANUS*) MOVEMENT IN THE SOUTHERN GULF OF ST. LAWRENCE.** Michel Comeau, Marc Lanteigne, Guy Robichaud, and Fernand Savoie, Science Branch, Department of Fisheries and Oceans, Maritimes Region, P.O. Box 5030, Moncton, N.B., Canada, E1C 9B6.

LoBster (*Homarus americanus*) tagging studies were conducted in 34 sites throughout the southwestern Gulf of St. Lawrence between 1980 and 1997. Results show relatively small traveled distances between the release and recapture position for animals ranging from 48 to 152 mm of carapace length. The average traveled distance ranged from 2 km in parts of Baie des Chaleurs and Cape Breton to 15 km in central Northumberland strait. No relationship was observed between the traveled distance and the number of days at liberty or the size of the animal. Furthermore, except for 6 out of 22 sites, no significant difference was observed for the average traveled distance between males, females and berried females. For the six sites where a significant difference was observed, the average traveled distance was farther for female lobsters. Geographical differences observed in the traveled distances seem to be related to the bottom topography. Long average traveled distances were observed in sites characterized by flat bottom with relatively smooth transition between shallow and deep areas (1 to 20 m). Short average traveled distances were observed in near shore lobster habitat characterized by a change in bathymetry (to depths >20 m) over a relatively short distance.

**DEVELOPMENT OF A LIPID CONDITION INDEX IN LOBSTERS (*HOMARUS AMERICANUS* H. MILNE EDWARDS, 1837) AND ITS APPLICATION IN THE INTERPRETATION OF LARVAL DISTRIBUTION IN CLOSE PROXIMITY TO GEORGES BANK, GULF OF MAINE.**

**Gareth C. Harding**, Department of Fisheries and Oceans, Bedford Institute of Oceanography, P.O. Box 1006, Dartmouth, N.S., B2Y 4A2, Canada; **A. J. Fraser**, 33 Battery Drive, Halifax, N.S., B3P 2G9, Canada.

The triacylglycerol/sterol condition index was applied to larval lobster populations in the vicinity of Georges Bank in the Gulf of Maine. This index is related to larval size by an increasing power function which explains around 40% of the variation. This poor fit can be explained by the uneven increase in triacylglycerol levels during development within each moult stage. Increased pigmentation is not related to larval condition, as measured by lipid storage, and masks the increased yellowish hue of lipids as development proceeds. The larval triacylglycerol/sterol index appears to undergo a diurnal cycle in stage IV and possibly stage III lobster, with lowest values at midday and highest values after dark. This pattern can not be explained completely by either nocturnal feeding or vertical diffusion by diurnal migrations, which leaves the possibility that healthy larvae might also be more likely to see the trawl and escape during daylight. Few lobster larvae were found in the vicinity of Georges Bank with a condition index less than 0.1, which is the level laboratory studies indicate approaches the "point-of-no-return". The condition of all developmental stages was found to be better in individuals located off Georges Bank. This is not ecologically significant in the case of the first two stages because such a small proportion of the population was actually located off the bank. It is not resolved how the third and fourth stages arrive off Georges Bank, since shoal water hatching is the norm, but their lipid reserves are significantly greater than identical developmental stages on the bank. Finally, the abundance of stage IVs per m<sup>3</sup> in the surface waters over Gulf of Maine is twice that found over Georges Bank which suggests that the lobster has evolved a life cycle in offshore waters in which the last two planktonic/pelagic stages either seek or are transported to, and by stage IV thrive in, the warmer stratified waters over greater depths.

**BEHAVIORAL THERMOREGULATION AND ITS EFFECTS ON THE MOVEMENT OF LOBSTERS IN THE FIELD.** **Steve Jury, W. Hunt Howell, and Winsor Watson.**

Center for Marine Biology & Zoology Dept., University of New Hampshire, Durham, N.H. 03824.

We have shown that lobsters, *Homarus americanus*, are capable of behaviorally thermoregulating in laboratory thermal gradients where males consistently preferred warmer temperatures than females, showing a final preferred temperature of 16.3°C (i.e., the temperature where acclimation temperature equals selected temperature), whereas females had a final preferred temperature of

14.3°C. Lobsters preferred significantly warmer temperatures in colder months ( $2.8 \pm 0.66$  warmer than ambient temperatures of  $8.2 \pm 0.3^\circ\text{C}$ ) than in warmer months ( $0.2 \pm 0.39$  warmer than ambient temperatures of  $15.5 \pm 0.4^\circ\text{C}$ ). Activity and avoidance assays also show that lobsters avoid water warmer than  $23.5 \pm 0.4^\circ\text{C}$  and at  $<5^\circ\text{C}$  they are relatively immobile and thus cannot behaviorally thermoregulate.

Catch per unit effort (CPUE) data from 1989–1991 in the Great Bay Estuary, New Hampshire show significant non-linear relationships with these laboratory data such that the highest catches tended to correlate with the preferred temperatures. In addition, a mechanistic model of the behavioral response to temperature based upon laboratory data results in movements comparable to those estimated from estuarine tag-recapture data from 1989–1991 in terms of direction and relative distance moved. These results indicate that laboratory and field derived estimates of thermal preference of lobsters are comparable in several ways and that models predicting lobster movements, distribution and abundance using temperature as a parameter may benefit by incorporating sex and acclimation effects.

**LOBSTER (*HOMARUS AMERICANUS*) COMMERCIAL CATCH COMPOSITION FLUCTUATIONS BASED ON A TIGHT TEMPORAL AND GEOGRAPHICAL SEA SAMPLING PROGRAM.** **Marc Lanteigne**, Science Branch, Department of Fisheries and Oceans, Maritimes Region, P.O. Box 5030, Moncton, N.B., Canada, E1C 9B6.

A weekly lobster (*Homarus americanus*) commercial catch sampling program was conducted in 35 landing sites along the coast of Prince Edward Island (PEI), Canada, in 1998. By sampling onboard commercial fishing vessels, information was collected on the prevalence of lobsters under the minimum legal size and of egg bearing females, which are both returned at sea during regular fishing activities.

Results show important temporal and geographical size composition variability. The removal of commercial size lobsters, and the accumulation of sub-legal size and egg bearing females as they are returned at sea, explains the temporal changes observed in the size compositions. The prevalence of egg bearing females in the commercial catches shows areas of abundance along the coast of PEI. These observations are suggesting that some areas may have higher levels of lobster egg production compared to neighbouring areas.

A distinct size composition gradient is observed in the Northumberland Strait. The central section of the Strait is characterized by the presence of larger lobsters and an overall multiple mode wide size range compared to the single mode narrow size range in both extremities of the Strait. These observations corroborate with the information obtained from fisheries statistics and suggest a phenomenon that may be explained by a particular lobster movement pattern within the Northumberland Strait.

**EGG PER RECRUIT AS A MANAGEMENT TARGET FOR AMERICAN LOBSTER FISHERIES.** R. J. Miller and K. F. Drinkwater, Bedford Institute of Oceanography, Dartmouth, Nova Scotia B2Y 4A2.

Management targets for American and spiny lobsters have been defined using the ratio of eggs per recruit in fished to unfished populations. Chosen values not to be exceeded range from 0.05–0.2. For 13 areas in eastern Canada we found no correlation between eggs/recruit ( $e/r$ ) and fishery yield/km<sup>2</sup> of fishing ground. Adjusting for possible larval drift between adjacent fishing areas did not improve the correlation. This result suggests that management targets using egg production are not useful, or should be area specific. Using the  $e/r$  ratio for fished and unfished populations presents other problems. Data for calculating  $e/r$  for unfished (virgin) populations seldom exist. The concept of  $e/r$  in fished and unfished populations is difficult to communicate to stakeholders. There is little or no theoretical basis for choosing a particular ratio. The calculation is based on eggs per lobster rather than eggs per geographic area; we usually manage lobster fisheries by area. Inputs to calculating  $e/r$  (growth, fishing mortality, size at maturity) can change temporally.

**SPATIAL SCALING OF HABITAT DISTRIBUTIONS IN THE AMERICAN LOBSTER.** Robert W. Rangeley and Peter Lawton, Biological Station, Department of Fisheries and Oceans, St. Andrews, New Brunswick, Canada EOG 2X0.

American lobster (*Homarus americanus*) preferences for near-shore cobble habitats are well known yet little is understood about the effects of spatial scaling of habitat patches on lobster distribution patterns. We studied patterns of lobster distributions for a range of spatial scales in two regions in Atlantic Canada immediately following heavy fisheries exploitation. In the southern Gulf of St. Lawrence habitat patches were extremely complex and fragmented at large spatial scales yet the flat cobble and bedrock substrates yielded relatively little crevice space at the scale of individual lobster shelters. Densities in the nearshore were low and heavily skewed towards juveniles. Exceptions were in relic stream beds which were small in area but contained the most productive habitats. In contrast, at our Gulf of Maine sites patch sizes were very large and fragmentation was low. Small scale structural complexity was high and supported greater densities of all sizes classes of lobsters. In addition, adult lobsters were also distributed on soft sediments and on large patches of horse mussels far from shelter patches. Our study demonstrates the value of analysing distribution patterns at multiple spatial scales.

**DAILY MOVEMENTS OF LOBSTERS FROM ULTRASONIC TRACKING.** M. John Tremblay and R. Duggan, Invertebrate Fisheries Division, Bedford Institute of Oceanography, P.O. Box 1006, Dartmouth, N.S. B2Y 4A2; R. O'Dor, C. Curtis, D. Webber, and Y. Andrade, Department of Biology, Dalhousie University, Halifax, N.S.

The daily range and periodicity of lobster (*Homarus americanus*) movement is of interest from several perspectives, including the likelihood of entry to traps, and the size of marine protected areas. Data are reviewed from studies that have located lobsters intermittently, and from 2 studies on the east coast of Nova Scotia where lobsters were tracked continuously. In Jeddore Harbour (early summers of 1989 and 1997), lobsters were tracked with ultrasonic transmitters fixed to the carapace. An array of 3 acoustic buoys received pulses from the transmitters and a base station calculated position based on pulse arrival time. Five ovigerous females (CL > 100 mm) were tracked in 1989; four immature females (72–88 mm CL) in 1997. For several reasons the 1989 and 1997 studies are not directly comparable but the results suggest differences in activity and movement related to size or maturity. Compared to the ovigerous females, the immature females tended to move less, particularly during daylight. Between the hours of 0800 and 2000 h immature females moved 32–48% as far as during the night (2100–0700 h). Ovigerous female movement during the day was 58–98% of that during the night. The greater daylight activity of large female lobsters may result in increased catchability which has been observed elsewhere at this time of year. The generality of size-related movement differences needs further study.

## MODELLING SHELLFISH ECOSYSTEMS

**A PHYSIOLOGICALLY-BASED MODEL OF THE GROWTH AND DEVELOPMENT OF *CRASSOSTREA GIGAS* LARVAE.** E. A. Bochenek, New Jersey Sea Grant Extension Program, Rutgers Cooperative Extension, 1623 Whitesville Rd., Toms River, NJ 08755; E. N. Powell, Haskin Shellfish Research Laboratory, Rutgers University, 6959 Miller Ave., Port Norris, NJ 08349; E. Hofmann and J. Klinck, Center for Coastal Physical Oceanography, Crittendon Hall, Old Dominion University, Norfolk, VA 23529.

A physiologically-based model was used to investigate the processes that control the growth and development of larvae of the Japanese oyster, *Crassostrea gigas*. The model is structured around the nonlinear relationship between larval caloric content and larval length; larvae reach a minimum in caloric content at 130–160  $\mu$ m. Formulations used to model larval filtration, ingestion and respiration rates were based upon laboratory and field studies. Simulations of larval growth show that under environmental conditions typical of most temperate estuaries, larval develop-

ment is completed in 20–30 days, depending upon ambient water temperature and food concentration. Periods of reduced food concentration, especially as the larvae are approaching their minimum caloric value, result in the larvae reaching and exceeding their metabolic point-of-no-return. In these simulations, providing food later in the larval development does not enhance larval survivorship. Thus, predictions of *C. gigas* larval survival from only adult egg condition will be in error if environmental conditions during the early larval life history are not considered.

**A BAYESIAN APPROACH TO SHELLFISH ECOSYSTEM MODELLING.** Michael Dowd, Satlantic Inc., Halifax, Canada; Renate Meyer, Dept. of Statistics, University of Auckland, Auckland, New Zealand; W. Carlisle Thacker, Atlantic Oceanographic and Meteorological Laboratory, Miami, FL.

Limited ecosystem models provide a useful framework for predicting shellfish growth and for addressing aquaculture concerns related to stocking density and carrying capacity. Such models predict the time evolution of that portion of the ecosystem directly important to shellfish growth and are coupled to circulation or particle dispersion models. For practical or management purposes, it is imperative that the uncertainty in the predictions of these intrinsically nonlinear models be quantified. Towards this end, we argue for a stochastic framework to predicting shellfish growth which optimally combines observations and models. Specifically, we offer a Bayesian approach to data assimilation which is applicable to time-dependent, nonlinear, and non-Gaussian ecosystem models. We focus on the practical aspects of implementing the methodology and provide an illustration based on a simple model describing bivalve growth in a coastal inlet. Extensive use is made of available software that takes advantage of recent advances in Monte Carlo integration and provides a computational environment suitable for the non-statistician. Our overall goal is to assess the promise (and pitfalls) of this Bayesian approach to unifying statistical and mechanistic models in order to better describe and predict the dynamics of shellfish ecosystems.

**EFFECT OF SPAWNER DENSITY AND DISTRIBUTION ON FERTILIZATION SUCCESS IN THE SEA SCALLOP, *PLACOPECTEN MAGELLANICUS* GMELIN.** Jean-François Dumas and Xavier Boespflug, Université du Québec à Rimouski, 310 allée des Ursulines, Rimouski, Québec, G5L 3A1 Canada; Dominique Baudinet and Marcel Fréchette, Institut Maurice-Lamontagne, Ministère des Pêches et des Océans, C.P. 1000, Mont-Joli, Québec, G5H 3Z4 Canada.

We model critical spawner concentration for preventing recruitment overfishing (RO) in sea scallops, *Placopecten magellanicus*. We assume that fertilization rates are dependent on ga-

mete concentration as measured in the laboratory. Fertilization rate *in situ* varies along gamete diffusion plumes downstream of spawners. The criterion for RO is that reproductive effort of an individual female is insufficient to balance total mortality during larval, juvenile and adult life. Natural mortality estimates are taken from the literature. Preliminary runs of the model suggest that for the particular settings used (regular spatial distribution, size of scallop groups = 4, 36 individuals, 10 cm shell height females, current speed = 0.2 m/s, etc.), results are sensitive to group size, which implies that small scale spatial patterns are important in fertilization success. Populations with the highest natural mortality rates tested do not persist. At median natural mortality rates and annual exploitation rate = 0.1, 0.2 and 0.3, RO occurs at spawner population density lower than ca. 0.004/m<sup>2</sup>, 0.45/m<sup>2</sup> and 5/m<sup>2</sup>, respectively. In groups with low natural mortality rate, RO does not occur at annual exploitation rates lower than 0.5.

**MODELLING RESUSPENSION AND ITS EFFECTS ON BI-VALVE FOOD SUPPLIES.** Jon Grant, Department of Oceanography, Dalhousie University, Halifax, NS B3H 4J1; Cedric Bacher, Centre de Recherche en Ecologie et Aquaculture (CREMA), BP 5, l'Hommeau, 17300, France.

Many models of shellfish growth in culture are based on food supplies being a primary determinant of growth. The representation of suspended food in these models is difficult because seston is a mixture of phytoplankton, detritus, and other material with complex temporal dynamics. In shallow coastal systems, resuspension contributes significantly to the particle load, although the material injected into the water column is of variable quality, depending on the abundance of benthic microalgae, macrophyte detritus, etc. Rates of resuspension are regulated by substrate type (sand and mud), shear stress due to waves and currents, and biological processes such as bioturbation. For cohesive muds, a model of resuspension and its effect on water column turbidity is superficially straightforward. However, there are a variety of pitfalls including critical erosion threshold, depth of erosion, deposition, food quality, and groundtruth data all of which complicate model formulation. A model of tidal resuspension is applied to a mussel aquaculture site in a shallow muddy bay (Upper South Cove, Nova Scotia). Previous studies in the cove have examined erosion rate and threshold as well as near-bottom suspended particulate matter, providing an unusually complete data set for model verification. Considerations of erosion formulation and its assumptions are presented for this model. The implications for applying resuspension to overall models of bivalve culture carrying capacity are then explored.

**MODELLING POPULATION DYNAMICS OF PACIFIC OYSTER *CRASSOSTREA GIGAS* IN KOREA.** Kymng-Hoon Hyun, Ig-Chan Pang, and Kwang-Sik Choi, College of Ocean Science, Cheju University Korea, Cheju-Do 690-756, Korea; Eric N. Powell, Haskin Shellfish Research Laboratory, Rutgers University, Port Norris, NJ 08349; John M. Klinck and Eileen E. Hofmann, Center for Coastal Physical Oceanography, Old Dominion University, Norfolk, VA 23529.

To determine the growth relationships of Pacific oyster, *Crassostrea gigas*, shell length, weight and gonadal condition of post-settlement oysters in mariculture fields in the Kamakman Bay, Choun-Nam Province, Korea, were measured from June 1997 to May 1998. Environmental factors, including food levels, were also measured. Dry weights were used to determine the rate of oyster growth in the field, and these were compared to the simulated growth rates obtained from a model of oyster population dynamics. The Kamakman Bay oysters with a shell length of 17 mm and a dry weight of 0.3 g that settled in June had increased to 73 mm and 3.2 g by May of the next year. This growth rate is slow compared to the growth rate of the same species grown in Japanese mariculture fields. No significant spatial variations in oyster growth were observed within one oyster farm sampled in detail, suggesting that flow speed through the farm is sufficient to prevent a local reduction in food supply produced by oyster filtration. The oyster model predicts lower growth curves than observed in the field when chlorophyll-a is used as a measure of oyster food supply. This is not unusual. Adding a correction factor from Soniat et al. (in press) based on measures of non-chlorophyll explained food produces a much better fit between simulation and observation. Using labile carbohydrates+protein as a food source results in higher growth rates than observed. Reducing filtration efficiency by assuming that most of the food is present in small particles results in simulated growth rates that are similar to field conditions.

**RAPANA VENOSA IN THE CHESAPEAKE BAY: CURRENT STATUS AND PROSPECTS FOR RANGE EXTENSION BASED ON SALINITY TOLERANCE OF EARLY LIFE HISTORY STAGES.** Roger Mann, Juli Harding, and Stephanie L. Haywood, Virginia Institute of Marine Science, Gloucester Point, VA 23062.

The Veined Rapa Whelk, *Rapana venosa*, has recently been identified as present in the Hampton Roads region of the Chesapeake Bay. The species is native to the Sea of Japan, but was introduced to the Black Sea in the 1940's, and has since spread to the Aegean and Adriatic Seas. There is strong evidence that range extension is mediated by transport of early life history stages in ballast water. The current status of knowledge of distribution of *R. venosa* in the Chesapeake Bay is described. There is concern over the potential impact of *R. venosa* on local shellfish populations and the industry that they support. Egg cases of *R. venosa* have been collected from the field, and larval forms cultured in the labora-

tory. Estimates of the salinity tolerance of the larval stages of *R. venosa* are described as a precursor to using current 3-D modeling of Chesapeake Bay circulation to estimate a potential range of distribution of the species within the Chesapeake Bay and its sub-estuaries. Such estimates are crucial to establishing which shellfish resources are potentially susceptible to predation by local *R. venosa* populations.

**LINKING WATER QUALITY AND LIVING RESOURCES: A COUPLED SUSPENSION FEEDER-EUTROPHICATION MODEL.** Mark B. Meyers, HydroQual, Inc., 1 Lethbridge Plaza, Mahwah, NJ 07430; Dominic M. Di Toro, HydroQual, Inc. and Dept. of Environmental Engineering, Manhattan College, Riverdale, NY; James J. Fitzpatrick, HydroQual, Inc.

Modern eutrophication models explicitly simulate many aspects of the carbon, nutrient, and oxygen dynamics of complex estuaries, including the interactions of settling organic matter, sediment diagenesis, and fluxes across the sediment-water interface. At the same time resource managers are seeking insight regarding connections between traditional eutrophication problems and living resource issues. We have coupled a model of suspension-feeding bivalve production to the Chesapeake Bay Water Quality Model, providing a dynamic feedback in the eutrophication-production-anoxia loop and a link between traditional water quality concerns and the food resources of higher trophic levels. Incorporation of suspension-feeding bivalves into the model improved calibration in regions where extensive bivalve populations are known to exist. Predicted bivalve biomass compared well with an extensive 12-year benthic monitoring program data set, in response to temporal and spatial variations in food and bottom water dissolved oxygen conditions.

**MODELING THE MSX PARASITE IN EASTERN OYSTER (*CRASSOSTREA VIRGINICA*) POPULATIONS: MODEL DEVELOPMENT, IMPLEMENTATION AND VERIFICATION.** Eric N. Powell and Susan E. Ford, Haskin Shellfish Research Laboratory, Rutgers University, Port Norris, NJ 08349; Eileen E. Hofmann and John M. Klinck, Center for Coastal Physical Oceanography, Crittenton Hall, Old Dominion University, Norfolk, VA 23529.

A model simulating the host-parasite-environmental interactions of Eastern oysters (*Crassostrea virginica*) and the pathogen, *Haplosporidium nelsoni*, which causes the disease MSX, has been developed. The model is physiologically-based and is structured around proliferation and death rates of *H. nelsoni* under different environmental conditions. Equations describing these rates were constructed using data from long-term field observations and field and laboratory experiments. Simulations that use environmental conditions characteristic of Delaware Bay reproduce the observed seasonal *H. nelsoni* cycles and consequent oyster mortality. These

simulations show the effect of environmental factors, such as salinity and cold temperatures, on controlling the intensity and prevalence of *H. nelsoni* infections. However, biological controls from density-dependent feedback on *H. nelsoni* proliferation and *H. nelsoni* sporulation events also greatly affect disease prevalence and intensity. The oyster-*H. nelsoni* model provides a quantitative framework for guiding future laboratory and field studies, as well as management efforts.

**A SIMPLE MODEL FOR ESTIMATING TIME TO CRITICAL LEVELS OF PERKINSUS MARINUS IN EASTERN OYSTERS, CRASSOSTREA VIRGINICA.** Thomas M. Soniat, Department of Biological Sciences, Nicholls State University, Thibodaux, LA 70310; Enrique V. Kortright, Department of Computer Science, Nicholls State University, Thibodaux, LA 70310; Sammy M. Ray, Department of Marine Biology, Texas A&M University at Galveston, Galveston, TX 77553.

A simple mathematical model is presented which uses an Excel spreadsheet to estimate a time to a critical level ( $t_{crit}$ ) of *Perkinsus marinus*, in eastern oysters *Crassostrea virginica*. The estimate assumes that a weighted incidence (WI) of disease of 1.5 is critical. It converts measured WI values and the critical WI to parasite number, calculates a rate of change ( $r$ ) of the parasite population using measured values of water temperature (T) and salinity (S), and solves for  $t_{crit}$  by simulation. Estimates of  $t_{crit}$  and  $r$  using a long-term data set of T, S, and WI from the Terrebonne estuary of Louisiana are provided. The model does not predict future values of WI since it cannot predict future trends in T and S; however, regularly determining T and S, considering their interaction in a model, measuring WI at reasonable intervals, and iteratively estimating  $t_{crit}$ , should be useful to oyster management. Users will have access to the model through the worldwide web. Estimates of  $t_{crit}$  would support decisions concerning transplanting infected oysters to lower salinity areas, harvesting heavily-infected populations early, and diverting freshwater into high-salinity estuaries.

**QUANTITATIVE ASPECTS OF OYSTER REEF BROOD-STOCK ENHANCEMENT IN THE GREAT WICOMICO RIVER, VIRGINIA.** Melissa Southworth and Roger Mann, Virginia Institute of Marine Science, Gloucester Point VA 23062.

The Great Wicomico River is a small, trap type estuary on the western shore of the Chesapeake Bay. Resident oyster populations were eliminated by the combined effects of Tropical Storm Agnes in 1972, and subsequent disease mortalities related to *Perkinsus marinus* and MSX. Oyster broodstock enhancement was initiated in June 1996 by the construction of a three dimensional intertidal reef with oyster shell, followed by the "seeding", in December 1996, of that reef with high densities of large oysters from disease challenged populations in Pocomoke and Tangier Sound. Estimated egg production of the reef population is within an order of magnitude of total egg production in the Great Wicomico River

prior to Tropical Storm Agnes. Field studies in 1997 indicate spawning by reef oysters from July through September. MSX was absent. *Perkinsus* prevalence increased from 32% in June to 100% in July, while intensity increased from June to September. Plankton tows recorded oyster larval concentrations as high of  $37,362 + 4,380 \text{ m}^{-3}$  on June 23—orders of magnitude higher than typically recorded in Virginia subestuaries of the Chesapeake Bay in the past three decades. Drifter studies suggest strong local retention of larvae, a suggestion reinforced by marked increases in local oyster spatfall on both shellstring collectors and bottom substrate in comparison to years prior to 1997.

## REPRODUCTION AND RECRUITMENT

**TWO-YEAR COMPARISON OF SPAWNING PATTERNS IN SOFT-SHELL CLAMS (MYA ARENARIA).** Linda A. MacLean, Atlantic Veterinary College, UPEI, 550 University Avenue, Charlottetown, PE C1A 4P3; Neil G. MacNair, Prince Edward Island Department of Fisheries and Tourism, P.O. Box 2000, Charlottetown, PE C1A 7N8; T. Jeffrey Davidson and Gerald G. Johnson, Atlantic Veterinary College, UPEI, 550 University Avenue, Charlottetown, PE C1A 4P3.

On Prince Edward Island, Canada, soft-shell clam (*Mya arenaria*) fishing is characterized by licensed seasonal commercial fishers harvesting between 125 and 400 tonnes on an annual basis in the last few years. Recent interest in aquaculture development for *Mya arenaria* requires understanding of the characteristics of spawning since the supply of soft-shell clams is entirely dependent on natural reproduction. The spawning of *Mya arenaria* was monitored and the environmental conditions under which this process occurred were recorded for the field seasons of 1997 (May–Nov.) and 1998 (Apr.–Sept.). Samples of 30 clams greater than 50 mm (2") and samples of 30 clams between 35–50 mm (1.4"–2") in overall length were collected on a weekly basis from three sites. Sites were chosen from both the north and south shores based on different water temperature profiles, tidal flushing, and sediment type. The two north shore sites are located in the same estuary, one in the sheltered upper reaches and the second in an exposed location near the mouth of the estuary. At the exposed site, clams greater than 50 mm in length were not consistently available. Steamed meat yields were performed and provided indicators of physiological changes associated with spawning in *Mya arenaria*. Temperature probes were left *in situ* at each site over the field season (both years) to regularly record water temperature. The spawning performance at the three sites indicate differences in the spawning behavior between locations, and the pattern varied between years. In July of 1997, a large single spawning event occurred in each site whereas, in 1998, there were continuous small spawning events at most sites over the entire field season. This paper will show some of the natural variation in the spawning cycle of *Mya arenaria*.



**THE INFLUENCE OF ESTRADIOL ON VITELLOGENESIS IN THE GREEN SEA URCHIN, *STRONGYLOCENTROTUS DROEBACHIENSIS*.** Nature A. McGinn, Michael P. Lesser, and Charles W. Walker, Department of Zoology, University of New Hampshire, Durham, NH 03824.

Sea urchins store nutrients in specialized gonadal cells called nutritive phagocytes. The size and contents of these cells fluctuate predictably during the reproductive cycle. A major element of the large nutritive phagocytes at the onset of gametogenesis is a yolk related protein. Little is known about the origin and accumulation of this protein in sea urchins. Yolk protein synthesis, part of vitellogenesis, is initiated by estradiol in some animals. Treatment with estradiol may increase yolk related protein production in *S. droebachiensis*. Green sea urchins were collected in June 1998 and maintained in flowing seawater. They were fed a prepared diet either untreated (= control) or treated with 17 $\beta$ -estradiol (1  $\mu$ g/g feed). Test and gonad measurements were made and gonad index was determined for monthly urchin collections through October 1998. Histological sections from fixed and resin-embedded gonads were examined by light microscopy. We are determining the amount and location of yolk related protein in developing urchin gonads using Western blot analysis and immunohistochemistry with rabbit polyclonal anti-urchin major yolk protein antibody. Implications for sea urchin aquaculture will be discussed. Supported by CMB grant to NAM and Sea Grant to CWW and MPL.

**TEMPORAL PATTERNS OF LARVAL AND POST-SET DISTRIBUTIONS OF THE BLUE MUSSEL (*MYTILUS EDULIS/M. TROSSULUS*) AND THE STARFISH (*ASTERIAS VULGARIS*) ON NEWFOUNDLAND MUSSEL CULTURE SITES.** Miranda Pryor, G. Jay Parsons, and Cyr Couturier, Fisheries and Marine Institute of Memorial University of Newfoundland, St. John's, NF, Canada A1C 5R3.

As the blue mussel culture industry in Newfoundland grows, farmers are experiencing problems with the predatory starfish species (*Asterias vulgaris*). Temporal and spatial patterns of planktonic larvae and post-set stages of both species have been studied in-depth to determine if a consistency in the timing of appearance and abundance exists between these organisms. Four geographically distinct sites were chosen throughout the province, with weekly larval samples and spat/juvenile collector retrievals taken from May through November, 1998. Mussel larvae were abundant at three sites, located on the North coast, from mid-June through late August; starfish larvae were present from late July through late August at these sites. Mussel spat and starfish juvenile settlement subsequently occurred at varying rates for all three sites, with peak starfish set occurring about 2–3 weeks after peak mussel set. At the fourth site, located on the southern shore of the island, mussel spawning was sporadic resulting in low settlement on collectors.

As well, no planktonic starfish larvae or settling juveniles were observed on this site. Environmental (temperature, salinity, chlorophyll) and hydrographic conditions were examined in relation to timing of appearance and larval and post-set abundances. Prediction of starfish juvenile settlement, in relation to mussel spat settlement, will provide mussel growers with the ability to make decisions regarding collector deployment in an effort to reduce the potential impact of this predatory species.

**EVIDENCE FOR FALL SPAWNING OF NORTHERN BAY SCALLOPS, *ARGOPECTEN IRRADIANS IRRADIANS* (LAMARCK, 1819), IN NEW YORK.** Stephen T. Tettelbach, Southampton College, Long Island University, Southampton, NY 11968; Roxanna Smolowitz, Laboratory for Aquatic Animal Medicine and Pathology, University of Pennsylvania, Marine Biological Laboratory, Woods Hole, MA 02543; Christopher F. Smith, Kim Tetrault, and Sandra Dumais, Marine Program, Cornell Cooperative Extension, Riverhead, NY 11901.

Spawning of *Argopecten irradians irradians* is generally believed to occur between late May–August; however, some literature reports and our personal observations have suggested that ripe individuals may be present well into the fall. In this study, we examined samples that we had archived from different bay scallop populations in eastern Long Island, New York waters, from different years, to determine if there was any histological evidence of fall spawning. At two sites, a spawning peak in September followed a discrete spawning peak in early summer (late June/early July). Scallops at four different sites were conclusively shown to spawn well into the fall (late September, October or early November) during three different years (1993, 1994, and 1995). Fall spawning of *A. i. irradians* in New York waters does not appear to be uncommon and may be very important during certain years.

**AN INVESTIGATION OF *MYA ARENARIA* (SOFT-SHELL CLAM) RECRUITMENT IN MAINE.** Tracy Vassiliev and William Congleton, Department of Bio-systems Science and Engineering, University of Maine, Orono, ME 04469; Brian Beal, University of Maine Machias, Machias, ME 04654; Stephen Fegley, Maine Maritime Academy, Castine, ME 04420.

The declining *Mya arenaria* recruitment in Eastern Maine contrasts with increasing clam harvests on Maine's Southwestern coast. Core samples (0.0133 m<sup>2</sup>) were collected in the fall/winter from mudflats in Eastern Maine and Southwestern Maine for two years. Densities of juveniles (1.8–6.0 mm) averaged 16.95 clams/m<sup>2</sup> in 120 samples in Eastern Maine and 204.46 clams/m<sup>2</sup> in 120 samples in Southwestern Maine. In May through October 1998

spat bag systems were deployed in Mason Bay (Eastern Maine) and Jones Creek (Southwestern Maine) to determine the density of *Mya* larvae in near-shore waters. Spat bags were replaced and the contents sieved with a 125  $\mu$  mesh each month. Twenty, four-inch flowerpots were filled with mason sand and placed on the intertidal mudflats near the spat bag systems. Pots were replaced monthly and *Mya* settlement was determined by sieving. Preliminary observations indicate a larger number of bivalves collected at the Southwestern site in both spat bags and intertidal pots versus the Eastern sites. These results indicate the reduced number of clam spat, on the mudflats in Eastern Maine, is due to reduced densities of larvae in near-shore waters rather than location rejection by larvae for settlement on the mudflats.

## SCALLOP FISHERIES: ECOLOGY AND APPLIED BIOLOGY

**EFFECTS OF A NEW BITUMEN FUEL SOURCE ON THE GROWTH AND ENERGETICS OF SEA SCALLOPS.** **Shelley L. Armsworthy** and **Peter J. Cranford**, Fisheries and Oceans Canada, Bedford Institute of Oceanography, P.O. Box 1006, Dartmouth, NS, B2Y 4A2; **Kenneth Lee**, Fisheries and Oceans Canada, Maurice Lamontagne Institute, P.O. Box 1000 Mont-Joli, PQ, G5H 3Z4.

A newly developed bitumen-based fuel is presently being used by thermal power plants as a cost-effective alternative to traditional heavy fuels. Based on what is known of the physical properties (relatively high density), chemical composition (large proportion of polyaromatic hydrocarbons), and behaviour of this fuel in seawater (formation of fine bitumen droplets), an accidental spill could pose a threat benthic suspensions-feeding organisms. Laboratory experiments were conducted to determine the lethal and sublethal effects of this fuel to a commercially important benthic suspension feeder, the sea scallop, *Placopecten magellanicus*. Toxicity was determined from acute and chronic mortalities, effects on tissue growth, and changes in the animals energy status (scope for growth: SFG). Mortalities during 43-day chronic exposures increased with increasing fuel concentration (0, 0.01, 0.1, 1 and 10 mg l<sup>-1</sup>), but were generally low with 82% survivorship at the highest concentration tested. Scallop energy budget measurements provided insight into the fuel's impact on growth and the physiological mechanism(s) responsible for the impact. Scallop SFG was reduced by 80% at 0.01 mg l<sup>-1</sup> and was negative for animals exposed to concentrations greater than 1 mg l<sup>-1</sup>. Growth reductions resulted mainly from effects on clearance rate, which was reduced by 90% at fuel concentrations greater than 1 mg l<sup>-1</sup> (EC<sub>50</sub> value was between 0.01 and 0.1 mg l<sup>-1</sup>). Respiration rate and absorption efficiency of scallops exposed to dispersed bitumen increased significantly relative to controls. Research on a new formulation of this fuel source is underway to assess manufacturer claims of reduced toxicity.

**MODELLING POTENTIAL EFFECTS OF DRILLING WASTES ON GEORGE'S BANK SCALLOP STOCKS.** **Peter J. Cranford, Donald C. Gordon, Jr., Charles G. Hannah, John W. Loder, Timothy G. Milligan, and Dwight K. Muschenheim**, Fisheries and Oceans Canada, Bedford Institute of Oceanography, P.O. Box 1006, Dartmouth, NS, B2Y 4A2.

Moratoria on oil and gas activities on Georges Bank are currently in place until 2000 (Canada) and 2012 (USA). If not extended, exploration drilling could take place with the attendant risks to the marine ecosystem and aquatic resources. A numerical benthic boundary layer transport model (*bblt*) was developed to provide estimates of the suspension, drift, dispersion and concentration of water-based drilling mud which could be released from a hypothetical 92-day exploration well at different sites on Georges Bank. Simulations predict that highest near-bottom concentrations of drilling mud would occur in the relatively deep Side region (>100 m) as a result of relatively low suspension, dispersion and drift. Lowest concentrations would occur in the central Mixed region (<65 m) because of high dispersion, while intermediate concentrations would occur in the Frontal region. Laboratory experiments show that adult scallops are highly sensitive to drilling mud, and the near-bottom concentration time series from *bblt* simulations provide a basis for estimating impacts. The region of greatest potential impact on scallop growth is the Side region where mud concentrations from the hypothetical release scenario are predicted to prevent scallop growth for 2–40 days depending upon the settling velocity used and area over which results are averaged. Scallop stocks in this region are relatively small but dense aggregations are found in some areas. Growth losses in the Frontal region, which has the densest scallop stocks, are predicted to be more localized and confined to a range of 0–15 days. Predicted growth loss in the central Mixed region is predicted to be negligible (<2 days).

**MANAGEMENT ADVICE OF GIANT SCALLOP *PLACOPECTEN MAGELLANICUS* BASED ON GONAD MATURATION.** **Leslie-Anne Davidson**, GFC, 343 Archibald St. Moncton, N.B. E1C 9B6; **Yves Poussart**, Université de Moncton, Moncton, N.B. E1A 3E9.

The giant scallop, *Placopecten magellanicus*, which is commercially fished off the east coast of Canada and the United States is also a cultured species. Management strategies to optimize harvest of both wild and cultured scallop, must not adversely affect stock replenishment. The reproduction cycle of the commercial size scallops (>80 mm) has been extensively studied, however, gametogenesis of pre-recruit size groups (<80 mm) has not been studied in detail until the present study. Weekly samples during the reproduction season were retained from the following shell height size intervals: 5–20 mm, 21–25 mm, 26–30 mm, 31–40 mm, 41–50 mm, 51–60 mm, 61–70 mm and 71–80 mm. Histological gonadal section of every scallop sampled were obtained and ob-

served. Scallops in size classes 61–70 mm and 71–80 mm filled their follicles with mature sex cells and released the majority of these cells during the spawning period. Scallops in the size classes between 21 mm and 60 mm accumulated the sexual cells however did not release the majority of these cells during the spawning period. Scallops are fished with dredges made up of rings and washers and the selectivity of the dredge is dependent on the ring size. If the rings are too small, it is possible to harvest a scallop before it effectively contributes to future generations. In addition, if an aquaculturist collects his own spat and also markets half-shell or princess size scallop (60–70 mm), he/she must retain some scallops >60 mm to maintain a viable broodstock on the culture site to assure a successful spat collection.

**JET-PROPELLED SWIMMING IN SCALLOPS.** M. Edwin DeMont, Biology Department, St. Francis Xavier University, Antigonish, N.S. Canada B2G 2W5.

A comprehensive study on the mechanics of jet-propelled swimming in the scallop *Placopecten magellanicus* has been completed. The work integrated unsteady and steady-state internal and external fluid forces, the physical properties of the hinge, and the whole dynamic system. Jet-propelled swimming is initiated by contraction of the adductor muscle. The two shells are rapidly pulled together, which generates fluid pressure in the mantle cavity. Jets of water are generated, which propel the animal. Refilling of the mantle cavity is powered by both fluid forces, and release of energy stored in the hinge during the contraction of the muscle. The results showed that the cost of keeping the shells oscillating is very low, so that most of the work done by the muscle is used to generate useful mantle cavity pressure. Scale effects were examined, and the results showed that inferior hydrodynamic characteristics of the shells are enhanced as the animals grow. Potential environmental effects on swimming ability were examined, and the results showed that the physical properties of the hinge are independent of temperature. Riblets on the external surface of the shells may reduce friction drag.

**SCALLOP GEAR SELECTIVITY AND SCALLOP BIOLOGY: A MISMATCH IN RESOURCE MANAGEMENT.**

William D. DuPaul, James E. Kirkley, and David B. Rudders, Virginia Institute of Marine Science, Gloucester Point, VA 23062.

Regulated changes on sea scallop (*Placopecten magellanicus*) harvesting gear configuration since 1994 have resulted in significant changes in size selectivity patterns. Sequential increases in dredge ring size and trawl mesh size have shifted length frequency modes that reflect the differential selectivity of the gear to larger animals but not to an equal extent. Dredges with 88.9 mm rings inexplicably capture a greater number of animals >120 mm which are larger than the internal diameter of the rings and greater than

the inter-ring spaces. In this presentation, we compare the selectivity patterns of various ring and mesh sizes with various size and age related biological and stock parameters such as yield per recruit (YPR), age and size at first capture, fecundity, and number and biomass at age when fishing mortality (F) equals 0.0. We also examine whether or not the present gear requirements would be beneficial in terms of enhanced growth and reproduction which would likely occur with closed area management strategies. Results of the comparison indicate that previous and present gear sizes and configurations are inadequate for optimizing the potential for gains in growth and reproduction, nor would they be sufficient to take full advantage of gains resulting from area closures. We conclude that area management strategies should include the use of a more size selective harvesting strategy in order to maximize benefits related to increases in YPR. Recognizing the broad selectivity pattern and inherent inefficiencies of harvesting gear, additional regulatory measures may be needed to maximize the biological potential of the resource.

**GENETIC VARIATION IN *PLACOPECTEN MAGELLANICUS* WITH IMPLICATIONS FOR FISHERIES MANAGEMENT.**

Ellen Kenchington, Fisheries and Oceans Canada, Bedford Institute of Oceanography, P.O. Box 1006, Dartmouth, NS, B2Y 4A2; Carolyn J. Bird, Institute for Marine Biosciences, National Research Council of Canada, 1411 Oxford St., Halifax, NS, CANADA B3H 3Z1; Eleferios Zouros, Institute of Marine Biology of Crete, P.O. Box 2214, 710 03 Iraklio Crete, Greece.

Six microsatellite variable nucleotide regions (VNTRs) have been developed for the sea scallop *Placopecten magellanicus*. These markers have been used in a survey of ten sea scallops beds ranging from New Jersey (U.S.A.) to St. Pierre Bank (Nfld.) and including the Gulf of St. Lawrence. We present data on allele frequencies which identify significant differences between the scallop beds. Additionally, four year classes from the Digby scallop grounds in the Bay of Fundy were analyzed for differences in allele frequency by locus, and a significant year class effect was observed. These results are discussed with respect to sea ranching, transfers of scallop spat for aquaculture purposes, forensic identification and fisheries management.

**EFFECT OF DEPLOYMENT DATE ON SEA SCALLOP GROWTH AND SURVIVAL.**

Lorelei A. Levy and G. Jay Parsons, Fisheries and Marine Institute of Memorial University of Newfoundland, St. John's, NF, Canada A1C 5R3; Patrick Dabineft, Ocean Sciences Centre, Memorial University of Newfoundland, St. John's, NF, Canada A1B 3X9.

Growth and survival rates of hatchery-reared sea scallop spat (*Placopecten magellanicus*) (1.7 mm shell height) deployed on a farm-based nursery are variable. For a commercial operation the

problem arises of when intermediate-sized scallops (7 mm SH) are available. Commencing in July, deployment of scallops over five consecutive 18-day intervals on a farm-based nursery provided the opportunity to determine the effects of deployment time on scallop growth and survival. Shell height was measured at the end of each interval, and again in November and the following June. Water quality and sea star settlement were monitored also. Scallops deployed in August 1997 reached intermediate size by November 8, 1997, while scallops deployed in September 1997 reached intermediate size by June 24, 1998. Scallops deployed on October 19, 1997, however, had not reached intermediate size by June 24, 1998. Growth rates were significantly different between intervals (ANOVA,  $F = 95.162$ ; d.f. 4,11,  $P < 0.001$ ) and correlated with temperature ( $r = 0.840$ ;  $P < 0.001$ ) and total phytoplankton levels ( $r = 0.994$ ,  $P < 0.001$ ). Survival declined over the study period. Survival was significantly different between intervals (ANOVA,  $F = 47.129$ , d.f. 4, 11,  $P < 0.001$ ) and negatively correlated with sea star settlement ( $r = -0.796$ ,  $P < 0.001$ ). Reduced growth and survival of scallops over different time intervals at a farm-based nursery illustrates the need to deploy scallops in July and August when temperature and food conditions are high. Management of spat with respect to the settlement of sea stars in September is also important.

**RETENTION OF SCALLOP VELIGERS AND CONSEQUENCES FOR STOCK ENHANCEMENT PROGRAMS, AQUACULTURE AND STOCK MANAGEMENT.** Joan L. Manual, Department of Biology, Dalhousie University, Halifax, Nova Scotia, Canada. B3H 4J1.

The vertical migration behavior of the veliger larvae of the giant scallop (*Placopecten magellanicus*) is examined as a model for the retention of small organisms in off shore habitat. Veliger responses to thermoclines similar to natural conditions (as low as 1.2°C) varied with time of day and size of the veliger. Veligers from different populations (Georges Bank, Passamaquoddy Bay and Mahone Bay) exhibited different vertical distributions. I propose that vertical migration behavior of the veligers is in part determined by the horizontal transport consequences of the migration, and that population differences are the result of the different hydrographic properties in home regions. Veligers of *P. magellanicus* appear to migrate in response to both light and tidal stimuli. Moon rise early in veliger life may set an internal clock that controls migration at a tidal period, while the diel migration results from responses to changes in light at dawn and dusk. Such a mechanism could be widespread among marine populations, allowing planktonic organisms a means of utilising tidal cycles for horizontal transport in offshore regions. I discuss the consequences of such population differences for management of commercially important stocks, aquaculture, and stock enhancement programs.

**AN EXAMINATION OF THE LINKAGE BETWEEN THE EARLY LIFE HISTORY PROCESSES OF THE SEA SCALLOP AND LOCAL HYDROGRAPHIC CHARACTERISTICS.** Shawn M. C. Robinson, James D. Martin, and Ross A. Chandler, St. Andrews Biological Station, Dept. Fisheries and Oceans, St. Andrews, New Brunswick, Canada, E0G 2X0; G. Jay Parsons, Marine Institute of Memorial University, P.O. Box 4920, St. John's, Newfoundland, A1C, 5R3.

The sea scallop, *Placopecten magellanicus*, is similar to many other scallop species around the world in that large recruitment fluctuations can occur on an annual basis. There have been speculations in the literature on why these recruitment pulses occur, but there have been few long-term studies set up to examine this phenomenon. Our team has been studying temporal and spatial scallop recruitment patterns in Passamaquoddy Bay, an enclosed body of water at the mouth of the Bay of Fundy, since 1989. Annual spawning patterns for local populations were monitored through weekly sampling of the gonadosomatic index (GSI) during the spawning season and biomass surveys using Digby scallop drags were done annually. The resulting spat settlement was monitored through the use of standard Japanese onion bags deployed in a uniform grid pattern for 25 sampling stations. These stations were also sampled monthly for temperature, salinity and chlorophyll *a* using a CTD. Results indicated that the spatial patterns of settlement were highly consistent from year to year and that the areas with the highest settlement also had the largest animals. The number and size of animals correlated well with warmer areas of the bay as well as with higher chlorophyll *a* levels. The distribution of larvae (estimated by spat settlement) was different than the distribution of adults and appeared to be more related to physical circulation patterns. Inter-annual variability in the GSI did not appear to explain inter-annual differences in spat settlement. Implications to the fishery and culture will be discussed in the presentation.

**THE USE OF RNA/DNA RATIOS AS AN INDEX OF HEALTH FOR THE SEA SCALLOP (*PLACOPECTEN MAGELLANICUS*).** Dale Roddick, Ellen Kenchington, and Stephen Smith, Fisheries and Oceans Canada, Bedford Institute of Oceanography, P.O. Box 1006, Dartmouth, NS, B2Y 4A2; Jon Grant, Oceanography Department, Dalhousie University, Halifax, Nova Scotia, Canada. B3H 4J1.

The use of RNA/DNA ratios as an index of health has been an active field of research in fish larvae. It has been proposed as a means of monitoring the health of wild *Placopecten magellanicus* stocks after a die-off occurred in the Digby Nova Scotia stock in 1989. Previous studies have shown both an inter-annual and spatial variation in the RNA/DNA ratios for this stock. This study used a combination of field measurements and laboratory experiments to

examine 3 key pieces of information necessary to determine the suitability of the RNA/DNA ratio in such a monitoring program: 1) the seasonal variation present in the scallop population; 2) the response time before a change due to nutritional stress can be detected; and 3) the influence of temperature on the RNA/DNA ratio. Seasonal variations were found in the RNA/DNA ratios and could be modeled with a sine function. This model could only account for 22% of the total variation over a 37 month period. Inter-annual variations were also significant, and deviations from seasonal RNA/DNA ratios were significantly correlated with subsequent deviations from seasonal growth. Under laboratory conditions the RNA/DNA ratio of the adductor muscle provided a detectable response to nutritional stress in 2 to 3 weeks, and growth rates were significantly correlated with RNA/DNA ratios. The RNA/DNA ratio of the mantle tissue did not provide a suitable index of nutritional stress. The RNA/DNA ratio of the adductor muscle does provide an index of health for *Placopecten magellanicus*, but the precision of this index may not be fine enough to discriminate between populations that are under critical stress from those that are stressed but not in any danger of increased mortality rates.

**PHYSICAL AND BIOLOGICAL VARIABLES INFLUENCING THE SPATIAL DISTRIBUTION OF THE GIANT SCALLOP *PLACOPECTEN MAGELLANICUS*.** Kevin Stokesbury, Center for Marine Science and Technology, University of Massachusetts Dartmouth, 706 South Rodney French Boulevard, New Bedford, MA, 02744-1221.

The purpose of this study was to determine the physical and biological variables influencing the spatial distribution of the giant scallop, *Placopecten magellanicus*. Scallops were aggregated on both a large (km) and a small (cm) scale. Large scale aggregations were strongly associated with gravel substrates while small scale aggregations (clumps) were not. The short distance between scallops within clumps, the high proportion of clumps with both sexes present, and an average of 3 scallops per clump suggested high fertilization success within clumps. Comparisons of the physical and biological conditions within scallop beds and in adjacent areas with low scallop densities indicated that gravel substratum, low decapod predation, and presence of filamentous flora and fauna were critical factors determining scallop aggregation location. Contrary to previous experimental results, scallops were not safe from decapod predation once they attained a large size. Scallop movement reduced predation rates. Scallop movement was random, and scallops did not appear to migrate from unsuitable to suitable habitats. However, scallops may move to form clumps resulting in increased fertilization success. If this is true, disturbing this small scale distribution prior to a spawning event may decrease reproductive success.

**RE-INTRODUCING THE BAY SCALLOP *ARGOPECTEN IRRADIANS* INTO CHINCOTEAGUE BAY, MD.** Mitchell L. Tarnowski and Mark L. Homer, Maryland Department of Natural Resources, Tawes State Office Building, B-2, Annapolis, MD 21401.

Nearly 70 years ago, the bay scallop disappeared from Chincoteague Bay, coincident with a destructive disease that wiped out the region's eelgrass beds. During the past decade, however, seagrasses have made a remarkable recovery in this area. With thousands of acres of seagrass meadows now in existence and stable, relatively high year-round salinities afforded through the stabilization of the Ocean City (Md.) Inlet foremost among several positive parameters, conditions appear to be optimal for the return of the bay scallop.

In October 1997, 533,000 seed scallops (8 mm mean length) were introduced into Chincoteague Bay and placed in predator exclusion pens. By the end of the 1997 growing season, the scallops had tripled in size and survivorship was about 85%. Overwintering mortality was extremely low, less than 10%, and by May 1998, the scallops had grown to an average shell length of 30 mm. Survivorship was compromised in August 1998 due to a severe thermal event in the shallower water pen. Overall survival was estimated to be 45% in September and 20% in late November. The surviving scallops attained a size of about 50 mm by November. Two distinct spawning events occurred in 1998, one in May–June, followed by another in August–September. Water column sampling revealed the presence of scallop larvae during the summer of 1998. In late October 1998, an additional 610,000 seed scallops were placed into pens in Chincoteague Bay. Initially 20 mm in size, these scallops attained mean shell lengths of 26 mm by early December. Initial survivorship was better than 95%. Samples were taken in December 1998 to determine recruitment success. "Wild" scallops were collected in a number of areas some 8–11 km south of the enclosure pens, although at this time it is not known if these were progeny from scallops released in Virginia or from the penned scallops held in Maryland.

**EVALUATING BAY SCALLOP STOCK ENHANCEMENT EFFORTS WITH MOLECULAR GENETIC MARKERS.** Ami E. Wilbur, William S. Arnold, and Theresa M. Bert, Florida Marine Research Institute, 100 8th Ave S.E., St. Petersburg, FL 33701.

Overfishing, habitat degradation, and toxic algal blooms have all contributed to the collapse of bay scallop (*Argopecten irradians*) populations in nearshore waters off west-central Florida. Management efforts to halt this decline (regional closures, reduced bag limits) have not resulted in any significant increase in scallop abundance. This lack of natural recovery has led to heightened interest in the enhancement of decimated areas with hatchery-

produced seed. Although such enhancements are becoming a well-recognized component of environmental remediation, few have been objectively evaluated for their efficacy. We have implemented an extensive genetic monitoring program to assess the impact of hatchery-seed outplantings on local recruitment in the nearshore waters off west-central Florida. We have developed an array of genetic markers (mtDNA, microsatellites and introns) that can function as a "genetic tag" for the hatchery stocks, and will allow unambiguous identification of the progeny of the outplanted scallops. We are currently monitoring recruitment in and around two sites where approximately 25,000 scallops were planted in the spring of 1998. The new recruits will be typed genetically and the results will allow an assessment of the efficacy of this enhancement strategy for facilitating the recovery of bay scallop populations.

## SHELLFISH BIOCHEMISTRY

**IDENTIFICATION AND OCCURENCE OF A NOVEL FATTY ACID IN PECTINIDS.** Yanic Marty,<sup>1</sup> Philippe Soudant,<sup>2</sup> Sébastien Perrotte,<sup>1</sup> Jeanne Moal,<sup>2</sup> Jean François Samain,<sup>2</sup> Jacques Dussauze<sup>3</sup>; <sup>1</sup>UMR/CNRS 6521, Université de Bretagne Occidentale, BP 809, 29285 Brest; <sup>2</sup>Laboratoire de Physiologie des Invertébrés, Ifremer Centre de Brest BP 70, 29280 Plouzané, France; <sup>3</sup>Laboratoire d'analyse Brest-Océan, Pôle Analytique des Eaux, 29280 Plouzané, France.

A new fatty acid discovered from the scallop *Pecten maximus* was isolated as a methyl ester using silver nitrate liquid chromatography and silicic high performance liquid chromatography. This fatty acid was determined to be a 22:4 geometrical isomer [ $\Delta 4?, 7?, 10?, 13?$ ] (X) using gas chromatography-mass spectrometry analysis of their 2-alkenyl-4-4-dimethylloxazoline derivatives (GC/MS DMOX). The number of trans double bounds of this fatty acid was determined on silver ion high-performance liquid chromatography by comparison with the mono-trans geometrical isomers of 22:4 [ $\Delta 4cis, 7cis, 10cis, 13cis$ ] (S), obtained through partial hydrazine reduction of all-cis-22:6(n-3) followed by a *p*-toluenesulfonic isomerization of the derivatives. Comparing the four trienes (22:3) obtained through partial hydrazine reduction of the two compounds (X) and (S) determined that the trans double bound was located in  $\Delta 13$ .

This fatty acid identified as *cis*-4,7,10,*trans*-13-docosetetraenoic has not been reported previously in any organisms. It is believed to be specific for the pectinid family. Mainly in polar lipids, it was found in high proportion in gills and mantle. The new identified fatty acid showed an apparent association with the phosphatidylserine.

**FATTY ACIDS FOR REPRODUCTION AND LARVAL DEVELOPMENT IN TWO BIVALVES MOLLUSCS: POLAR LIPID APPROACH.** Jean-François Samain, Philippe Soudant, Yanic Marty, and Jeanne Moal, Laboratoire de Physiologie des Invertébrés, Ifremer Centre de Brest BP 70, 29280 Plouzané, France, Laboratoire de Chimie Marine, URA CNRS 322, UBO 29200 Brest.

The aim of this study is to develop an approach of lipid requirements for aquacultured marine bivalves during reproduction and larval development. Specificity in PUFA composition of polar lipid classes of gonads, eggs and larvae, was studied during the reproductive cycle in two bivalve species, the scallop *Pecten maximus* and the oyster *Crassostrea gigas* in different lipid food compositions.

All the six separated phospholipid classes, demonstrated a specificity in their PUFA composition relatively similar in the two species. Some identical phospholipid classes resisted more to food composition changes of the diet than others. So, compensatory mechanisms exist in peculiar phospholipids, leading to a selective retention of specific essential fatty acids, suggesting their biological importance for the two species.

Specific events occurred in the fatty acid composition of the PL classes during gametogenesis and larval development, opening a possible way to characterize essential fatty acid requirements for these two important biological phases. Differences between the two species were also observed and discussed.

**COMPARISON OF ESSENTIAL FATTY ACID ACCUMULATION BETWEEN A REPRODUCTIVE CYCLE IN NATURE AND A HATCHERY CONDITIONING OF CRASSOSTREA GIGAS.** P. Soudant,<sup>a</sup> K. Van Ryckeghem,<sup>a</sup> J. Moal,<sup>b</sup> Y. Marty,<sup>c</sup> J. F. Samain,<sup>b</sup> and P. Sorgeloos.<sup>a</sup> <sup>a</sup>Laboratory of Aquaculture & Artemia Reference Centre, University of Gent, Rozier 44, B-9000 Gent, Belgium; <sup>b</sup>DRV/A, Laboratoire de physiologie des mollusques, IFREMER centre de Brest, BP 70, 29280 Plouzané, France; <sup>c</sup>UMR/CNRS 6521, Université de Bretagne Occidentale, BP 809, 29285 Brest, France.

Hatchery techniques have been developed based on empirical trials and a basic knowledge of key aspects of artificial reproduction, including broodstock nutrition and timing of gametogenetic cycles, are lacking even for the leading commercial species *C. gigas*. Lipids deposited in the eggs during broodstock conditioning play a major role as source of energy and essential fatty acid for embryonic and early larval development.

In the present study, the lipid content increased and accumulated in the gonads during the reproductive phase of the oysters from natural and artificial conditioning but to a higher extend in the naturally-conditioned animals. During that period the neutral lipid percentage of total lipids in the gonad plus mantle was stable

and high (>70%) and equal under both conditions, underscoring that the lipid reserves were preferentially located in that organ.

The fatty acid (FA) composition of the neutral lipids in all organs was influenced by changes in the diet but differently according to organs: high dietary impact occurred in the digestive gland whereas the muscle was less affected. The total polyunsaturated fatty acids (PUFA) content of the neutral and polar lipids in the gonads changed little whatever the conditions but the respective proportions of (n-3) and (n-6) PUFA differed drastically as a result of diet composition. In all organs, there was a clear evidence for a specific accumulation of 22:6(n-3) and 20:5(n-3) in the polar lipids for both conditionings. Nevertheless the percentages of 22:6(n-3) and 20:5(n-3) from neutral and polar lipids in the artificial conditioning were significantly lower than those in nature.

## SHELLFISH DISEASE

**RECENT TRENDS IN INFECTION OF THE EASTERN OYSTER *CRASSOSTREA VIRGINICA* BY THE PARASITE *PERKINSUS MARINUS* IN THE PATUXENT RIVER ESTUARY.** Brian W. Albright and George R. Abhe, Academy of Natural Sciences Estuarine Research Center, St. Leonard, MD 20685.

*Perkinsus marinus* is currently distributed throughout oyster populations in Chesapeake Bay and its tributaries, having spread into most low salinity areas of Maryland by the fall of 1992. Since the late 1980s, *P. marinus* has been the most virulent pathogen of the eastern oyster (*Crassostrea virginica*) in the Patuxent River owing to its widespread distribution and persistence in low salinity areas. Oysters from ten natural oyster beds in low- to high-salinity areas of the Patuxent River were analyzed annually since 1995 for *P. marinus* using rectal tissue assays incubated in Rays Fluid Thioglycolate Medium. All sampling was conducted in September yielding maximum values for both prevalence and intensity which are known to occur immediately following maximal summer temperatures. No natural oyster beds presently exist in the Patuxent River that are completely free of infection, although salinity continues as the primary environmental factor controlling local distribution and intensity.

To compare individual beds within the Patuxent the development of an infection index was needed. The infection index selected is the percent prevalence times the weighted intensity (weighted intensity is defined as the mean intensity of the infected individuals in a sample) which yields a number from zero (no infection) to 700 (all individuals maximally infected). Although the overall river wide infection index decreased from 336 in 1995 to 239 in 1996 to 150 in 1997, this decrease was not uniform throughout the river. Beds with initially low levels of infection

(principally the most upriver locations) were unable to rid themselves further of the parasite. While summer salinities and temperatures have remained relatively similar over the study period, the abnormally warm winter of 1997 allowed *P. marinus* to proliferate resulting in a river wide mean infection of 333 in 1998.

**DIVERSITY AMONG *PERKINSUS MARINUS* ISOLATES FROM THE CHESAPEAKE BAY.** Gwynne D. Brown, S. Kottob, and M. Faisal, Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062.

*Perkinsus marinus*, the causative agent of Dermo, is the primary oyster pathogen of the Eastern oyster (*Crassostrea virginica*) in the Chesapeake Bay. Recent developments in *P. marinus* cell culture have enabled scientists to produce several isolates, all believed to be the same species. Variations in virulence among *P. marinus* isolates from the Chesapeake Bay remain to be elucidated. In this study, seven isolates from the Chesapeake Bay were examined for genetic variations within the internal transcribed spacer regions (ITS1 and ITS2) and 5.8S region of the ribosomal gene unit. Extracellular proteins (ECPs) collected from the seven isolates were also compared by SDS-PAGE and analyzed for proteolytic activity. Sequence analysis of the ITS-5.8S region showed little variation when compared to the published sequences of *P. marinus*, confirming that the isolates are *P. marinus*. Protein profiles of ECP, revealed by silver staining, showed subtle differences between isolates. Variations in protease activity were detected utilizing hide powder azure assay. In addition, differences in the average size of protozoal cells and variations in growth rate were observed. We are currently investigating other phenotypic variations among isolates.

**TRANSMISSION OF *PERKINSUS MARINUS* TO INTERTIDAL OYSTERS.** David Bushek and A. J. Erskine, Baruch Marine Field Laboratory, University of South Carolina, Georgetown, SC 29442; Richard F. Dame, Department of Marine Science, Coastal Carolina University, Conway, SC 29528; Loren D. Coen and Nancy Hadley, Marine Resources Research Institute, South Carolina Department of Natural Resources, Charleston, SC 29442-2559.

Our basic understanding of the processes that control dispersal and transmission of the oyster pathogen *Perkinsus marinus* is limited. Some studies show that transmission rates decrease rapidly with distance (within 15 m) from infected oysters while others imply that transmissible stages are widely dispersed over long distances (km). A recent model by Powell and co-workers predicts that heavy oyster recruitment can stall an epizootic by diluting the *per capita* infective 'dose'. Conversely, do reductions in the size of

oyster populations enhance infection rates and stimulate epizootics by concentrating the per capita infective 'dose' among remaining oysters? We examined the roles of dispersal distance and local host population density on infection rate. Specific pathogen free (SPF) oysters were deployed seasonally at distances of 0, 50, 100 and 150 m from the mouths of eight small intertidal creeks, and then monitored for the onset of *P. marinus*. All native oysters were removed from four of the eight creeks to determine the effect of local host density. Parasites were detected in SPF's in as few as two weeks regardless of season, the presence of native oysters, or distance from the creek mouth. These results indicate that transmission occurs throughout the year in South Carolina and that *P. marinus* is ubiquitously dispersed in this system.

**UPTAKE, DISTRIBUTION, AND BIOCONVERSION OF FLUORESCENT LIPID ANALOGS IN THE OYSTER PROTOZOAN PARASITE, *PERKINSUS MARINUS*.** Fu-Lin E. Chu, Philippe Soudant, Yongqin Huang, Aswani K. Volety, and Georgeta Constantin, Virginia Institute of Marine Science, School of Marine Science, College of William and Mary, Gloucester Point, VA 23062.

It has been established that host lipids play a unique role for long term survival and life cycle completion in endogenous parasites. Parasites exploit fatty acids and lipids from the host, not only for membrane synthesis, but also for modification of their surface integrity to avoid host defense. To study lipid utilization by this parasite, we followed the uptake of 'fluorescent-labeled' fatty acid and phospholipid analogs in the merozoite/meront and prezoosporangia stages of *P. marinus*. After 24 hr incubation at 28°C with fluorescent labeled phosphatidylethanolamine (PE), phosphatidylcholine (PC), and free fatty acid (FFA, C12:0) analogs, yellow-gold fluorescence was present primarily in the discrete lipid droplets throughout the parasite. Analysis of lipid class composition using thin layer chromatography showed that the merozoite/meront stage incorporated PC and transformed it into PE. FFA, triacylglycerol (TAG) and one component tentatively identified as phosphatidylserine (PS) and PE was also metabolized to PS, PC and TAG in meront/merozoite. Incorporation of PC and FFA (C12:0) was observed in prezoosporangia, but there was no conversion of PC to PE or PS, instead PC in prezoosporangia was metabolized into two components presumptively to be FFAs. The fluorescent-labeled C12:0 in prezoosporangia was incorporated in TAG. These results suggest that *P. marinus* incorporated and modified lipids from exogenous sources and that the metabolic modes of meront differed from prezoosporangia. The uptake and bioconversion of fluorescent lipid analogs in the parasite are currently being quantified.

**IDENTIFICATION OF A NEW *PERKINSUS* SPECIES ISOLATED FROM *MACOMA BALTHICA* BY CHARACTERIZATION OF THE RIBOSOMAL RNA LOCUS. EVIDENCE OF ITS PRESENCE, SIMULTANEOUS WITH *P. MARINUS*, IN *CRASSOSTREA VIRGINICA*, *MACOMA MITCHELLI* AND *MERCENARIA MERCENARIA*†.** Cathleen A. Coss, José A. F. Rohledo, and Gerardo R. Vasta, Center of Marine Biotechnology, University of Maryland Biotechnology Institute, 701 East Pratt Street, Baltimore, MD 21202; Gregory M. Ruiz, Smithsonian Environmental Research Center, Edgewater, MD 21037.

A *Perkinsus* species from the Baltic clam *Macoma balthica* was isolated and an *in vitro* culture established under culture conditions previously optimized for *P. marinus* (Gauthier and Vasta, 1995). Examination of the cell morphology and proliferation behavior of the cultured isolate revealed differences with *P. marinus*. Regions of the rRNA locus (SSU, ITS1 and ITS2) of this isolate were cloned, sequenced, and compared with those available for other *Perkinsus* species and isolates. Sequence data from the rRNA locus indicates not only that *Perkinsus* sp. from *M. balthica* is not *P. marinus*, but also that it is different from *P. atlanticus* and *P. olseni*. The degree of difference is comparable or even greater than differences between all three accepted *Perkinsus* species, *P. marinus*, *P. atlanticus*, and *P. olseni*. Therefore, we formally designate the *Perkinsus* sp. from *M. balthica* as a separate species, *Perkinsus balthicae*, after the bivalve host from which it was isolated and cultured. Using a PCR-based assay specific for *P. balthicae* and the *P. marinus*-specific PCR assay, we found that *P. balthicae* can be present in the eastern oyster *Crassostrea virginica*, the clams *M. mitchelli* and *Mercenaria mercenaria*, and can coexist with *P. marinus* in all four bivalve species. †This study was supported by DOC Cooperative Agreement No. NA46RG0091 awarded by NOAA through the Maryland Sea Grant to G.R.V. and a Smithsonian predoctoral fellowship to C.A.C.

**INTEGRATED MONITORING OF MARINE DISEASE AND MORTALITY.** William S. Fisher, U. S. Environmental Protection Agency, National Health and Environmental Effects Research Laboratory, Gulf Ecology Division, Gulf Breeze, FL 32561; Benjamin H. Sherman, University of New Hampshire, 211 Nesmith Hall, Durham, NH 03824.

There have been apparent increases over the last several decades in disease and mortality of marine and estuarine organisms, including shellfish, presumably due to greater anthropogenic stress generated both in watersheds and coastal areas. These events are investigated from a local perspective even though they may have been equally driven, or at least influenced, by regional or global conditions. An ability to link the events with co-occurring physical and chemical disturbances, biophysical characteristics (water quality, harmful algal blooms), hydrographic characteristics, and ma-



rine-related human diseases can promote understanding of the effects of larger scale and interactive factors, and has potential value for indicating cause and/or environmental condition. Establishing these linkages will require a comprehensive program for collecting, documenting, compiling and integrating data collected from a variety of local sources over long periods of time. Two existing programs illustrate the potential to overcome challenges related to data collection, data quality, and database development, as well as establishing and maintaining continuity over time: (1) The Gulf of Mexico Aquatic Mortality Network, a program of state resource managers, demonstrates the ability to develop consistent investigative approaches, standard protocols, and standard data formats that are required to composite data, all the while maintaining state autonomy and ownership of the data. (2) The Health, Ecological and Economic Dimensions of Global Change Program is a retrospective compilation of disease and mortality events. This relational data framework offers the potential to link events with multiple environmental characteristics (water quality, temperature anomalies, etc.) within the same or external databases, and should serve as a template for future entries.

**WITHERING SYNDROME IN FARMED RED ABALONE, *HALIOTIS RUFESCENS*.** James D. Moore, Thea T. Robbins, and Carolyn S. Friedman. Bodega Marine Laboratory, P.O. Box 247, Bodega Bay, CA 94923.

Withering Syndrome (WS) is a chronic wasting disease responsible for mass mortality in wild populations of black abalone (*Haliotis cracherodii*). The etiology of WS is uncertain with limited evidence for the role of a gastrointestinal rickettsia-like pro-caryote (RLP). Signs of WS and accompanying mortality have been reported in other abalone species, including wild and cultured red abalone (*H. rufescens*). In the current study, 60 juvenile red abalone (8 cm) were randomly selected from a farmed population raised at 14°C and having low intensity RLP infections. The abalone were held in triplicate containers receiving either relatively cool (14.7°C, CW) or warm (18.5°C, WW) flowing seawater and fed equal amounts for 220 days. Survival was 100% (30/30) for the CW group and 66% (20/30) for the WW group. WW animals had higher RLP infection intensities, showed more clinical signs of WS, and fed at less than half the rate of CW animals. For data from both groups combined, RLP infection intensity was negatively correlated with WS signs including total weight, condition index and digestive gland condition. During 1997–98, several abalone farms in California noticed a dramatic increase in the number of shrunken animals, in conjunction with ENSO-elevated seawater temperatures. Examination of 70 abalone from four farms revealed highly significant correlations between RLP infection intensity and WS clinical signs, validating the laboratory study and strengthening the hypothesis that temperature-enhanced RLP infection plays a direct role in the etiology of Withering Syndrome. Supported in part by Saltonstall-Kennedy grant NA76FD0046 and the California Department of Fish and Game.

**PLASMA OF *CRASSOSTREA SPP* POSSESS A LOW MOLECULAR WEIGHT INHIBITOR OF *PERKINSUS MARI-NUS* PROTEASE.** Jacques L. Oliver, Mohamed Faisal, and Stephen L. Kaattari, Department of Environmental Sciences, Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, Virginia 23062.

*Perkinsus marinus* is a protozoan pathogen of the eastern oyster, *Crassostrea virginica*, and is the cause of severe mortalities in eastern oysters throughout the Chesapeake Bay. The eastern oyster is known to be susceptible to *P. marinus*, however, it has been demonstrated that the Pacific oyster, *Crassostrea gigas*, is tolerant to this infection. The mechanism(s) of this differential susceptibility to *P. marinus* is not known. Recent research has implicated serine proteases of the pathogen as likely virulence factors in the progression of the disease. The ability of oysters to produce inhibitors to pathogen proteases might alter disease progression and thus, be important in oyster defenses against *P. marinus*. Recently, we have detected the presence of low molecular weight protease inhibitory activity in the plasma of both *C. virginica* and *C. gigas*. The role of this inhibitory activity in oyster humoral defense is not presently known. However, we have observed high anti-proteolytic activity in Pacific oysters as well as eastern oysters that have exhibited high survival following natural challenges with *P. marinus*. These results suggest that protease inhibitors might play a role in oyster host defense mechanisms. Work in our laboratory has focused on the effects of this inhibitory activity on *P. marinus* proliferation *in vitro* and the characterization of these putative inhibitors. This research has been supported by NOAA-Sea Grant NA56RGO141.

**DIAGNOSTIC SCREENING OF OYSTER PATHOGENS: PRELIMINARY FIELD TRIALS OF MULTIPLEX PCR.** Soledad Penna and Richard A. French, University of Connecticut, Department of Pathobiology, 61 N. Eagleville Rd., Storrs, CT 06269; John Volk, John Karolus, and Inke Sunila, Connecticut Department of Agriculture, Bureau of Aquaculture and Laboratory, Milford, CT 06278; Roxanna Smolowitz, University of Pennsylvania, Coastal Research Laboratory, Woods Hole Oceanographic Institution, 193 Oyster Pond Rd., Woods Hole, MA 02543.

Parasitic diseases are one of the greatest obstacles threatening the success of the oyster (*Crassostrea virginica*) industry. Two protozoal infections, particularly *Haplosporidium nelsoni* (MSX) and *Perkinsus marinus* (Dermo) are the major causes of oyster mortalities and declining oyster production along the Atlantic coast. This situation has necessitated heightened diagnostic testing and initiated the development of disease-resistant oyster strains.

However, oyster populations are infrequently monitored for the presence of pathogens. The lack of routine surveillance is attributable to reliance on histopathology for *H. nelsoni*, *Haplosporidium costale* (SSO) and the Ray/Mackin assay for *P. marinus* diagnosis. The continued success of the oyster industry requires a practical strategy for diagnosing, monitoring and screening oyster diseases. This strategy would rely on diagnostic aids which are sensitive, rapid, cost effective and convenient. This study presents the development of the multiplex PCR for application in the screening and surveillance of disease agents of the eastern oyster. Multiplex PCR allows for the simultaneous testing of two or more pathogens in a single test reaction. Preliminary results have identified three PCR primer sets which amplify 564-bp, 150-bp, and 304-bp products of *H. nelsoni*, *H. costale*, and *P. marinus*, respectively, under equivalent conditions. In multiplex reactions, products of control cloned small subunit rRNA of MSX and SSO, and Dermo DNA extracts can be differentiated. The multiplex PCR test has recently been applied to field samples and preliminary findings indicate a high specificity and sensitivity for *H. nelsoni*, *H. costale* and *P. marinus*.

**ANALYSIS OF THE GEOGRAPHIC DISTRIBUTION OF PERKINSUS MARINUS STRAINS.** Kimberly S. Reece, Virginia Institute of Marine Science, The College of William and Mary, Gloucester Point, VA 23062; David Bushek and Karen L. Hudson, Baruch Marine Field Laboratory, University of South Carolina, Georgetown, SC 29442.

Recently developed *in vitro* culture and cloning methods have facilitated molecular genetic analyses of the oyster pathogen *Perkinsus marinus*. We have examined the genetic relatedness of *P. marinus in vitro* cultures and their clonal composition using isolates collected from throughout the U.S. Atlantic and Gulf coasts. Genetic relatedness of 63 primary cultures and 88 clonal cultures derived from these isolates was examined at six polymorphic loci by restriction fragment length polymorphism analysis. Comparison of clonal culture and primary culture genotypes indicated that primary isolate cultures from a single oyster can be polyclonal, evidence that oysters can be infected with multiple strains. Heterozygous genotypes for several clonal cultures suggested that cultured *P. marinus* cells were diploid, a fact confirmed by DNA sequence analysis of alleles at two anonymous single copy loci in heterozygous clonal cultures. Allele sequences were identical in isolates from geographically distant sites, but allelic and genotypic frequencies differed significantly among the Northeast, Southeast and Gulf coast regions. Overall, there were fourteen different composite genotypes detected with more than 80% of the isolates possessing one of three predominant genotypes. One of the major composite genotypes was unique to Gulf coast isolates. Genetic distance analysis indicated three major genotypic clades, each containing one of the three major genotypes.

**LONG-TERM SURVIVAL OF PERKINSUS MARINUS CELLS OUTSIDE ITS HOST.** Adel A. Shaheen, Faculty of Vet. Med. Zagazig University (Benha branch)—Moshtohor—Egypt.

Although *Perkinsus marinus* has been associated with severe mortalities among eastern oyster (*Crassostrea virginica*) populations of the Chesapeake bay, little is known about its survival in vegetative form outside host tissues. In the present study, effects of different salinities and temperatures on long-term survival of *Perkinsus marinus* cells in seawater are investigated. *Perkinsus marinus* cells were cultured in artificial sea water (ASW) at three temperatures (4, 18 and 27°C at 22 ppt salinity) and four salinities (10, 22, 28 and 35 ppt at 27°C temp). Viability, growth rate, zoosporulation, ability to regrow on growth culture medium, and to form hypnospores in Ray's fluid thioglycolate medium (RFTM) are investigated. Our results suggest that *P. marinus* culture kept in ASW in 22 ppt salinity at 27°C can survive without exogenous supply of nutrients for more than six weeks. During this time, the parasite divided by both binary fission and successive bipartition. Viability was decreased by 25%. The average cell diameter was increased 30% in ASW than the original cell size. Low percent of sporulation has occurred (0.5–1.5%) with failure of zoospores to be released. Currently, effects of exposure to other different temperatures and salinities are investigated.

## SHELLFISH IMMUNOLOGY: ADAPTATION AND MODULATION

**XENOBIOTIC-INDUCED IMMUNOTOXICITY IN THE PACIFIC OYSTER, CRASSOSTREA GIGAS: FIELD AND LABORATORY EXPERIMENTS.** Michel Auffret and Radouane Oubella, Laboratoire BioFlux UMR 6539, Institut Universitaire Européen de la Mer, Université de Bretagne Occidentale, F-29280 PLOUZANE, France.

As a part of a monitoring programme of a coastal ecosystem submitted to chronic anthropic influxes, immunomodulation has been studied in a potential sentinel species, the Pacific oyster *C. gigas*. A double approach has been conducted, including a study of immunotoxic effects of selected environmental contaminants by controlled exposure in the laboratory, and investigations on possible alterations of hemolymph parameters in caged individuals.

Standardized biological parameters have been measured in hemolymph samples, including immunopathological parameters (total and differential counts, cell viability, cellular toxicity, serum proteins) and functional parameters of hemocytes (aggregation, phagocytosis, intracellular bactericidal activities). Exposure of oysters in the laboratory to contaminants identified in estuarine sites (trace metals, pesticides) resulted in heavy changes in hemolymph, as hemocytosis and inverted subpopulation proportions. Several hemocyte functions related to internal defense were altered. These experiments indicate that most of the xenobiotics tested have potential immunotoxic effects depending on the con-

centrations applied. In caged individuals, responses identified in laboratory experiments were also observed. However, most of these responses did not indicate an homogenous trend towards depression since cases of stimulation were found. When examining all the parameters together, an altered overall status of the internal defense system could be established in the sites with the higher contaminant levels.

In conclusion, several proofs of immunomodulation revealed in this study indicate that many components and functions of hemolymph in the Pacific oyster are targets for environmental xenobiotics, depressing defense capacities of the individuals. However, as already mentioned by other authors, any study on immunotoxicity in such organisms should include a panel of measures and assays to establish if immunosuppression really occurs.

**EFFECT OF THE NADPH OXIDASE INHIBITOR DIPHENYLENEIODONIUM ON THE BACTERICIDAL ACTIVITY OF *CRASSOSTREA VIRGINICA* HEMOCYTES.** Lisa H. Bramble and Robert S. Anderson, University of Maryland, Center for Environmental Science, Chesapeake Biological Laboratory, Solomons, MD 20688.

Reactive oxygen species (ROS) produced by eastern oyster hemocytes are hypothesized to serve as bactericidal agents. Inhibition by diphenyleneiodonium (DPI) of the putative NADPH oxidase responsible for initiating the hemocyte ROS-generating pathway allowed testing of this hypothesis. DPI caused a dose-dependent inhibition of hemocyte ROS produced in response to zymosan; 0.5  $\mu\text{M}$  DPI abrogated ROS stimulation. Hemocyte phagocytic capability was unaffected by this DPI concentration, suggesting that the effect of DPI on ROS production was specific to NADPH oxidase function. Bactericidal assays were conducted in the presence of 0.5  $\mu\text{M}$  DPI, using *Bacillus megaterium* and *Pseudomonas fluorescens* as target species. DPI had no effect on the ability of hemocytes to kill *B. megaterium* and reduced bactericidal activity towards *P. fluorescens* by only 4%. These data suggest that ROS produced by eastern oyster hemocytes do not function as effective bactericidal agents. An alternative hypothesis for the role of ROS in eastern oyster immunology—with interesting evolutionary implications—is as immunoregulatory molecules, as has recently been demonstrated in mammalian models.

**THE EFFECTS OF HYPOXIA AND HYPERCAPNIA ON CELLULAR DEFENSES OF OYSTERS, SHRIMP, AND FISH.** Lonis E. Burnett, John Boyd, Chris Milardo, Tina Mikulski, and Libby Wilson, Grice Marine Laboratory, University of Charleston, 205 Fort Johnson, Charleston, SC 29412; Karen Burnett, Medical University of South Carolina, 217 Fort Johnson, Charleston, SC 29412.

Organisms living in estuaries have long been known to have mechanisms that enable them to exist in water containing low amounts of oxygen. The consumption of  $\text{O}_2$  that generates hypoxia

is also responsible for producing significant amounts of  $\text{CO}_2$  (hypercapnia). An elevation of water  $\text{CO}_2$  causes an acidosis in aquatic organisms. Thus, the combination of low  $\text{O}_2$  and elevated  $\text{CO}_2$  represents a significant environmental challenge to organisms living in estuaries. The effects of hypercapnic hypoxia on cellular defenses of estuarine organisms has been investigated. Hemocytes of the oyster *Crassostrea virginica* produce significantly less Reactive Oxygen Intermediates in response to stimulation when held under physiological conditions simulating hypoxia compared to well-aerated normoxia. This response is due separately to specific effects of  $\text{O}_2$  and the accompanying low pH. Similar results have been obtained using the macrophages of the fish, *Fundulus heteroclitus*. These results suggest that hypercapnic hypoxia depresses the cellular defense mechanisms. To extend these observations to whole organisms, a pathogen challenge model was used to test the hypothesis that hypercapnic hypoxia makes shrimp more susceptible to pathogenic infections of *Vibrio parahaemolyticus*. The shrimp *Palaemonetes pugio* and *Penaeus vannamei* showed lower survival when injected with *Vibrio* and held under 30% air saturation compared with controls held in well-aerated water. These studies suggest that the generalized innate immune response is depressed in hypoxia and that this contributes to animal mortality. (Supported by SC Sea Grant R/ER-14.)

**EFFECTS OF TEMPERATURE, SALINITY, AND ENVIRONMENTAL POLLUTANTS ON CELLULAR AND HUMORAL RESPONSES IN OYSTERS (*CRASSOSTREA VIRGINICA*).** Fu-Lin E. Chu, Virginia Institute of Marine Science, School of Marine Science, College of William and Mary, Gloucester Point, VA 23062.

This paper reviews our recent studies about the effects of temperature, salinity, field contaminated sediments (CS) and related soluble water soluble fraction (WSF) on the cellular and humoral responses in relation to *Perkinsus marinus* infection and progression in oysters. Results suggest that oyster hemocytes, which are believed to be the primary line in the defense system, are not active in defense against the oyster protozoan parasite, *P. marinus*. Higher numbers of circulating hemocytes, % of granulocytes, and phagocytic activity in oysters maintained at high temperature occurred concomitantly with higher *P. marinus* prevalence. Salinity did not produce significant effects on the above cellular parameters. Neither temperature nor salinity affected the plasma protein level in oysters. Oysters maintained at different temperatures had relatively similar hemagglutination titers. Plasma lysozyme activity was negatively and significantly correlated with salinity, temperature, and *P. marinus* prevalence. *In vitro* exposure of hemocytes to water soluble fractions derived from field contaminated sediments (CS) reduced hemocytes' chemotactic, phagocytic, and chemiluminescent responses. CS exposure stimulated neutral red uptake, mitochondrial dehydrogenase production, and

<sup>3</sup>H-leucine incorporation in hemocytes, but did not affect the concentrations of total circulating hemocytes, plasma protein and lipid, plasma lipid class composition, and plasma lactate dehydrogenase level. Exposure of oysters to 15, and 30% dilutions of WSF for 33 days or 1.0, 1.5, or 2.0 g CS for 30 days significantly elevated the expression/progression of latent *P. marinus* infection in oysters in a dose-dependent manner. No direct relationship was observed between cellular responses and *P. marinus* expression in oysters.

**THE ROLE OF PROTEASE-ANTIPROTEASE INTERACTIONS IN *PERKINSUS MARINUS* INFECTION IN *CRASSOSTREA* SPP.** Mohamed Faisal, School of Marine Science, Virginia Institute of Marine Science, The College of William and Mary, Gloucester Point, VA 23062.

*Perkinsus marinus* causes devastating losses in populations of the eastern oyster (*Crassostrea virginica*). Of particular importance to the invasiveness of protozoan parasites is their elaboration of a spectrum of tissue-disruptive proteases. In this vein, our previous studies have demonstrated that *P. marinus* secretes extracellular serine proteases which enhance parasite propagation and compromise host defenses. *Crassostrea virginica*, however, has been found to possess several inhibitors of these proteases. Among these, a serine protease inhibitor (serpin) has been identified which specifically blocks proteolytic digestion of oyster proteins. The Pacific oyster (*C. gigas*) also possesses protease inhibitors with higher specific activities.

Interestingly, *Crassostrea* spp. themselves, elaborate metalloprotease activities which can be detected in their plasma, and are increased during *P. marinus* infections. Together our work suggest that there may be a broad spectrum of humoral host defenses that is brought to bear on *P. marinus* infections by these two *Crassostrea* species.

**EXAMINATION OF THE CELLULAR IMMUNE RESPONSE OF BLACK ABALONE, *HALIOTIS CRACHERODII*, WITH AND WITHOUT WITHERING SYNDROME.** Carolyn S. Friedman, California Department of Fish and Game and Department of Medicine and Epidemiology, Bodega Marine Laboratory, P.O. Box 247, Bodega Bay, CA 94923; Thea Robbins and Jacqueline L. Jacobsen, Bodega Marine Laboratory, P.O. Box 247, Bodega Bay, CA 94923; Jeffrey D. Shields, Virginia Institute of Marine Science, Gloucester Pt., VA 23062.

Withering syndrome (WS) is a chronic disease that has resulted in dramatic declines in black abalone abundances along the southern and central California coast. A rickettsiales-like procaryote has recently been identified as the likely etiological agent. We hypoth-

esized that differences in hemocyte cellular functions may be affected by WS and may serve as indicators of early disease. We examined the chemotactic, phagocytic and chemiluminescent abilities of hemocytes from abalone with and without WS. Hemocytes from abalone with WS were more chemotactically active than those from asymptomatic abalone ( $n = 35$ ,  $p < 0.01$ ). However, hemocytes from diseased abalone were less able to engulf foreign particles ( $n = 59$ ,  $p < 0.01$ ), engulfed fewer particles ( $n = 52$ ,  $p = 0.00$ ), and produced a reduced respiratory burst ( $n = 26$ ,  $p = 0.00$ ) relative to those from asymptomatic abalone. The immune capability of the hemocytes correlated with degree of WS. Thus, hemocytes from abalone with WS may be more chemotactically active as a result of degeneration of the digestive gland and utilization of the foot muscle as an energy source. However, the capability of these stimulated cells to engulf and destroy foreign particles appears to be compromised and may contribute to mortality associated with this disease.

**MODULATION OF EASTERN OYSTER HEMOCYTE ACTIVITIES BY *PERKINSUS MARINUS* EXTRACELLULAR PROTEINS.** Jerome F. La Peyre, Department of Veterinary Science, Louisiana State University, Baton Rouge, LA 70808; and Aswani K. Volety, EPA, Gulf Breeze, FL 32561.

The oyster pathogen *Perkinsus marinus* produces many extracellular proteins (ECP) *in vitro*. Analysis of this ECP revealed a battery of hydrolytic enzymes. Some of these enzymes are known to modulate the activity of host defense cells. Although information on the effects of *P. marinus* ECP on oyster hemocytes is limited, it has been shown that ECP can inhibit hemocyte motility and hemocyte chemiluminescence response. Moreover, ECP effects on Gulf coast oyster hemocytes are of special concern since these oysters harbor human pathogens such as *Vibrio vulnificus* and *Vibrio parahaemolyticus*. *Perkinsus marinus* infections may thus be associated with higher numbers of *Vibrio* bacteria in oyster tissues. The objective of this initial study was to better characterize the effects of *P. marinus* ECP on oyster hemocyte activities *in vitro*.

Increasing concentrations of ECP caused increase degranulation and vacuolization of granulocytes (granular hemocytes). The normal spreading of the granulocytes and large hyalinocytes (agranular hemocytes) was also reduced and hemocyte clumping was increased. Phagocytosis of zymosan particles was significantly decreased at the highest concentration of ECP tested. More important, the capacity of hemocytes to kill *Vibrio parahaemolyticus* was decreased in a dose dependent manner within 30 min of exposure to ECP. *P. marinus* can clearly be detrimental to oyster hemocytes *in vitro*. The potential effect of ECP on oyster hemocytes *in vivo* and the possible relationship between *P. marinus* infection intensities and oyster tissue *Vibrio* load in Gulf coast oysters remain to be investigated.

**CHEMICAL EFFECTS ON OYSTER (*CRASSOSTREA VIRGINICA*) HEMOCYTE MICROBICIDAL ACTIVITY.** Leah M. Oliver, Aswani K. Volety,<sup>2</sup> and William S. Fisher, U.S. Environmental Protection Agency, National Health and Environmental Effects Research Laboratory, Gulf Ecology Division, 1 Sabine Island Drive, Gulf Breeze, FL 32561-5299. <sup>2</sup>National Research Council Post-Doctoral Associate.

Oyster (*Crassostrea virginica*) hemocytes, or blood cells, perform important internal defense functions such as phagocytosis and intracellular destruction of pathogens and bacteria. Using techniques such as phagocytosis and chemiluminescence assays, potential impairment of hemocyte immunocompetence resulting from *in vitro* and *in vivo* exposure to anthropogenic chemicals has been demonstrated. A new microbicidal assay recently optimized for oyster hemocytes shows promise for this type of investigation, and may better measure the integrative cidal function of hemocytes compared to measuring discrete portions of the phagocytic process. Hemocytes were exposed *in vitro* for 3–18 h to various chemicals including metals, organics, and biotoxins. Tributyltin (TBT), previously shown to exacerbate *Perkinsus marinus* infections when administered to oysters *in vivo*, inhibited killing of *Vibrio parahaemolyticus* and cultured *P. marinus*, at *in vitro* concentrations exceeding 32 ppb. The lowest TBT concentration caused a slight elevation, or hormesis, of hemocyte killing activity. Although *in vitro* results suggest immunosuppression by chemical exposure, previous assessment of defense activities of indigenous oysters collected from Tampa Bay, FL, suggested that these activities were elevated in oysters with high tissue burdens of certain metals. In a separate study, oysters were deployed at different sites in Pensacola Bay, FL, to test the effect of exposure to chemical mixtures on hemocyte microbicidal activity. Oysters deployed at contaminated habitats tended to have higher hemocyte bactericidal activity (21%) compared to oysters from relatively clean areas (0%).

**PHAGOSOMAL MECHANISMS IN EASTERN OYSTER (*CRASSOSTREA VIRGINICA*) BLOOD CELLS.** Kennedy T. Paynter, Chesapeake Biological Laboratory and Department of Biology, University of Maryland, College Park, MD 20742.

Oyster blood cells appear to have much in common with vertebrate macrophages. Like their vertebrate brethren, oyster blood cells are motile cells which locate, engulf and destroy foreign entities including particles, cells and viruses. Studies in our laboratory have demonstrated that 1) chemiluminescence produced by blood cells is likely caused by the production of hypochlorous acid

(HOCl), 2) oyster blood cells contain a myeloperoxidase-like activity which has an acidic pH optimum and may be responsible for the production of HOCl, 3) phagosomal pH becomes extremely acidic (pH < 4.0) shortly after engulfment of a target particle or cell for extended periods of time, and 4) acidification of the phagosome lumen can be blocked by bafilomycin A<sub>1</sub>, a specific inhibitor of vacuolar (V-type) proton-pump.

While some of these observations are consistent with the mechanisms described in vertebrate macrophage cells, others appear to be more protozoan in character. For instance, the high degree of acidity we have observed in oyster blood cells is matched only by the pH of protozoan acidosomes. By comparing and contrasting mechanisms observed in oyster blood cells with those reported in other organisms, we may gain insight into the nature of immune cell function in general and perhaps trace the evolution of phagocytosis from a purely digestive function into one of defense.

**INFLUENCE OF SEASONAL FACTORS ON OYSTER HEMOCYTE KILLING OF *VIBRIO PARAHAEOLYTICUS*.** Aswani K. Volety, National Research Council, c/o US EPA; James T. Winstead and William S. Fisher, US Environmental Protection Agency, Gulf Ecology Division, 1 Sabine Island, Gulf Breeze, FL 32561.

Seasonal variation of cellular defenses of oyster *Crassostrea virginica* against *Vibrio parahaemolyticus* were examined from June 1997 to December 1998 using a recently developed bactericidal assay that utilizes a tetrazolium dye. Mean hemocyte numbers, plasma lysozyme, and *P. marinus* infection in oysters were also examined. Hemolymph was sampled from oysters collected at Bayou Texar, Pensacola, Florida once every month. To determine if gametogenic cycle and/or energy reserves affect hemocyte activity, gonadal stage, tissue total lipids and lipid classes in digestive diverticulum tissue were also determined. Hemocyte bactericidal activity and mean hemocyte numbers in oyster hemolymph was higher in warmer months (40–80%) compared to winter months (0–30%). In contrast, total lipid and triglyceride concentration in digestive diverticulum tissue was higher in winter months and decreased with spawning in summer months. Lysozyme activity was also higher in winter than in summer months. The role of temperature and salinity on hemocyte killing capacity and plasma lysozyme in oysters is being investigated with the recognition that a variety of influences, including seasonal reproductive changes and potential environmental stimulation, are possible.

## SHELLFISH-MICROBIAL INTERACTIONS: ECOLOGICAL AND HUMAN HEALTH PERSPECTIVES

**IN VIVO TRANSFECTION OF ADULT OYSTERS.** John T. Buchanan, Department of Oceanography and Coastal Sciences, Louisiana State University, Baton Rouge, LA 70803; Ta Chi Cheng, Jerome F. La Peyre, and Richard K. Cooper, Department of Veterinary Science, LSU Agricultural Center, Baton Rouge, LA 70803; Terrence R. Tiersch, Aquaculture Research Station, LSU Agricultural Center, Baton Rouge, LA 70820.

We are developing techniques for gene transfer in the eastern oyster, *Crassostrea virginica* to enhance disease resistance. We transfected oysters with two genes, red-shifted green fluorescent protein (rsGFP), commonly used as a reporter gene, and the lytic peptide cecropin, known to have antimicrobial properties. In a preliminary study, oysters were assigned to three groups: the first was injected with the rsGFP gene mixed with superfect transfecting reagent (Qiagen); the second was injected with the cecropin gene mixed with superfect transfecting agent. The third group was injected with saline (control group). Hemolymph was collected at 4 and 10 days after injection. DNA was extracted for polymerase chain reaction (PCR), and hemocytes were examined by flow cytometry and fluorescence microscopy for detection of green fluorescence due to rsGFP expression. The rsGFP gene was detected by PCR in hemocytes of 13 of 15 oysters injected with this gene at day 4, and 15 of 15 at day 10. The cecropin gene was detected by PCR in 12 of 15 oysters at day 4, and 13 of 15 at day 10. No oysters from the control group were positive for either gene at day 4 or 10. Green fluorescence was detected by flow cytometry in oysters injected with rsGFP at significantly higher levels ( $P < 0.5$ ) than other oysters. This report indicates the ability to introduce DNA into adult oysters with subsequent gene expression.

**FACTORS INFLUENCING IN VITRO KILLING OF BACTERIA BY HEMOCYTES OF THE EASTERN OYSTER (CRASSOSTREA VIRGINICA).** Fred J. Genthner, Leah M. Oliver, and William S. Fisher, U.S. Environmental Protection Agency; Aswani K. Volety, National Research Council, National Health and Environmental Effects Research Laboratory and Gulf Ecology Division, 1 Sabine Island Drive, Gulf Breeze, FL 32561.

*Vibrio parahaemolyticus* strains altered in motility or colonial morphology (opaque versus translucent), *Listeria monocytogenes* mutants lacking catalase, superoxide dismutase, hemolysin, or phospholipase activities, and *Vibrio vulnificus* strains, possessing and lacking capsules were exposed to oyster hemocytes. Tetrazolium dye reduction was used to quantify bacterial killing by the hemocytes. Higher killing by hemocytes was observed in summer than winter. *Listeria monocytogenes* was more resistant to the

bactericidal activity of hemocytes than *V. parahaemolyticus* or *V. vulnificus*. No differences in hemocyte killing were observed between the different *L. monocytogenes* mutants. Translucent strains of *V. parahaemolyticus* showed higher susceptibility to killing by hemocytes than the parental opaque strain. No significant differences in killing by hemocytes were observed between encapsulated and nonencapsulated pairs of *V. vulnificus*. No seasonal differences (winter versus summer) were observed in activities of 19 hydrolytic enzymes associated with hemocytes.

**TOTAL BACTERIA AND VIBRIO VULNIFICUS LOAD IN DIPLOID AND TRIPLOID EASTERN OYSTERS IN LOUISIANA.** Jerome F. La Peyre, Richard K. Cooper, Department of Veterinary Science, John E. Supan, Office of Sea Grant Development, Louisiana State University, Baton Rouge, LA 70808; Aswani K. Volety, EPA, Gulf Breeze, FL 32561.

Advantages of triploidy in oysters include greater growth rate and better meat quality. It has also been postulated that triploid oysters have better host defenses; energy allocated to reproduction in diploid oysters may be allocated to host defenses in triploid oysters with impaired gonad development. The extended spawning season of Gulf coast oysters and the occurrence of the human pathogen *Vibrio vulnificus* (*V.v.*) make triploidy attractive for this region. In this preliminary study we compared the total bacteria and *V.v.* load between diploid and triploid oysters. The capacity of hemocytes to kill *V.v.* and *Vibrio parahaemolyticus* (*V.p.*) was also measured in these oysters.

Oysters were obtained from the Grand Isle Oyster Hatchery, LA, and divided into three groups, each composed of 10 diploid and 15 putative triploid oysters. The first group was kept overnight on ice, a second group was left outside of the water for 42 h at 25°C and a third group was maintained for one week at 20°C in U.V.-treated seawater. Each oyster was then homogenized and the number of colony forming units (cfu) of total bacteria and *V.v.* was determined. In all groups, triploid oysters had significantly lower total bacteria and *V.v.* cfu than diploid oysters. Hemocytes from triploid oysters had significantly greater bactericidal activity against *V.v.* and *V.p.* than hemocytes from diploid oysters. This data must be interpreted with caution since diploid and triploid oysters did not originate from the same broodstock and were not raised at the same site for most of their grow-out period.

**REGULATION AND MANAGEMENT OF WATER QUALITY TO PRESERVE SHELLFISH HARVESTING AND HUMAN HEALTH IN TOMALES BAY, CALIFORNIA.** Paul G. Olin, University of California Sea Grant Extension, Santa Rosa, CA 95403; Gregg Langlois, California Department of Health Services, Berkeley, CA 94704.

Shellfish growers in Tomales Bay, California experience significant economic losses annually as a result of rainfall triggered harvest closures. As a result, in 1993 the California State Legis-

lature passed the Shellfish Protection Act to protect water quality in shellfish growing areas. The act requires formation of a technical advisory committee to identify and remediate pollution sources. In 1994, the Tomales Bay Shellfish Technical Advisory Committee was formed and a subsequent study identified excessive coliform loading from animal agriculture as the primary source of pollution. The committee has developed research and management objectives designed to improve water quality in the Bay that include fencing, manure pond treatments, increased holding capacity and adjusted wastewater irrigation schedules.

The focus on water quality broadened to include human fecal contamination in 1998 when a viral outbreak involving over 150 people was linked to consumption of raw oysters contaminated with a human pathogen. This resulted in a closure of Bay waters to shellfish harvest for 26 to 81 days. The outbreak triggered an intense examination of water quality and potential pollution sources. These activities and other steps were undertaken to insure consumer safety prior to resuming shellfish harvesting. This illustrates the need to be proactive in preserving water quality in our heavily used coastal areas.

**HEMATOLOGY OF BLUE CRABS, *CALLINECTES SAPIIDUS*, INFECTED WITH THE PARASITIC DINOFLAGELLATE *HEMATODINIUM PEREZI*.** Jeffrey D. Shields and Christopher M. Squyars, Department of Environmental Sciences, Virginia Institute of Marine Science, The College of William & Mary, P.O. Box 1346, Gloucester Point, VA 23602.

Along the eastern seaboard, the blue crab, *Callinectes sapidus*, experiences recurring epizootics of the parasite, *Hematodinium perezii*. We investigated host mortality due to the disease, assessed differential hematological changes in infected crabs, and examined proliferation of the parasite. Healthy, mature female crabs were injected with  $10^3$  or  $10^5$  cells of *H. perezii*. Mortalities began 14 d after infection, with a median time to death of  $30.3 \pm 1.5$  d (se), and a mortality rate of 86% in infected crabs. Hemocyte densities declined by 60–80% within 3 d of infection and exhibited differential changes in subpopulations of granulocytes and hyalinocytes that lasted throughout the infection. Injected crabs that did not present infections exhibited significant long-term (21–27 d) granulocytopenia. Patency increased from approximately 30% after 14 d to 60% after 21 d to 100% after 35 d. Plasmodial stages were, however, detectable in histological preparations of the heart within 3 d of infection with significant increases over 5 and 7 d. The mortality studies indicate that *H. perezii* represents a significant threat to the blue crab fisheries in high salinity estuaries, and may have a greater effect on mature females that move to higher salinities to breed.

**DETECTION OF PROTOZOA PATHOGENS IN THE EASTERN OYSTER TAKEN FROM THE GREAT BAY ESTUARY.** Kim M. Stowell, Stephen D. Torosian, and Aaron B. Margolin, University of New Hampshire, Durham, NH 03824.

Many outbreaks of human gastroenteritis are caused by the consumption of raw or undercooked shellfish in which protozoa pathogens such as *Giardia lamblia* and *Cryptosporidium parvum* are present. These pathogens are introduced into the waterways via direct human fecal contamination, animal reservoirs and farm runoff. Adult oysters filter approximately 100 gallons of water per day and concentrate protozoa on gills, in hemocytes and in the intestinal track. These pathogens remain in the oyster for at least a month after exposure and remain infectious in mice one week after ingestion.

The water in Great Bay has the disadvantage of being the drop off site for many rivers that carry wastewater from treatment plants. There are several oyster beds located in the vicinity of these rivers as well as a major site abutting a wildlife refuge.

The current method of detection is the immunofluorescence assay (IFA), which requires microscopy to distinguish between the two organisms and may also antigenically cross-react with various species of algae. This research utilizes a molecular approach to detection by the development of a multiplex polymerase chain reaction (PCR), which detects *Giardia* and *Cryptosporidium* simultaneously. Inhibitors to PCR are overcome by the use of streptavidin-coated paramagnetic beads and biotinylated primers. Results derived from seeded oyster extract have shown that using multiplex PCR is an efficient, and extremely specific, means of protozoa detection. Eventually a multiplex can be designed which will detect both viral and protozoa pathogens simultaneously. This data and methodology will be used to improve shellfish testing and combat a public health threat.

**VIBRIO FLUVIALIS IMPLICATED IN RECENT OUTBREAKS AMONG AMERICAN LOBSTERS.** B. D. Tall,<sup>1</sup> M. Crosby,<sup>3</sup> D. Prince,<sup>3</sup> J. Becker,<sup>5</sup> G. Clerge,<sup>1,4</sup> D. Lightner,<sup>2</sup> L. Mohny,<sup>2</sup> M. Dey,<sup>1</sup> F. M. Khambaty,<sup>1</sup> K. A. Lampel,<sup>1</sup> J. W. Bier,<sup>1</sup> B. E. Eribo,<sup>4</sup> and R. Bayer,<sup>3</sup> <sup>1</sup>JIFSAN, U.S.FDA, Washington, DC 20204, <sup>2</sup>Univ. of Arizona, Tucson, AZ 85721, <sup>3</sup>The Lobster Inst., Univ. of Maine, Orono, ME 04469, <sup>4</sup>Howard Univ., Washington, DC 20059, <sup>5</sup>Maine Lobster Technologies, Hancock, ME 04640.

An unexplained, highly invasive disease has emerged in *Homarus americanus* (American lobsters) harvested from Atlantic coastal waters. Economic losses exceeding \$2.5 million threaten the \$136 million-a-year industry. Gram-negative bacilli were observed in hemolymph samples from diseased lobsters; results from antibiotic therapy studies showed that enrofloxacin was highly effective (100% survival) in abating illness in naturally diseased lobsters and lobsters experimentally infected with hemolymph from diseased animals. Culture of hemolymph samples from 5 of

6 diseased lobsters yielded bacteria, of which *Vibrio fluvialis* (*Vf*) was the predominant microorganism. The isolates were highly susceptible to a variety of antibiotics tested. PFGE analysis showed that most of the isolates either shared a common DNA fingerprint or possessed minor variants thereof; two could not be typed. Seven isolates possessed plasmids. A sheep hemagglutinin was found to be expressed by 93% of the isolates. This suggests the presence of cell-associated proteases or adherence factors. Invasion studies using Atlantic menhaden liver cells demonstrated that the *Vf* strains were capable of invasion, irrespective of plasmid presence. Our results indicate that this illness was likely caused by a cohort of highly related, invasive *Vf* strains that expressed a sheep hemagglutinin. The emergence of this pathogen, capable of infecting fish and humans, and reported now for the first time in *Crustacea* poses a significant health and economic threat that merits additional studies.

**DIFFERENTIAL EFFECTS OF OYSTER (*CRASSOSTREA VIRGINICA*) DEFENSES ON CLINICAL AND ENVIRONMENTAL ISOLATES OF *VIBRIO PARAHAEEMOLYTICUS*.**

**Aswani K. Volety**, National Research Council, c/o US EPA; **Fred J. Genthner** and **William S. Fisher**, US Environmental Protection Agency, Gulf Ecology Division, 1 Sabine Island, Gulf Breeze, FL 32561; **Susan A. McCarthy**, US Food and Drug Administration, PO Box 158, Dauphin Island, AL 36528; **Kirk Wiles**, Texas Department of Public Health, 1100 West 49th Street, Austin, TX 78756.

Three clinical (2030, 2062, and 2107) and three environmental (1094, 1163, and ATCC 17802) isolates of *Vibrio parahaemolyticus* were exposed to hemocytes and plasma collected from oysters (*Crassostrea virginica*) to determine their susceptibility to putative oyster defenses. Clinical strains were isolated from patients who became ill from the June 1998 food poisoning outbreak of *V. parahaemolyticus* associated with oysters harvested from Galveston Bay, Texas. Detection of thermolabile direct hemolysin (*tlh*) and thermostable direct hemolysin (*tdh*) genes in isolates was conducted using alkaline phosphatase- and digoxigenin-labeled probes. All isolates of *V. parahaemolyticus* possessed the *tlh* gene while only the clinical isolates had the *tdh* gene. Although pulse-field gel electrophoresis revealed that all clinical strains were identical, isolate 2062 was more susceptible to killing by oyster hemocytes than the other clinical isolates (2030, 2107). Overall, environmental isolates were more susceptible to hemocyte killing than clinical isolates. However, environmental isolates 1163 and ATCC 17802 had higher susceptibility to hemocyte killing than isolate 1094. When grown on nutrient agar plates, all strains displayed different colonial morphologies. Examination of cells of clinical isolates using electron microscopy did not reveal differences in degree of encapsulation. Bacterial susceptibility to hen egg white lysozyme and oyster plasma was investigated. Results

indicate that while clinical strains are genetically identical, they may differentially express putative factors responsible for protection against killing by oyster hemocytes.

## POSTER SESSION

**BAFILOMYCIN A<sub>1</sub> INHIBITS ACIDIFICATION OF GRANULAR AND AGRANULAR OYSTER HEMOCYTE PHAGOSOMES.** **Amy E. Beaven**, University of Maryland, College Park, MD 20742; and **Kennedy T. Paynter**, Chesapeake Biological Laboratory, Solomons, MD 20688.

Recently, we have shown that the phagosomes of eastern oyster (*Crassostrea virginica*) hemocytes, like vertebrate macrophages, become acidified after engulfment of foreign particles. Acidification of intracellular compartments in a variety of cell types and organisms is typically accomplished by proton-pumping mechanisms. The vacuolar (V-type) ATPase, which is found in macrophage phagosomes and lysosomes, generates a proton gradient with the concomitant hydrolysis of ATP. In order to determine the mechanism of phagosomal acidification in oyster hemocytes, we exposed hemocytes to bafilomycin A<sub>1</sub> (Baf) a specific inhibitor of the V-type ATPase. Oyster hemocytes were incubated with Baf and then challenged with zymosan labeled with both rhodamine and fluorescein. The pH of internalized zymosan was determined using the ratio of the emission intensities of rhodamine and fluorescein. Zymosan internalized within phagosomes of both granular and agranular hemocytes exposed to Baf became much less acidified than the control hemocytes. Maximal inhibition of both granular and agranular hemocyte phagosomal acidification occurred at 5 μM, and partial inhibition (40%) occurred at doses as low as 50 nM. Inhibition of hemocyte phagosomal pH reduction suggests a V-type ATPase may be responsible for acidification of the phagosomal lumen in both granular and agranular oyster blood cells.

**AN ECOSYSTEM MODEL OF *PERKINSUS MARINUS*.** **Jodi Brewster** and **David Bushek**, Baruch Marine Field Laboratory, Baruch Institute for Marine Biology and Coastal Research, University of South Carolina, Georgetown, SC 29442; **Richard F. Dame**, Department of Marine Science, Coastal Carolina University, Conway, SC 29528.

The mechanisms that drive and maintain *Perkinsus marinus* epizootics are obscure. Relationships to temperature and salinity have been well documented and predictive models using these parameters exist. Still, much uncertainty remains. Modeling provides a tool to develop a better understanding of the forces that control epizootics. An important use of models is to highlight areas that lack solid information. Most studies have considered the in-



interactions between the eastern oyster and *P. marinus* outside of the framework of the ecosystem in which they co-exist. We constructed a box model with Stella 5.0 software to show the flow of *P. marinus* through a salt marsh oyster dominated ecosystem—the North Inlet Estuary, SC. Our goal was to identify major processes that control parasite transmission and the development of epizootics in order to identify those processes where management strategies can be designed to minimize the risk or impact of an epizootic. The model reiterates the importance of temperature and salinity, but also highlights the role of estuarine flushing in maintaining a balanced equilibrium between host and parasite. The model also indicates that information is lacking or considerably limited on the seasonal concentration of transmissible stages in the water column and, perhaps more importantly, measures of the relative importance of the various potential fates of water borne *P. marinus*.

**EVALUATION OF A GLUCOSE OXIDASE/PEROXIDASE METHOD FOR INDIRECT MEASUREMENT OF GLYCOGEN CONTENT IN OYSTERS (*CRASSOSTREA VIRGINICA*).** Shelley Burton, Allan MacKenzie, T. Jeffrey Davidson, and Audrey Fraser, Atlantic Veterinary College, University of Prince Edward Island, 550 University Ave., Charlottetown, PEI, Canada, CIA 4P3.

A colorimetric method for indirect measurement of glycogen (utilizing glycogen conversion to glucose by amyloglucosidase) in tissue homogenates of oysters (*Crassostrea virginica*) was evaluated. The procedure was optimized as to extracting buffer pH (5.0) and amyloglucosidase concentration (5 mg/ml). Coefficients of variation ( $n = 10$ ) for oyster homogenates with mean glycogen concentrations of 84 and 242 mg/dL had within-run values of 3.28 and 3.65%, and between-run values of 4.49 and 3.15%, respectively. When mean glycogen concentrations of thawed oyster homogenates were compared to those of initial fresh homogenates, no significant ( $P \leq 0.05$ ) differences were detected in samples thawed after 1 hour, 1 day, 1 week or 1 month. Glycogen recovery percentages of 104.1, 103.7 and 104.5% were obtained with mixed solutions containing 111, 94 and 19 mg/dL. As dilutions of an oyster homogenate with a high glycogen concentration (430 mg/dL) gave observed results within 5% of expected, the assay was considered to be linear to 430 mg/dL. The lower limit of sensitivity was approximately 14 mg/dL. An initial laboratory range (mean  $\pm 2$  SD = 3–50 mg/g) for tissue glycogen based on wet weights (1.6–3.9 g) was determined for 49 two-year-old oysters obtained during July from the Covehead region of Prince Edward Island, Canada. It was concluded that the colorimetric assay offered a reliable indication of tissue concentrations of glycogen in *Crassostrea virginica*.

**TUNICATE FOULING IN NOVA SCOTIA AQUACULTURE: A NEW DEVELOPMENT.** Debbie Cayer and Marli MacNeil, Aquaculture Association of Nova Scotia, Halifax, Nova Scotia; Andrew G. Bagnall, Nova Scotia Department of Fisheries and Aquaculture, Halifax, Nova Scotia.

A significant fouling problem with the tunicate *Ciona intestinalis* developed on a suspended blue mussel (*Mytilus edulis*) aquaculture site during the 1997 growing season. *Ciona intestinalis* has subsequently colonized several aquaculture sites at varying levels of intensity. The infestation has had a serious, negative impact on at least one farm and could potentially affect the economic viability of other operations.

The Aquaculture Association of Nova Scotia and the Nova Scotia Department of Fisheries and Aquaculture have been networking internationally, documenting the impacts of *C. intestinalis* on aquaculture production and searching for strategies to manage its effects. Information has been collected and contacts have been made around the world—a solution has not been found.

Methods to manage this fouling problem will be species and site specific. Attempts to remove the tunicates have been cost prohibitive on a large scale. Farm management strategies must be developed as an economically viable solution. Part of this process involves studying the behaviour of *C. intestinalis* and its interactions with Nova Scotia operations. *Ciona intestinalis* biology and ecology are also being examined in an effort to prevent the further spread of these tunicates to previously unaffected aquaculture sites and to assist operations with implementing mitigative measures.

**USE OF FLOW CYTOMETRY AND HISTOLOGY TO ASSESS GAMETOGENESIS IN TRIPLOID *CRASSOSTREA ARIAKENSIS*.** Whitney Chandler, Aimee Howe, and S. K. Allen, Jr., Aquaculture Genetics and Breeding Technology Center, Virginia Institute of Marine Science, Gloucester Point, VA 23062.

Triploid *Crassostrea ariakensis* were produced by treating fertilized diploid eggs with cytochalasin B. Seventy-seven 5-year-old adults were conditioned and sacrificed at peak gametogenesis. Flow cytometry of gill samples confirmed that all animals were triploid. Eighty four percent ( $n = 65$ ) were male and the rest hermaphrodites. Almost all-male follicles still had spermatogonia, but the majority of cells were spermatids. Spermatozoa were in all “ripe” follicles. Flow cytometry confirmed that the majority of cells in 63 of 65 male triploids were fully reduced, 1.5 n. No haploid gametes were detected. Most of the hermaphrodites had female like follicle structure, characterized by small undeveloped follicles with undifferentiated gametic cells. In these “female-like” individuals, hermaphroditism was detected only by the presence of completely reduced (1.5 n) cells via flow cytometry. Four hermaphrodites had typical female follicle structure, but some of the follicles had “erupted into maleness.” One hermaphrodite was

clearly a male, with a number of follicles containing one to a few ripe ova. Flow cytometry and histology in combination were powerful tools in assessing gonadal and gametic maturation, in particular, the detection of hermaphrodites.

**IMPROVEMENT OF THE WHOLE-OYSTER PROCEDURE FOR ENUMERATING *PERKINSUS MARINUS* IN OYSTER TISSUES.** Gregory M. Coates, School of Forestry, Wildlife and Fisheries, Richard K. Cooper and Jerome F. La Peyre, Department of Veterinary Science, Louisiana State University, Baton Rouge, LA.

The whole-oyster procedure for enumerating *P. marinus* in oyster tissues is the most reliable technique to date to determine infection intensity. This procedure depends on the enlargement of the parasites in Ray's fluid thioglycollate medium (RFTM), their subsequent isolation from tissue debris and finally their quantification after staining with Lugol's solution. Each one of these steps however has yet to be optimized adequately. Insufficient parasite enlargement, incomplete tissue digestion during isolation, parasite clumping and adherence to the walls of centrifuge tubes can lower parasite counts. In this initial study, we report on the use of various chemicals to alleviate some of these problems and increase both precision and accuracy of the whole-oyster procedure.

Parasite enlargement was significantly enhanced by addition of pronase or a lipid mixture (i.e., cod liver oil) to RFTM. The size range of parasites also decreased with these two treatments. Neither pronase nor the lipid mixture caused proliferation of cultured parasites in RFTM. Tissue debris remaining after sodium hydroxide digestion of oyster tissues could be further eliminated by rinsing the parasite suspension in hydrochloric acid solution. Moreover, the acid treatment intensified staining of the parasite with Lugol's solution and eliminated fading. Finally, mucin was effective in preventing parasite clumping. The use of these chemicals made counting of the parasite easier and increased the number of parasites detected. Future work should focus on developing a technique to expedite parasite counting.

**POTENTIAL USE OF RAY'S FLUID THIOGLYCOLLATE MEDIUM TO DETECT AND QUANTIFY *PERKINSUS MARINUS* IN ENVIRONMENTAL WATER SAMPLES.** Rebecca C. Ellin and David Bushek, Belle W. Baruch Institute for Marine Biology and Coastal Research, University of South Carolina, P.O. Box 1630, Georgetown, South Carolina 29442.

*Perkinsus marinus* is a major pathogen of the Eastern oyster, *Crassostrea virginica*. Direct transmission of *P. marinus* occurs via the water column, however, processes that influence planktonic transmission dynamics remain poorly understood. Accurate methods for quantification of planktonic *P. marinus* are necessary to

study transmission dynamics. Available methods include quantitative competitive PCR and immunoassay flow cytometry. These methods are expensive and technologically complex. Moreover, cross-reactivity of the antibody probe with other estuarine organisms has recently been confirmed. We examined Ray's Fluid Thioglycollate Medium (RFTM) assay as an alternative.

The RFTM assay is an inexpensive, accurate methodology to assess *P. marinus* infection intensities within oyster tissue, hemolymph, feces, and pseudofeces. We detected planktonic stages of *P. marinus* in environmental water samples using RFTM. Recovery of *in vitro* cultured *P. marinus* from spiked water samples was low, but significantly higher using filtration ( $743,667 \pm 106,444$ ) rather than centrifugation methods ( $37,339 \pm 9,385$ ;  $p < 0.05$ ) to capture parasites. The low cell recovery could not be attributed to various stages of the RFTM method but may be explained by the inability of cultured *P. marinus* to enlarge in RFTM. This is supported by a significantly higher cell recovery rate with the addition of lipids to RFTM ( $p < 0.05$ ). Differences between cultured and environmental *P. marinus* limit the application of the RFTM method recovery efficiency to natural populations. Therefore, the recovery of naturally occurring planktonic *P. marinus* in environmental water samples using the RFTM methodology will be compared with existing methodologies to determine efficiency and to investigate other sources of cell loss.

**CORRELATION BETWEEN THE LEVEL OF PROTEASE INHIBITORS AND INTENSITY OF *PERKINSUS MARINUS* INFECTION IN EASTERN OYSTER (*CRASSOSTREA VIRGINICA*).** Ehab Elsayed and Mohamed Faisal, School of Marine Science, Virginia Institute of Marine Science, The College of William and Mary, Gloucester Point, VA 23062.

*Perkinsus marinus* is a protozoan parasite that causes a severe loss in eastern oyster (*Crassostrea virginica*) populations. Proteases of *P. marinus* are believed to play an important role in its pathogenicity. Recent studies in our laboratory demonstrated the presence of PI in the plasma of eastern oysters. The role these inhibitors play in *P. marinus* infection remains to be elucidated. In this study, the correlation between *P. marinus* infection intensity and protease inhibitors (PI) level in oyster plasma has been investigated. *Crassostrea virginica* naturally infected with *P. marinus* were collected from Points of Shoal, James River, VA. Parasite burden was determined by Ray's fluid thioglycollate medium (RFTM). PI activities against *P. marinus* extracellular proteases were measured. A statistically significant increase in levels of PI was observed in oysters with low infection burden. Heavily infected oyster showed no measurable PI activities. In another set of experiments 10 *Crassostrea virginica* families were compared for levels of PI. Our results suggest a correlation between these oyster plasma PI levels, parasite burden and mortality rates.

**EFFECTS OF BROODSTOCK AND LARVAL DIETS ON LIPID AND FATTY ACID COMPOSITION OF SEA SCALLOP (*PLACOPECTEN MAGELLANICUS*) EGGS AND LARVAE IN RELATION TO CULTURE OPTIMIZATION.** Scott C. Feindel, Ray Thompson, Pat Dabinett, and Christopher Parrish, Ocean Sciences Center, Memorial University of Newfoundland, St. John's, NF A1C 5S7.

Previous studies have indicated the potential importance of lipids and essential fatty acids (EFA) in the diet of some marine bivalves. To investigate this link with regard to egg quality and larval performance in sea scallops (*Placopecten magellanicus*), feeding trials using live algal cultures with broadly differing EFA composition, specifically eicosapentaenoic acid (20:5n3), docosahexaenoic acid (22:6n3), and arachidonic acid (20:4n6), were carried out on broodstock and larvae. Preliminary results indicate that lipid and EFA composition of eggs and larvae was influenced by diet. However, egg quality in terms of hatchability to D-stage did not correlate with the treatments. This could be due to the transfer of previously stored nutrients into the developing gonad and/or to the conservation and preferential incorporation of EFA in the eggs in the required amounts for normal early development. Differences in larval growth and survivability are partly related to lipid and EFA composition of the diet. Although the treatments consisted of only four species of algae and the biochemical composition of algae is known to vary with culture conditions, the results may be indicative of a diet with an appropriate EFA ratio and lipid composition that can be used to enhance culture success. Optimal broodstock conditioning may depend on other factors.

**GROWTH, MORTALITY AND BIOCHEMICAL CONTENT OF THE PACIFIC OYSTER, *CRASSOSTREA GIGAS*, DURING SPAT-ADULT DEVELOPMENT.** Zaul Garcia-Esquivel, Marco A. Gonzalez-Gomez, and Dahen L. Gomez-Togo, Instituto de Investigaciones Oceanológicas, Universidad Autónoma de Baja California, Ensenada, Baja California, 22800, Mexico.

Changes in shell height, tissue dry weight (TDW), mortality and biochemical content were evaluated in *Crassostrea gigas* during the spat to adult development in San Quintin Bay (SQB), NW Mexico. Shell growth rate was 1.5× greater at the mouth (0.36 mm/d) than the head of SQB and tissue growth rate was also 5-fold higher at the mouth (8.6 mg TDW/d). Oysters located at the head of SQB consistently suffered longer emersion periods (17%) than the ones at the mouth. The market size (9 cm) was reached after 8 mo. (mouth) and 13 mo (head) post-setting. Cumulative mortality during the study ranged between 63 and 87% but the highest mortality (50 to 60%) took place within the first mo. post-setting, during the rearing period. Proteins, lipids and carbohydrates were present in proportions of 48–64%; 2–9% and 1–22% respectively,

with lower values associated to winter months and longer aerial exposure. Proteins and lipids were the most abundant biochemical components during early spat development, but lipids were replaced by carbohydrates as the main energy source in the Fall, when oysters reached 50 to 64 mm. Based on the growth rates and biochemical patterns, it is concluded that site-specific differences in developmental rates were mainly due to longer emersion times at the head of SQB.

**DISEASE DYNAMICS: MODELING THE EFFECT OF CLIMATE CHANGE ON OYSTER DISEASE.** Eileen E. Hoffmann and John M. Klinck, Center for Coastal Physical Oceanography, Crittenton Hall, Old Dominion University, Norfolk, VA 23529; Susan E. Ford and Eric N. Powell, Haskin Shellfish Research Laboratory, Rutgers University, Port Norris, NJ 08349.

Models that simulate the host-parasite-environmental interactions of Eastern oysters (*Crassostrea virginica*) and the pathogens, *Haplosporidium nelsoni*, which causes the disease MSX, and *Perkinsus marinus*, which causes the disease Dermo, have been developed. Both diseases have been epizootic in Delaware and Chesapeake Bays during the 1990s. The models are physiologically-based and are structured around the transmission, proliferation and death rates of the two parasites. Environmental conditions of temperature, salinity and oyster food supply provide the external forcing that results in variations in these biological rates. Simulations that use time series characteristic of warm and cool periods show the advantage given both parasites by warm conditions and demonstrate the importance of a sequence of warm and dry years in triggering wide spread epizootic conditions of the two diseases. Results of these simulations can be used to understand the causes underlying the northward spread of these two oyster diseases along the east coast of the United States, from Chesapeake Bay to Maine, in the decade of the 1990s. The models can also be used to restructure the practices of the oyster industry to maximize production under conditions where the life span of the commercial species is controlled by disease.

**REFERENCE RANGES FOR CHEMICAL AND CELLULAR CONSTITUENTS OF HEMOLYMPH FROM "HEALTHY" LOBSTERS (*LIOMARUS AMERICANUS*).** Barbara S. Horney, Allan L. MacKenzie, Richard J. Cawthorn, Claude C. Morris, Larry K. Hammell, and Robert MacMillan, Atlantic Veterinary College, University of Prince Edward Island, Charlottetown, PEI C1A 4P3.

Reference ranges for blood chemical and cellular components in the "healthy" population of interest are required to identify alterations in individuals in association with disease and disorders.

The purpose of this study was to determine reference ranges for various constituents of hemolymph from visually healthy lobsters (*Homarus americanus*). Hemolymph was collected from the ventral sinus from 5 different groups of freshly caught, pound held or laboratory maintained animals. The mean weight and carapace length of these groups ranged from 298 to 614 grams and 72 to 90 cm respectively. Chemical assays included measurement of the concentrations of sodium, potassium, chloride, calcium, phosphorus, magnesium, glucose, cholesterol, lactate and total protein and the activities of amylase, alanine amino transferase, aspartate amino transferase, alkaline phosphatase, lactate dehydrogenase and arginine kinase on an automated chemistry analyzer. Total solids protein was measured by refractometer and freezing point osmolality was also measured. Hemocyte numbers were evaluated manually and by an automated cell counter. The results are reported as mean  $\pm$  SD of each parameter for each group of lobsters. Comparisons of assay results are made between male and female animals, between groups, and between test methods. This collected data of hemolymph parameters in health is valuable in the identification of hemolymph alterations associated with disease or poor survivability of lobsters.

**PREDICTION OF BLUE MUSSEL (*MYTILUS EDULIS*) FAILURE LOAD.** William P. Ireland, Department of Anatomy and Physiology, Atlantic Veterinary College, University of Prince Edward Island, Charlottetown, PEI C1A 4P3; T. Jeffrey Davidson and Laurie McDuffee, Department of Health Management, Atlantic Veterinary College, University of Prince Edward Island, Charlottetown, PEI C1A 4P3.

Ten blue mussels (*Mytilus edulis*) were collected from each of seven mussel growing areas in Prince Edward Island, measured (length, width, height, thickness and weight) and subjected to a compressive load applied at a constant (0.07 cm/sec) rate to failure by a materials testing machine.

Elements of the machine include an hydraulic cylinder capable of exerting 5000 lbs force (>2,000,000 Newtons) mounted above a load cell connected via an AID interface to a 386 computer. Half shells were placed between the cylinder and loaded to failure by the cylinder. At 50 msec intervals the computer registered the compressive load and saved time step, distance traveled and load to a file.

Correlations were calculated between shell dimension and failure load because we predicted that shell geometry was a factor in shell strength. Regression analysis was done to derive an equation to predict breaking strength from shell measurements. Correlations of strength with shell dimension and weight were significant. Two measures, weight and length taken together significantly predicted shell failure load. This predictive ability was consistent between regions sampled.

**SPATIAL TRENDS FOR TOXIC CONTAMINANTS IN *MYTILUS EDULIS* FROM THE GULF OF MAINE.** Stephen Jones, Jackson Estuarine Laboratory, University of New Hampshire, Durham, NH 03824; Margo Chase, Department of Zoology, Miami University, Oxford, Ohio, 45056; John Sowles, Department of Environmental Protection, Augusta, ME 04333; Peter Hennigar and Peter Wells, Environment Canada, 45 Alderney Drive, Dartmouth, NS B2Y 2N6.

Gulfwatch is a program which has used *Mytilus edulis* for 7 years as the sentinel species for habitat exposure to toxic contaminants in the Gulf of Maine. Mussels were collected at 59 sites in all five jurisdictions for spatial analysis. Tissue was analyzed for trace metals, pesticides, PAHs and PCBs, and growth and condition index were measured. Results showed a southward trend of increasing concentrations for organic contaminants and both silver and lead, reflecting major local and regional pollution sources. Other trace metals showed relatively uniform distribution. Concentrations were elevated at sites previously assumed to be uncontaminated, while other areas appear to be hot spots. Gulfwatch has been useful to managers in a variety of ways, and it provides unique, Gulf-wide information that helps focus efforts to reduce contaminant loading.

**OYSTER RESTORATION IN MARYLAND: MEASURING PROGRESS AND PRODUCTIVITY.** Timothy Koles and Kennedy T. Paynter, Department of Biology, University of Maryland, College Park, MD 20742.

Shellfish restoration in coastal waters has become increasingly recognized as an important part of estuarine ecosystem management. In 1997, in cooperation with the Maryland Department of Natural Resources and the Army Corps of Engineers, five sites in both the Choptank and Patuxent Rivers, extending from the mouth of each river to approximately eight miles upstream, were identified for restoration. At each site, fossil oyster shells were deposited in a configuration of two .5 acre flat areas and one mound approximately three to four meters high. Some of these areas were then planted with hatchery reared spat (1 million/acre; 247/m<sup>2</sup>) while the rest were left unplanted. Monitoring of these sites was accomplished using divers to obtain quadrat samples from each of the flats and mounds and YSI 6000 continuous water quality monitors to measure ambient water temperature, salinity, pH, and dissolved oxygen. The samples were then analyzed for oyster size, abundance, mortality, fouling community, and parasite (*Perkinsus marinus*) prevalence and intensity.

Preliminary analyses have shown similarities among oysters from the two rivers as well as some differences. In both rivers, the oysters appeared to be growing vigorously. Parasite prevalence was very low with only a few oysters in each river infected with *P. marinus*. Mortality was also low and was mostly associated with small oysters that appeared to be overcrowded on the shells. In

addition, the unplanted mounds in both rivers recruited higher numbers of natural spat set than the unplanted flat areas nearby. These results will be used to evaluate the impact of differing bottom morphology (mound vs. flat) and differing water characteristics (i.e. salinity) on oyster recruitment, growth, mortality, and disease pressures.

**DETECTING THE PRESENCE OF *PERKINSUS MARINUS* IN THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA*, IN RHODE ISLAND WATERS.** Ken Leonard III and Marta Gomez-Chiari, Fisheries, Animal, and Veterinary Sciences, University of Rhode Island, Kingston RI 02881; Arthur Ganz, Rhode Island Department of Environmental Management, Coastal Fisheries Lab, Wakefield, RI 02879.

Dermo and MSX, caused by the parasites *Perkinsus marinus* and *Haplosporidium nelsoni* respectively, have been responsible for oyster mortalities throughout the east coast of the United States. The goal of our research is to survey the prevalence and intensity of Dermo and MSX in Eastern oysters from Rhode Island waters. Oysters (30 per site) were collected from 8 locations in Rhode Island from May to November 1998, including 2 aquaculture sites. The prevalence and intensity of Dermo infection were evaluated using the Ray's Fluid Thioglycolate Medium (RTFM) method and by examining histological sections. MSX infections were evaluated using histological examination. We have detected the presence of *Perkinsus marinus* and *Haplosporidium nelsoni* in Rhode Island oysters. The intensity and prevalence of Dermo disease significantly varied between locations and sampling season. Highest *Perkinsus marinus* infection levels were seen in the August sampling session, and may be responsible for the oyster mortalities observed at several sites. The information obtained through this study will be useful to regulatory agencies in their management of the oyster populations in Rhode Island waters.

**INVESTIGATIONS INTO TREATMENTS TO CONTROL FOULING ORGANISMS AFFECTING OYSTER PRODUCTION.** Neil MacNair and Matt Smith, Prince Edward Island Department of Fisheries and Tourism, Fisheries and Aquaculture Division, P.O. Box 2000, Charlottetown, Prince Edward Island, Canada, C1A 7N8.

Fouling organisms are an increasing challenge for oyster production on Prince Edward Island (PEI), Canada. A recently introduced green algae species, *Codium fragile* (the oyster thief), has the potential to interfere with the bottom culture and wild harvest of oysters. In an effort to slow down the transfer of the organism into *Codium* free areas, the Department investigated the effects of pre-transfer immersion treatments, on affected shellfish, using saturated brine and 4% hydrated lime in an attempt to kill *Codium*. Results indicate that the plant is very difficult to eradicate using these treatments and that it takes a long period of time to assess their efficacy. As well, the summer of 1997 proved to be an ex-

ceptional year for set of the sea grape, *Molgula sp.*, which fouled oyster spat collectors and resulted in spat mortalities. The Department investigated the use of saturated brine and 4% hydrated lime to control *Molgula sp.* The study concluded that a three minute dip in saturated brine or a one minute dip in 4% hydrated lime effectively killed *Molgula sp.* on collectors without causing mortalities of the oyster spat.

**SESTON DYNAMICS AND FOOD AVAILABILITY IN A MUSSEL SYSTEM (GULF OF GAETA, SOUTHERN TYRRHENIAN SEA, ITALY).** Antonio Mazzola, Tiziana La Rosa, Benedetto Savona, and Gianluca Sarà, Marine Biology and Resource Laboratory, Dept. Animal Biology, University of Palermo, Via Archirafi, 18, I-90123 Palermo, Italy.

Spatial and temporal changes in the biochemical composition of suspended organic matter in the Gulf of Gaeta (southern Tyrrhenian Sea, Italy), were investigated during a one-year period in order to assess the origin and nutritional value of POM for cultured suspension feeders (*Mytilus galloprovincialis*). Water samples were collected monthly from January to December 1997 at the surface and near the bottom (–12 m) and analyzed for total suspended matter, suspended pigments, particulate carbohydrate, protein and lipid concentrations. The biopolymeric fraction of particulate organic carbon was defined as the sum of carbohydrate, protein and lipid carbon and used as an index of the particulate organic matter readily available to benthic consumers. Mean total suspended matter concentration was  $4.8 \pm 3.9 \text{ mg l}^{-1}$ , while inorganic suspended material concentrations were high, indicating a possible dilution effect of the particulate organic matter. Phytopigment concentrations of  $1.2 \pm 0.91 \text{ } \mu\text{g l}^{-1}$  lead us to define the waters of Gulf as meso-tropical. Particulate biopolymeric organic carbon concentrations were quite low (on average  $120 \pm 85 \text{ } \mu\text{g C l}^{-1}$ ) and no significant difference was detected in comparison with a control station outside the Gulf. Proteins were the dominant class in the particulate matter (40%), followed by carbohydrates (34%) and lipids (26%); POM represented on average only 7% of total suspended matter. These results do not substantiate the fact that cultivation of mussels is a normal commercial activity in the Gulf (yielding about 200–300 t per year). The role of physiological compensation in the maintenance of a relatively constant food absorption rate in environments which are characterized by time-varying resources could be invoked.

**EFFECTS OF FOOD QUALITY ON THE PARTICLE HANDLING TIME IN BIVALVES.** Lisa M. Milke and J. Evan Ward, Department of Marine Science, University of Connecticut, Groton, CT, 06340; Sandra E. Shumway, Southampton College, LIU, Southampton, NY 11968.

Suspension-feeding bivalves may assume a large ecological role by linking benthic and pelagic systems. Therefore, a knowledge of feeding processes is necessary to fully understand bivalve

dominated environments. In this study, we examined the pallial cavity residence time for *Mytilus edulis* and *Crassostrea virginica*. By measuring residence times and subtracting previously calculated particle transport rates found on the gills of mussels and oysters, handling time on the labial palps can be determined.

Bivalves were offered one of three food types: *Rhodomonas lens* cells, *Spartina alterniflora* particles, or a 50/50 mixture of both. Once actively feeding, bivalves were delivered 10 µm polystyrene beads as a tracer and feeding continued. Bivalves were sampled at 30 s intervals for 0 to 150 s and placed in liquid nitrogen, ensuring the cessation of particle transport. Digestive systems were then isolated and examined for the presence of tracer beads. For mussels, it appears that food quality has little effect on handling time. At the 30 s interval, 10–30% of the animals had tracer beads in their gut, increasing to 90% by the 150 s time interval. Assuming a residence time of 60s (25–50% of animals) for mussels with average gill lengths of 2.8 cm (used in our study), handling time on the palps would be 12 s. Therefore, estimated labial palp transport rates are indicative of slurry as opposed to mucus transport. Results of this and parallel studies with oysters will be utilized to create a numeric model of pallial processes using the modeling software Stella.

**HOW TO PROVIDE ESSENTIAL NUTRIMENTS TO BIVALVES IN HATCHERY.** J. Moal,<sup>1</sup> C. Seguineau,<sup>1</sup> J. F. Sainain,<sup>1</sup> P. Soudant,<sup>2</sup> M. Cansell,<sup>3</sup> J. R. LeCoz,<sup>1</sup> H. Migaud,<sup>1</sup> M. Sanles,<sup>1</sup> B. Ponce,<sup>1</sup> C. Langdon.<sup>4</sup> <sup>1</sup>Ifremer, Centre de Brest, BP 70, 29280 Plouzané. <sup>2</sup>Virginia Institute of Marine Science, 1208 Great Road, Gloucester Point, VA 23062. <sup>3</sup>ISTAB, Université Bordeaux I, av des Facultés, 33405 Talence. <sup>4</sup>Hatfield Marine Science Center, 2030 S. Marine Science Drive, Newport, OR 97365.

A large variability is observed in essential nutrient composition between eggs from different spawnings and from different females of *Crassostrea gigas* oysters. It depends on the food regime of broodstock. We have tested different ways to modify the egg and the larval composition, either by supplementation of the food regime of adults and larvae by emulsions, liposomes or spray beads, or by direct modification of egg composition by lipofection technique.

Emulsions (INVE Aquaculture: ICES 30/0.6/C) were used for lipophylic supplementation. Marine liposomes of a size range adapted to bivalve ingestion capacities (enrichment possibility: lipids from their membrane and an aqueous internal volume (Cansell in prep.)). The similarity of their membrane lipid composition with that of mollusc membranes is a possible way to increase the lipofection process. Spray beads for food supplementation of aqueous and lipophylic molecules (Buchal and Langdon (in press)).

First experiments were conducted to determine if these different particules were ingested and digested by *C. gigas* at different developmental stages. Empty liposomes were radiolabeled on the membrane and mixed to an oocyte preparation to study the lipofection possibilities. Results are presented and discussed.

**EXPERIMENTAL DREDGING OF STARFISHES AND CRABS BEFORE COMMERCIAL SEEDING OF SEA SCALLOPS IN MAGDALEN ISLANDS (QUÉBEC, CANADA).** Madeleine Nadeau and Georges Cliche, Ministère de l'Agriculture, des Pêcheries et de l'Alimentation du Québec, Direction de l'Innovation et des Technologies, C.P. 658, Cap-aux-Meules, Québec, G0B 1B0; Denyse Hébert, Association des pêcheurs de pétoncles des Îles-de-la-Madeleine, C.P. 516, Étang-du-Nord, G0B 1E0.

Commercial bottom seeding of juvenile sea scallops (*Placopecten magellanicus*) are performed annually in the Magdalen Islands (southern Gulf of St-Lawrence) since 1993. In 1997, the areas seeded in 1993, 1994 and 1995 were re-opened to commercial fishing. About 6% of the scallops seeded in 1993 and less than 1% of those seeded in 1994 and 1995 seeding have been recaptured. Predation by starfishes and crabs appeared to be a major factor in the loss of scallops. Recapture rates in Japan were increased from 5.5% to more than 30% by removing with dredges predators before seeding. Therefore we tried such an operation of predators removal by dredging just before seeding in spring 1998. After 230 hours of dredging, more than 200,000 predators (92% starfishes and 8% crabs) were removed from a 2.25 km<sup>2</sup> during this operation. Starfishes were mostly represented by *Solaster endeca* (50%), *Leptasterias polaris* (25%) and *Crossaster papposus* (20%). *Hyas* sp. composed 70% of the crabs. An inventory realised before dredging with a video camera suggested that more than 25% of the predators were taken off during the operation. This capture level was comparable to Japanese results. The impact on scallops survival and the commercial interest of such operation will be evaluated more precisely at the reopening of this seeded area, in 4 years.

**MUSSEL CULTURE POTENTIAL IN SOUTHERN MOZAMBIQUE.** F. Ribeiro, F. Simoes, and L. Swenarchuk, Instituto de Investigação Pesqueira, P.O. Box 4603, Maputo, Moçambique.

From 1976 to 1989, the Mozambican government carried out a study into mussel (*Perna perna*) aquaculture, with support from CUSO-SUCO, a Canadian NGO. Natural coastal settlements, found south of Inhambane, served as seed for transfer to culture ropes and growout on long-lines or existing fixed structures in Maputo and Inhambane Bays. Growth was excellent in both sites.

Repeated failure of natural spatfall, however, proved to be a critical obstacle blocking development of industrial-scale culture.

This problem was subsequently resolved using hatchery techniques. Spawning of wild mature adults was artificially induced by temperature shifts in the Maputo Laboratory, and veliger larvae (5 larvae/ml) were fed on *Isochrysis galbana* and *Tetraselmis suecica*, growing to 300  $\mu$  as metamorphosed spat within 19–23 days, before settling on collectors made from discarded multifilament fishing nets. After incubation in outdoor 1m<sup>3</sup> tanks for 4–6 weeks, seed mussels had reached 2–3 mm, and were transferred to growout ropes, reaching 9–10 cm in 11 months.

Inhambane and Maputo bays are suitable sites for integrated enterprises including hatcheries and growout zones, with estimated minimum potential production of  $0.56 \times 10^6$  MT/Year and  $10 \times 10^3$  MT/Year (wet weight), respectively. Moreover, culture in the interior regions of Inhambane Bay may ultimately reduce the need for hatchery-supplied seed, as reproduction from the cultured animals becomes self-sustaining. Major target markets should be the coastal populations, already accustomed to consuming mussels, and the rapidly growing shrimp culture industry.

#### EUTROPHICATION CONTROL BY BIVALVES: POPULATION FILTRATION, SEDIMENTATION AND NUTRIENT REMOVAL THROUGH SECONDARY PRODUCTION.

**Michael A. Rice**, Department of Fisheries, Animal and Veterinary Science, University of Rhode Island, Kingston, RI 02881; **April Valliere**, **Mark Gibson**, and **Arthur Ganz**, Rhode Island Division of Fish and Wildlife, Coastal Fisheries Laboratory, 1231 Succotash Rd., RR#1, Wakefield, RI, 02897.

Filter feeding by populations of bivalves has been suggested as a means of reducing eutrophication in coastal estuaries by exerting control of phytoplankton populations in the water column. Frequently large populations of mature shellfish reside behind pollution closure lines in estuaries represent a large filter feeding biomass. The standing crop of quahogs, *Mercenaria mercenaria*, in the Providence River averages 9.1 clams/m<sup>2</sup> or about 26,400 tonnes, filtering about  $1.5 \times 10^7$  m<sup>3</sup> of water daily or a rate equivalent to 24% of the rate of water exchange during a tide cycle. The population of quahogs, however, is composed of mostly older adults with valve lengths in excess of 60 mm. These large animals are slow growing, have a low rate of secondary production in relation to standing crop biomass, and have a neutral nitrogen balance (organic-N assimilated = NH<sub>3</sub>-N excreted). These large adults increase sedimentation through filter feeding, but since they are neither harvested nor growing they do not directly remove much nitrogen from the system, although the increased sedimentation rates may result in increased sediment denitrification. Smaller more rapidly growing quahogs have the capability of incorporating organic nitrogen into growing tissues and if harvested regularly provide a mechanism for direct removal of nitrogen from

the estuary. As part of a Narragansett Bay wide shellfisheries management plan, 10% of the standing crop of quahogs in the Providence River is recommended for relay to management beds down bay for later harvest. At this level of relay effort, 2,640 tonnes of shellfish would be moved to harvest beds annually, representing about 25 tonnes of nitrogen removal from the estuary if these were eventually harvested. The removal of quahogs from the dense assemblages in the Providence River reduces the population filtration by only 10%, but it culls the population making room for faster growing juveniles and small adults. This is publication 3682 of the Rhode Island Agricultural Experiment Station, University of Rhode Island.

**DEVELOPMENT OF A PCR-BASED DIAGNOSTIC ASSAY FOR A NOVEL PERKINUS SPECIES ISOLATED FROM MACOMA BALTICA.** † **José A. F. Robledo**, **Cathleen A. Coss**, and **Gerardo R. Vasta**, Center of Marine Biotechnology, University of Maryland Biotechnology Institute, 701 East Pratt Street, Baltimore, MD 21202.

The non-transcribed spacer (NTS) at the rRNA locus of a new *Perkinsus* species isolated from the baltic clam *Macoma balthica* was amplified, cloned, sequenced, and compared to that of the sympatric species *P. marinus*. Length and sequence of the NTS are dramatically different from that of *P. marinus*, supporting the conclusion based on the analysis of the SSU, ITS1 and ITS2, that the clam isolate constitutes a distinct species. Based on the NTS sequence a set of primers was designed for a PCR-based diagnostic assay that specifically amplifies *Perkinsus* sp. from *M. balthica*. Because the host specificity of *P. marinus* and *Perkinsus* sp. has not been determined, this assay, together with the one previously developed and validated for *P. marinus*, should contribute to addressing not only this question, but issues related to the role of other invertebrate or vertebrate species as putative reservoirs, intermediate hosts or vectors for both parasite species. †This study was supported by DOC Cooperative Agreement No. NA46RG0091 awarded by NOAA through the Maryland Sea Grant to G.R.V.

**THE NEW WESTERN MEDITERRANEAN ENTRY BRACHIDONTES PHARAONIS (FISCHER P., 1870) (BIVALVIA, MYTILIDAE): CHANGES IN FILTRATION RATE UNDER VARYING NATURAL FOOD CONDITIONS.** **Gianluca Sarà**, **Chiara Romano**, and **Antonio Mazzola**, Marine Biology and Resource Laboratory, Dept. Animal Biology, University of Palermo, Via Archirafi, 18, I-90123 Palermo, Italy.

The hyperhaline bivalve mollusc *Brachidontes pharaonis* (*B. pharaonis*) is a common Indo-Pacific species that has recently reached the western Mediterranean through the Suez Channel. The

most western MED finding of *B. pharaonis* has been documented in a saltworks in western Sicily. Here, *B. pharaonis* has colonized hard substrates and now represents the most important suspension filter feeder of the area. There is very little information on the population dynamics, feeding habits and behaviour of *B. pharaonis*. Seasonal changes in food availability (May–December 1998) were investigated in order to assess the relationship with somatic growth and filtration rate of *B. pharaonis*. The filtration rate was measured seasonally in a flowing open system and calculated by taking the difference between the suspended organic matter concentrations in inflow and outflow water samples. During the study period, water temperature and salinity ranged from 8°C to 27°C in winter and summer respectively, and 44 psu and 58 psu in spring and summer respectively. Food concentration expressed as total suspended organic matter was found to be highly variable (from 2 to 200 mg l<sup>-1</sup>). Filtration rate was significantly and negatively correlated to food concentration, varying between 1.4 and 7.61 g mussel<sup>-1</sup> h<sup>-1</sup>. Lastly, the role of the quality and quantity of organic matter on the performance of *B. pharaonis* and the reasons for its absence in the adjacent basin (Stagnone di Marsala) are discussed.

**PARTIAL CULTURE AND CRYOPRESERVATION OF THE PARASITIC DINOFLAGELLATE *HEMATODINIUM PEREZI* FROM THE BLUE CRAB.** Jeffrey D. Shields, Department of Environmental Sciences, Virginia Institute of Marine Science, The College of William & Mary, P.O. Box 1346, Gloucester Point, VA 23602.

*Hematodinium perezi* is a parasitic dinoflagellate that infects blue crabs along the eastern seaboard of the United States. Currently, the parasite can only be maintained in the laboratory via serial injection. *In vitro* culture and cryopreservation of the pathogen were attempted. Isolates were held in a balanced salts buffer at temperatures of 4°, 15° and 20°C. The main stages in the life cycle of *H. perezi* were observed, but only partial completion of the life cycle was attained. Under culture conditions, plasmodia developed into schizonts but plasmodial budding and schizogony were not completed. Trophonts (vegetative stages) progressed to highly vacuolated presporonts which rarely sporulated to become dinospores. Primary cultures of the parasite lived for up to 16 d at 4°C, 28 d at 15°C and 7 d at 20°C. Naïve crabs acquired infections when inoculated with cultures that had been maintained *in vitro* for 14 d, but those inoculated with reconstituted cryopreserved samples did not acquire infections. Parasites reconstituted from cryopreservation were alive, but did not grow in culture, nor were they infectious. Recovery of live parasites was significantly higher in glycerol than in dimethyl sulfoxide. Successful culture and reconstitution of cryopreserved *H. perezi* from the blue crab will require a better support medium.

**INFECTION BY THE DINOFLAGELLATE PARASITE *HEMATODINIUM* IN THE NORWAY LOBSTER (*NEPHROPS NORVEGICUS* L.) ON THE WEST COAST OF SCOTLAND, UNITED KINGDOM.** Grant D. Stentiford and D. M. Neil, Division of Environmental & Evolutionary Biology, IBLs, University of Glasgow, Glasgow, G12 8QQ, Scotland, UK.; R. J. A. Atkinson, University Marine Biology Station Millport, Isle of Cumbrae, Scotland UK.

The Norway Lobster (*Nephrops norvegicus* L.) is the most commercially important shellfish in the UK, with a first sale value of ~£63 (~\$105m US) in 1997, the major fishery being in Scotland. Stocks of *Nephrops* off the west coast of Scotland are known to harbour infection by the parasitic dinoflagellate *Hematodinium*. The infection shows a clear seasonal pattern, with peak prevalence in the spring and may also show an overlying long-term epidemiology. Prevalence levels reach 80% in some years and seem to be greatest in post-moult animals. Infection staging is possible by observing the degree of parasite accumulation in a pleopod, and by immuno-cytochemistry using a polyclonal antibody raised against *Hematodinium*. Recently, certain biochemical parameters of the host haemolymph have been identified as infection indicators and work is continuing in this area. The parasite itself causes gross histopathological changes to most host tissues and organs, and it is suggested that severe infections lead to a considerable physiological compromise in *Nephrops*. Such effects on host metabolism may be expected to cause alterations in the behaviour of *Nephrops* in the field, and may reduce its ability to evade trawl capture or predation. Understanding of the life cycle of *Hematodinium* has increased following its successful culturing in the laboratory, and such knowledge may help to elucidate the as-yet unknown mode of infection in *Nephrops*. It may also help to identify possible secondary or alternative hosts of this parasite on *Nephrops* grounds and lead to measures for its control in both Scottish *Nephrops* and other commercially important crustacean fisheries around the world.

**SEARCHING FOR THE PUTATIVE MSX INTERMEDIATE HOST USING MOLECULAR DIAGNOSTICS.** Nancy A. Stokes, Brenda Sandy Flores Kraus, and Eugene M. Burrenson, Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062; Kathryn A. Ashton-Alcox and Susan E. Ford, Haskin Shellfish Research Laboratory, Rutgers University, Port Norris, NJ 08349.

Forty years after its introduction to the eastern United States, the complete life cycle of the oyster pathogen *Haplosporidium nelsoni*, the agent of MSX disease, still has not been elucidated. Attempts to infect oysters directly with *H. nelsoni* spores have been unsuccessful, leading to speculation that parasite transmission between oysters occurs via an obligate intermediate host. Our



*H. nelsoni*-specific polymerase chain reaction (PCR) and *in situ* hybridization (ISH) diagnostic assays have been optimized for use with environmental samples and are being used in the search for the putative intermediate host(s). Samples of water and sediment fractions and of macroinvertebrates were taken from MSX-endemic areas of York River, VA and Delaware Bay for three years. Total genomic DNA was extracted from each sample and subjected to PCR amplification. Samples that yielded *H. nelsoni* PCR product were more frequent from the York River than from Delaware Bay, consistent with MSX disease prevalence in oysters from these locations. PCR-positive samples were subjected to ISH to allow visualization of parasite infections to discriminate between true infections and those where *H. nelsoni* simply adhered to the external surface or passed through the gut.

**PRELIMINARY EVALUATION OF TRIPLOID AMERICAN OYSTERS, *CRASSOSTREA VIRGINICA*, ON A MID-ATLANTIC OYSTER FARM.** Stewart M. Tweed, Rutgers Cooperative Extension of Cape May County, Cape May Court House, N.J. 08210; Ximing Guo, Haskin Shellfish Research Laboratory, Rutgers University, Port Norris, NJ 08349.

In 1996, a commercial oyster farm was established in New Jersey with oysters from Rutgers Highly Selected Resistant Lines (HSRL). The success of these lines prompted interest in developing HSRL triploids in order to produce faster growing better quality market oysters.

Triploid and Diploid American Oysters, *Crassostrea virginica*, were produced from a HSRL stock at the Haskin Shellfish Research Laboratory in the summer of 1998. In July, 1 to 2 millimeter seed oysters were placed in upwellers at the Atlantic Capes Fishery Oyster Farm. These seed were maintained on the farm and moved to nursery areas in the Fall and growout sites in the Spring.

Growth and survival of Triploid and Diploid cohorts were compared when they were moved from the upweller, and at three

different nursery and growout sites at 5 and 8 month intervals. Results are being used by the Oyster Farm to evaluate growing areas and triploid potential for improved production and marketing.

**PROTEOLYTIC ACTIVITY FROM BLOOD CELLS OF THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA*.**

Gregory Ziegler, Marine, Estuarine, Environmental Sciences, University of Maryland, College Park, MD 20742; and Kennedy T. Paynter, Chesapeake Biological Laboratory and Department of Biology, University of Maryland, College Park, MD 20742.

A protease was isolated from the eastern oyster, *Crassostrea virginica*, hemocytes and characterized using substrate impregnated sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and spectrophotometric assays. Hemocytes were isolated by centrifugation and homogenized in the presence of SDS at pH values ranging from 4.4 to 7.4. Crude homogenates were loaded into vertical slab gels and run at 30 mA for approximately 1 hour. Proteolytic activity was revealed as clear bands on Coomassie Blue stained, substrate impregnated gels. Proteolytic activity was found in acrylamide gels impregnated with gelatin, but activity was absent in gels impregnated with other substrates including casein, fibrin, fibronectin and laminin. Molecular weight of the enzyme responsible for the single proteolytic activity band was estimated to be 87 kDa when analyzed by SDS-PAGE. Activity was maximal at a pH of 7.4 on gelatin impregnated gels. A suite of inhibitors was employed to determine the mechanistic class of the isolated protease and indicated that the proteolytic activity was likely a cysteine protease. Spectrophotometric assays were performed to confirm protease characteristics and to ascertain a  $V_{max}$  and  $K_m$  for the enzyme-catalyzed reaction. The lack of additional proteolytic activity, even at lower pH extractions and incubations, is surprising given that most cells are thought to have a suite of proteases that serve various and different functions.



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**COVER PHOTO:** Veined rapa whelk, *Rapana venosa*, courtesy of Juliana M. Harding

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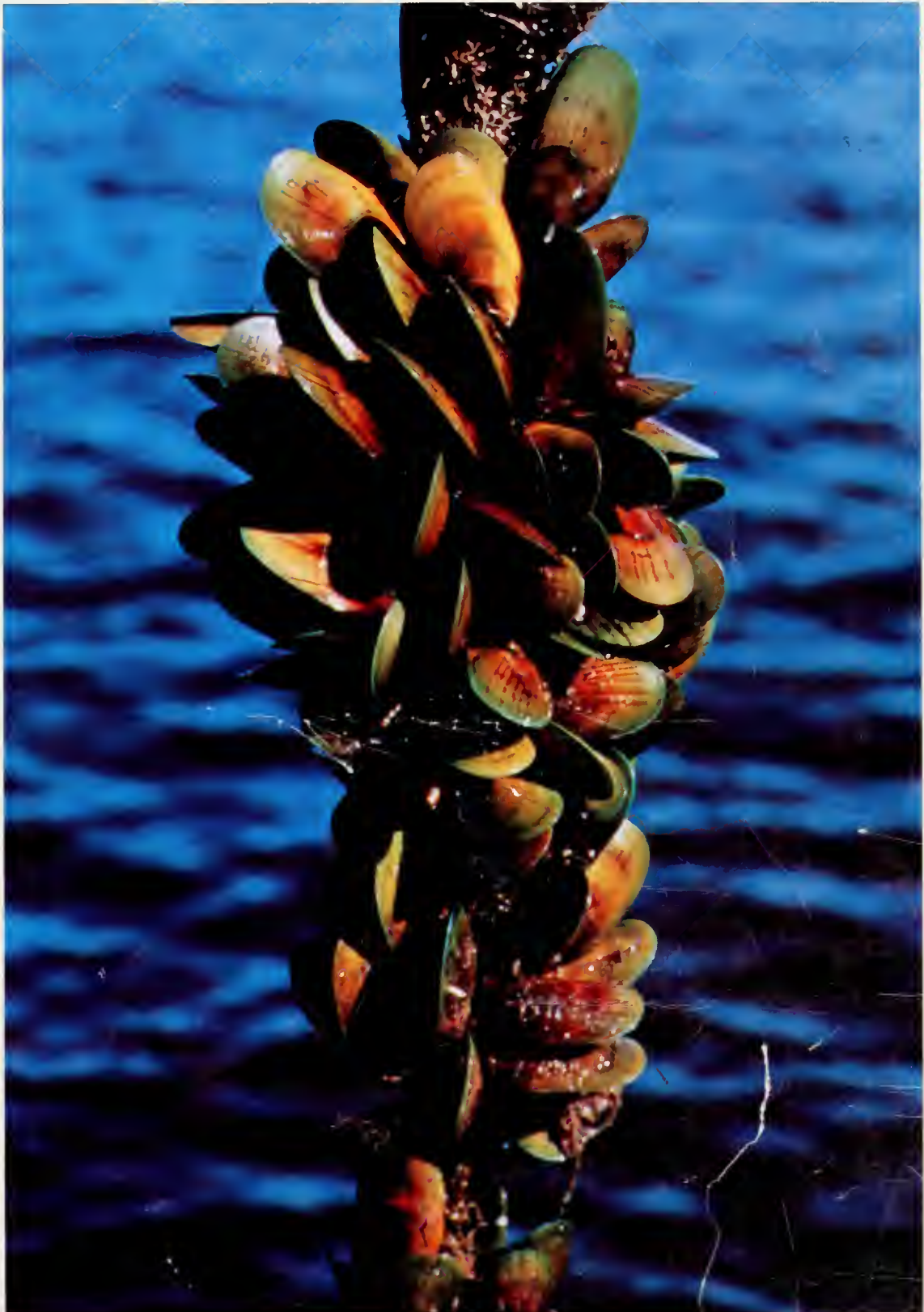
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**Harold Haley Haskin  
Honored Life Member**

Harold Haskin is affectionately known as “Doc” by his many students; by members of the shellfish industry who, over the years, came to respect and trust him in a way that few academics have ever been; and by state and federal government officials, who regularly sought his advice on matters pertaining to marine and estuarine environments. During his many years of service on the faculty of Rutgers University and to the shellfish industry of the US, he gained a reputation for bringing sound scientific reasoning to the often contentious issues concerning the use and protection of shellfish resources, and for teaching others to do the same.

Hal was born Harold Haley in Niagara Falls, NY on January 3, 1915, and was orphaned 3 years later during the influenza pandemic of 1918–1919. By his own admission, Hal was a bit of a “hellion” at this early age, and his future was in some doubt after his parents died. Fortunately, he was adopted by Frederick J. Haskin a family acquaintance who was a bachelor pipefitter. His adoptive father moved with Hal to southern New Jersey, where they lodged with retired farmers. This fortuitous arrangement introduced Hal to Delaware Bay, a focus of much of his professional life. The lifestyle instilled in Hal a lifetime love for the outdoors and an ethic of hard work. His father also strongly encouraged young Hal to advance himself, and Hal’s high school principal, sensing an unusual intellectual ability in the young student, helped him prepare for college. Because of his fascination with the water, Hal hoped to attend Annapolis, but he was also interested in science and finally entered Rutgers College (class of 1936) because of the reputation of its biology program. It was a professor of English, however, who encouraged Zoology Department Chairman Thurlow (T.C.) Nelson to offer Hal the scholarship and job that allowed him to remain in school during the lean Depression years. Hal was an outstanding student and the first graduate of Rutgers College to attain a straight A average. At the same time, he was a championship boxer and became the cadet colonel of the Rutgers ROTC. But it was his association with T.C., and particularly a summer job studying the biology of oyster drills in Barnegat Bay, that directed Hal toward his lifelong love affair with oysters.

On the basis of his outstanding academic record and his keen observations during field work, T.C. recommended Hal for graduate school to colleagues at Harvard, where he was awarded a fellowship to study marine biochemistry. There, he completed a master’s degree on chemoattractants, a PhD degree in algal physiology, and met his wife Peg (Smith College 1939). One month after graduating in 1941, Hal was inducted into the Army. He first commanded units guarding the coast from Long Island to the Virginia Capes and later was in charge of training infantry recruits—a job he did so well that the Army consistently refused his requests for overseas transfer. Major Haskin was discharged in early 1946 and spent the next 6 months as a research associate in coastal oceanography at WHOI, working

under his PhD advisor, Alfred C. Redfield. By the fall semester, however, he was back at Rutgers College where he began teaching general biology, limology, malacology, and oceanography, and resumed his research on shellfish problems. His early work on hard clam biology led to an offer to become director of the Virginia Fisheries Laboratory (now the Virginia Institute of Marine Science), but he decided to remain at Rutgers and in 1950 he succeeded T.C. as Chairman of the Department of Oyster Culture in Rutgers College of Agriculture and Director of the Oyster Investigation Laboratory. From 1951–1953, he took two years off from teaching and moved his growing family (eventually to include four daughters and a son) to southern New Jersey, near the “Oyster Lab” at Bivalve, where he directed a Navy-funded hydrography project within and offshore of Delaware Bay. There were few days during those years when Hal was not aboard the RV Julius Nelson sampling around the estuary. It was at this time that he met and formed lasting friendships with many of the local oyster planters, a feat made possible by his own “South Jersey” upbringing, his first-hand knowledge of the Bay, and his unpretentious manners.

In the early 1950s, while Hal was engaged in the “Navy Project,” the Delaware Bay oyster industry was facing serious problems. Planters relied on seed oysters from natural setting areas, but overharvesting and poor recruitment had severely depleted that supply. Hal not only recognized the gravity of the problem, but was willing to argue, essentially alone, for drastic action to turn the situation around. He convinced the state management agency that data needed to be gathered regularly and scientifically to document the status and trends of the seed oyster population and the factors affecting it. It was more difficult, however, to convince the oystermen that additional restrictions were needed on the quantity of seed being harvest and that the restrictions should be based on rigorously collected and analyzed data. Hal later recalled rancorous meetings and threats against the Laboratory, but his steadfastness, the high regard that the oyster-producing community had for him, and the volumes of data he presented eventually led to the establishment of the desired controls and improving conditions on the seed beds. More importantly, his efforts led to the establishment of an unusual tripartite management system, which is still in effect, consisting of the state management agency, the oyster industry, and the University researchers, each with defined roles and shared responsibility for managing the oyster resource. The concept of the University as an independent and unbiased supplier of data to industry and management agencies was later adopted, with Hal playing a leading role, in the management of the Atlantic surf clam fishery.

The MSX epizootic, which began in Delaware Bay in 1957, led Hal in new directions: oyster pathology and genetics. Working with numerous students and colleagues, Hal pursued the search for answers about this mysterious new disease, which was devastating oyster populations in Delaware and Chesapeake Bays. One of his most valued associates was Leslie A. Stauber, a parasitologist and fellow member of the Rutgers Zoology Department. The Haskin-Stauber team spawned generations of teachers and researchers (some now in the fourth generation) dedicated to studying disease processes and defense mechanisms in oysters and other marine bivalves.

One of Hal’s major contributions to science and to the oyster industry has been the development of oyster strains resistant to MSX disease. Beginning shortly after the MSX outbreak, he began breeding the survivors of the epizootic—to determine whether these individuals possessed a heritable trait that could be improved by selective breeding, and if so, to provide broodstock to the industry. The breeding program, which did produce resistant strains, is ongoing today and now includes selective breeding for resistance to Dermo disease. It is one of the longest sustained breeding programs for an aquatic species.

Hal’s work with oysters has sometimes overshadowed his contributions in other fields, which include research on hard clam depuration, oil and sewage pollution, oyster drill biology, and the effects of dredging, damming, and development on the estuarine environment. Hal has always insisted that basic and applied science are integral to each other. He pursued both, to their mutual benefit, even when this clashed with the prevailing philosophy of University administrators.

Hal is a dedicated educator, researcher and public servant. In addition to the large cadre of Haskin-trained students (including some Haskin children) who teach in colleges and universities in this country and abroad, many serve in state and federal agencies where they are involved in marine research and policy making. He is at his best when he combines his research interest with education, both formal and informal. He is famous for his graduate courses that involved field trips most every weekend, regardless of weather; hence another affectionate title “Hurricane Hal.” Students who took Hal’s courses in estuarine ecology and coastal oceanography can attest to being in the field on blustery days with Hal standing in icy water, seemingly oblivious to the cold, lecturing on one of his favorite subjects—oyster biology. Years later, former students would approach him at meetings or other functions and say that these classes were the most valuable they had taken in their graduate career.

Hal’s classroom lectures were fact filled and precise, reflecting his exhaustive preparation. A crew assigned to film lectures for General Biology at Rutgers found that most faculty lectures could easily be edited and considerably reduced, and many had to be refilmed. Hal’s, however, took the full time allotted and required virtually no editing. Hal’s teaching was by no means restricted to the classroom. Students were to be found everywhere: visitors to his office or lab who were entranced by his discourses on oysters, or clams, or estuarine processes; businessmen he met at social functions, who learned how the oyster industry operates; students and employees who were treated to impromptu dialogues in front of a shucked oyster; and the legions of undergraduates who spent a summer rearing oysters at the Cape Shore Laboratory or field sampling in Delaware Bay or off the New Jersey coast.

In recognition of his contributions to Rutgers, to state and federal governments, and to science and industry, Hal has received numerous awards. Until his retirement in 1984, he was the Julius Nelson Professor of Zoology at Rutgers, where he won the Lindback Award for Distinguished Faculty Research in 1980 and the University Medal for Distinguished Service in 1986. In 1980, he was the recipient of the Governor’s Earth Day Anniversary Award for outstanding contributions to the protection of New Jersey’s environment and in 1988, he was given an EPA Environmental Quality Award in recognition of commitment to environmental protection. He has been a member of the National Shellfisheries Association for his entire professional life and served as president from 1967 to 1969. He became an Honored Life Member in 1979 and received the David Wallace Award in 1984. Hal received a particular honor when, by a vote of the Board of Trustees of Rutgers University, the new, modern laboratory he worked so hard to build in Bivalve was named after him in 1990. This was the first time that a Rutgers building was named for a living person—a second “first” for Hal at the University.

Hal's many contributions to maintaining a safe and clean environment cannot be fully appreciated without acknowledging the major role played by his wife, Peg. The Haskins are known throughout New Jersey for their efforts to preserve water quality and to allow natural systems to operate while trying to integrate the many conflicting uses. It has been in these endeavors that the pair's access to state government served both science and the estuary well. In 1994, the "Hal and Peggy" team was honored by the Water Resources Association of the Delaware River Basin with the Dr. Ruth Patrick Excellence in Education Award for their "long-standing efforts to inform and create an awareness of the water resources of the Delaware River Basin." Their most recent award, given in 1999 on behalf of the people of New Jersey by the Governor, was in gratitude for their many efforts to "benefit the natural resources of the Delaware River Basin."

Hal Haskin's singular dedication and commitment to his mission has been extremely effective in the difficult task of integrating scientific information with political reality. Too often, science focuses on a single problem and loses perspective. We can all learn from Hal's dedication, his broad and balanced outlook, and his insistence on facts and good science. He said it best while speaking of the many interests that compete with oysters for use of the Delaware Estuary: "I am not one of those who believes in keeping everything just the way it is in the name of conservation. I think we can arrange things to serve many different needs, but we have to recognize we have a very valuable, renewable resource here, and the pressures on it are already great. We should be very watchful when we consider changes that could add new stresses."

John Kraeuter and Susan Ford  
Port Norris, New Jersey  
September 1999





**Carl James Sindermann  
Honored Life Member**

Among marine biologists and parasitologists around the world, the name Carl Sindermann is almost synonymous with books and articles on diseases of marine organisms. A background in fish and shellfish pathology, coupled with a talent for writing, propelled him into a successful career as a scientific author. Carl is a prolific and thoughtful writer, whose output includes not only scientific treatises, but books on the discipline of science and the scientists who practice it. Volumes that he has authored or co-authored are indispensable components of marine biology libraries worldwide.

Carl grew up in North Adams, Massachusetts, where he was born on August 28, 1922. After graduating from high school and working for two years for Pratt and Whitney, an airplane engine manufacturer in Hartford, Connecticut, he joined the US Army at the outbreak of World War II. Carl served as a medic in an infantry reconnaissance platoon, landing in Normandy a month after D-Day and moving with Patton's army through France, Germany, Austria, and Czechoslovakia before the war ended.

In 1946, Carl enrolled at the University of Massachusetts on the GI Bill. It was not until his senior year, however, that his interest in science was galvanized. A female faculty member, who, Carl recalls, was still an assistant professor after 20 years in the Zoology Department, assigned him a research project that became his senior honor's thesis: the study of an invasion of western Massachusetts by a large, predatory land planarian. Carl worked out the life cycle of this flat worm, which had been imported in soil from the tropics and was destroying natural earthworms in greenhouses—and launched into a career as a parasitologist. He had already been accepted at Purdue University when the head of the Zoology Department at the University of Massachusetts took Carl to visit colleagues at Harvard University. Carl was accepted on the spot to pursue a graduate program in parasitology.

Carl studied with the protozoologist, L. R. Cleveland, working on life cycles of parasites of wood-eating cockroaches. He shifted to the marine field for his PhD research, working with mycologist W. H. Weston. Carl's Dissertation was based on summer research at the US Fish and Wildlife Service Laboratory at Boothbay Harbor, Maine, where he studied a fungus disease of herring. While at Harvard, Carl became a teaching assistant in Parasitology and Tropical Public Health at the Harvard Medical School, and also an instructor at nearby Brandeis University where he taught undergraduate courses in biology and invertebrate zoology. He also continued his association with the US Fish and Wildlife Service, serving as the Chief of the North Atlantic Herring Investigations. After obtaining his PhD from Harvard in 1953, Carl remained on the Brandeis faculty until 1956, when he elected to return to the Boothbay Harbor Laboratory and become a research biologist.

Carl remained at Boothbay Harbor until 1962, by which time the Laboratory, along with all US fisheries programs, had been transferred to the newly created Bureau of Commercial Fisheries (BCF). Carl's administrative skills were recognized within the Bureau, and in 1963 he moved to Maryland's Eastern Shore to become Director of the new laboratory at Oxford (now the Sarbanes Cooperative Oxford Laboratory). The Oxford Laboratory was built as a consequence of the epizootic mortalities of eastern oysters, caused by MSX disease, that had begun in the Delaware and Chesapeake Bays a few years earlier. The new laboratory specialized in disease studies of commercially important fish and shellfish, and under Carl's direction, its scientists played important roles in the early days of oyster disease research and the laboratory's reputation became known world-wide. In 1968, Carl left the mid-Atlantic for a new post as director of the BCF's Tropical Atlantic Biology Laboratory in Miami, Florida, a job that he held for the next 4 years. In 1971, Carl returned to the mid-Atlantic, this time New Jersey, where he became director of the Middle Atlantic Coastal Fisheries Center with headquarters at the Sandy Hook Laboratory of the National Marine Fisheries Service (NMFS—the old BCF). While at the Sandy Hook Laboratory, Carl added to his administrative duties by becoming Assistant Director for Environmental Management of NMFS's Northeast Fisheries Center in 1976. While serving in these posts, Carl had written not only numerous articles and reports, but had also become a renowned book

author. In 1985, he withdrew from administration to devote full time to writing. From 1985 to 1990, he was an Intergovernmental Personnel Act Appointee, first at the University of Miami and later at the Maryland Department of Natural Resources, after which he returned to the Oxford Laboratory.

Throughout his career as a Federal employee, Carl retained close ties to academic institutions near his various postings. He held visiting or adjunct professorships at Georgetown, Florida Atlantic, Lehigh, and Cornell Universities, and the Universities of Miami, Guelph, and Rhode Island, where he taught invertebrate zoology, marine biology, fish pathology, and marine parasite ecology. He has served on the editorial boards of *Aquaculture*, *Chesapeake Science*, the *Journal of Fish Biology*, the *Journal of Invertebrate Pathology*, and the *Proceedings of the National Shellfisheries Association*. He was the Scientific Editor of the *Fishery Bulletin*.

The honors and awards that Carl has received are too numerous to list, but a sampling shows the breadth of activities and interests that have occupied him during the past half century: member, Bureau of Commercial Fisheries advisory group to NASA on back contamination from lunar exploration, 1967; recipient of the Department of Commerce Silver Medal for administrative and research activities, 1975; chairman, New Jersey Sea Grant Advisory Board, 1981–1985; keynote speaker for the Sixth Symposium on Pollution and Physiology of Marine Organisms, Charleston, SC, 1983. He served as the President of the World Mariculture Society in 1980–1981, and was chosen as an Honored Life Member of the National Shellfisheries Association in 1991.

Although Carl has been a member of various international fisheries bodies, his work with the International Council for the Exploration of the Sea (ICES) is perhaps the most important. His affiliation with that organization began in 1959 when he attended his first meeting in Copenhagen. In the 1970s and 1980s he served on a number of ICES working groups, including those for Fisheries Improvement, Marine Aquaculture, Marine Pathology, and Introduced Species (of which he was chairman for a decade). His ability to synthesize and analyze great quantities of material was critical to the preparation of numerous reports for these groups, some of which served as the basis for later publications. An important contribution of these working groups was the issuance of the ICES "Code of Practice," which lists steps to be taken during the transfer of aquatic species to reduce the risks of disease spread when aquatic organisms are moved to new locations. The guidelines are used at present by most European countries and many US states.

Although Carl devoted much of his career to laboratory administration, he is best known as a book author. His scientific writing began as papers describing his research on marine parasites and pathology. His first publication (1953) was on "clam digger's itch," a human problem, but caused by a trematode parasite with a marine snail intermediate host. Carl's interests subsequently turned to parasites of the marine organisms themselves. Because he was in charge of the Atlantic herring project, his studies focused on this species, with a number of publications in the 1950s describing parasites and diseases of herring. Several of Carl's early papers showed that parasites could be used as tags to trace the movement of fish stocks. At the Boothbay Harbor Lab, Carl's work on serology of fishes resulted in a series of papers ranging from comparative serotyping of different fish species to the effects of disease on blood characteristics.

In the early 1960's Carl's genius for synthesizing material became evident in an article entitled "Disease in marine populations" (1963). Not long afterward, he teamed up with Oxford Lab colleague Aaron Rosenfield, whom he had met while both were on the faculty of Brandeis University, to produce the now classic paper "Principal diseases of commercially important marine bivalve Mollusca and Crustacea," published in the *Fishery Bulletin* in 1967. In 1970, Carl expanded his earlier work in a volume entitled "Principal Diseases of Marine Fish and Shellfish" (Academic Press), which won the Wildlife Society of America award for best scientific publication in fisheries for 1970. Carl later updated this important work, which was re-issued in a 1990 two-volume set. These publications are acclaimed not only for the breadth of material included and the depth of analysis, but for the clarity of language and illustrations. His growing interest in aquaculture led to another indispensable book for the aquatic pathologist: "Disease Diagnosis and Control in North American Marine Aquaculture," edited by Carl and published in 1977 by Elsevier. This volume was also updated, in 1988, and in collaboration with Don Lightner.

While director of the Sandy Hook Laboratory, which is situated on the shore of the New York Bight, it was natural, perhaps inevitable, that Carl's attention would be drawn to the effects of coastal pollution on marine organisms. Once again, he meshed his surehanded grasp of disease processes in the marine environment with what he was learning about pollution in a series of publications showing links between environmental contaminants and disease in marine fish. One of his most recent books, "Ocean Pollution—Effects on Living Resources and Humans" (1996, CRC Press) is an outgrowth of these concerns.

Carl's enthusiasm for writing has led him into areas not often entered by scientists: writing about the scientists themselves. His first foray, entitled "Winning the Games Scientists Play" (1982, Plenum) elicited enthusiastic reviews, and some consternation among a few colleagues who recognized themselves in the vignettes he used as illustrations. "The Joy of Science" (Plenum) was published in 1985, followed in 1987 by "Survival Strategies for New Scientists" and in 1992 by "The Woman Scientist" (co-authored by Clarice Yentsch). Carl's most recent offering, written in collaboration with Tom Sawyer, is entitled "The Scientist as Consultant: Building New Career Opportunities" (1997). The books show Carl to be a keen observer of scientists and an accurate reporter of their behavior. They have an underlying theme: to analyze, often with a lighthearted touch, what makes a person successful in the scientific profession. They are realistic, discussing both pros and cons of certain career paths, and contain a wealth of practical advice—valuable not only for those considering or just embarking on a scientific career, but with admonitions that individuals well along in their professions would do well to follow.

Carl and his wife Joan are the parents of two daughters (both social scientists) and three sons (all in construction). When Carl retired in 1991, he and Joan decided to remain on the Eastern Shore near the Sarbanes Cooperative Laboratory. Carl continues to come into his office at the Laboratory, to use the library, to chat with Aaron Rosenfield over lunch, and to work on yet another addition to the long list of publications under the Sindermann name. His current work in progress is tentatively titled "Rhyme of an Ancient Scientist: the Aging Scientist in Today's Society."





**Aaron Rosenfield  
Honored Life Member**

His unassuming and modest demeanor belies the fact that Aaron Rosenfield has been one of the most important movers in the field of shellfish pathobiology over the past 40 years. At the Oxford, Maryland Laboratory, he led a team of researchers that made outstanding contributions in this field. Studies on the distribution, causes, and effects of diseases on aquatic biota have been his main research specialty. Information and technical transfer have also been a particular strength. He has played a major role over the past three decades in bringing together talented individuals with creative minds for symposia, workshops, and conferences. These assemblies have resulted in the synthesis of scientific information into reports and books for use by resource managers, industry regulators, and other decision makers, particularly in relation to the control of biological and anthropogenic pollution, fisheries conservation, and aquaculture.

Aaron was born in Boston Massachusetts on October 14, 1924. He grew up in nearby Cambridge, where he attended public schools, graduating from Cambridge Latin School in 1942. He then enlisted in the Navy and served for the remainder of World War II as a quartermaster on P.T. boats in the South Pacific, where he saw action in the Solomon and Philippine Islands, New Guinea, and Palau.

After his discharge from the Navy in 1946, Aaron entered the University of Massachusetts. His interest in science had been stimulated in high school by the Sinclair Lewis novel "Arrowsmith," about a microbiologist, and he pursued a degree in bacteriology and public health which he received in 1950. He remained at U. Mass to study for a Master's degree in bacteriology and food science which he received in 1951. Diploma in hand, Aaron entered the world of industry where he worked as an analytical chemist for a company that produced emergency rations for the military. He quickly decided that industry was not for him and in the fall of 1952, he was awarded a Sara Hays teaching fellowship at Brandeis University where he taught laboratory courses in general biology, botany, microbiology, and plant physiology. At the same time, he began graduate work at Boston University. During this period he worked summers with Carl Sindermann, whom he had met at Brandeis, at the Maine Department of Sea and Shore Fisheries Laboratory at Boothbay Harbor where they collaborated on a herring parasite project.

Aaron became a full-time graduate student in 1953 when he enrolled at the University of Texas at Austin. From 1954 to 1956, Aaron returned to teach at Brandeis with his new bride Clarice, whom he had married in 1953. In 1956, he returned to Texas to complete his doctoral degree while Clarice worked at the University's Chemistry Department to provide living expenses for the family, which soon included a daughter, Sandy. Despite his early interest in bacteriology, Aaron switched focus during his PhD research to a project examining the physiological consequences of heterosis in corn. The quantification of DNA to standardize other measures was a newly emerging technique, which Aaron adopted and which inspired his life-long fascination with cell and molecular biology. After obtaining his PhD in 1960, Aaron remained for several months at the University of Texas as a postdoc studying mechanisms of bioluminescence in marine organisms. At the end of 1960, he returned to the Boothbay Harbor Laboratory where he became Microbiology Program Leader with the US Bureau of Commercial Fisheries (BCF). His research projects included *in vitro* tissue culture of molluscs, mechanisms of disease transmission and resistance, and cytogenetics. In 1962, Aaron was invited to take charge of the Shellfish Mortality Program at BCF's new Oxford Laboratory on Maryland's Eastern Shore. At Oxford, Aaron became deeply involved in research on the newly emerging oyster diseases, MSX and SSO, as well as Dermo. He and his research team discovered and described the elusive spore stage of the MSX parasite (*Haplosporidium* [then *Minchinia*] *nelsoni*). During this period, Aaron also made occasional forays across

Chesapeake Bay to the Washington headquarters of BCF where he held temporary duty assignments, including Chief of the Bureau of Shellfisheries and Chief of the Division of Resource Research and Management.

Aaron's roots in New England showed themselves forcefully when he first came to the Oxford Lab. He had a pronounced accent. So did this technician, only she came from South Carolina and had a heavy southern accent. The two often had difficulty understanding each other and colleagues recall having to act as interpreters in the laboratory. Aaron's accent has diminished over the years, but one is never in doubt about his origin.

In the early 1970s, the BCF was transferred to the National Oceanic and Atmospheric Administration (NOAA) and renamed the National Marine Fisheries Service (NMFS). The Oxford Laboratory was then assigned to the Middle Atlantic Coastal Fisheries Center, with headquarters at Sandy Hook, New Jersey. Aaron became its Officer-in-Charge of the Oxford Laboratory and its Director of Pathobiology Investigations. In this position he had the responsibility for establishing and supervising major national and international research programs in coastal aquatic animal health, biomedicine, and comparative pathobiology. His work put him in close contact with the shellfish industry, university and government researchers, and resource managers. These contacts became increasingly important as diseases and die-offs of fish and shellfish, and pollution associated problems, began to increase not only in North America, but in other parts of the world as well. Meanwhile, the Oxford Laboratory's pathology program expanded to include projects and personnel at the Milford, Connecticut and Sandy Hook, New Jersey Laboratories. Pathobiology research also expanded to encompass studies on other invertebrates, especially crustaceans, and finfish, as well as studies on microbial pathogens and tumors in "lower animals." In the mid 1970s, with still another NMFS reorganization, Aaron continued to head the Oxford Laboratory as its Director and as Chief of the Northeast Fisheries Research and Science Center's Division of Pathobiology.

One of the most important contributions of the Pathobiology Program under Aaron's direction was the establishment of cooperative alliances with other federal agencies, with state agencies and commissions along all U.S. coasts, with foreign governments, and with scientific and professional organizations around the world. The Oxford Laboratory's reputation grew until it was recognized world-wide as one of the foremost institutions in the detection and control of marine fish diseases. Students and established researchers from all over the U.S. and from dozens of foreign countries traveled to Oxford to study pathology of marine fish and shellfish, or to engage in joint studies with the Laboratory's expert staff.

Because the Laboratory's activities had such a wide geographic scope, Aaron's outlook on the problems of diseases in marine organisms also became global. The potential transmission of disease organisms among marine ecosystems developed into a major concern and focus of efforts. Aaron and his staff were instrumental in the design of strategies and programs for aquatic animal health, including quarantine and inspection to prevent the spread of pathogens, pests, predators, and competitors in previously unaffected areas. Early in his career, he urged all of the state fishery commissions (Atlantic, Gulf, Pacific) and the International Council for the Exploration of the Sea (ICES) to consider the consequences of introduced genes (both hosts and parasites) on indigenous populations. These efforts have resulted in guidelines for the movement of fish and shellfish, which are now used by many states and foreign countries. In another move to foster global communications and data analysis among fish and shellfish pathologists, and to house and catalogue the growing collection of pathology specimens found by the Oxford Laboratory's own investigators or sent to them by distant collaborators, Aaron conceived of the Registry of Marine Pathology, now incorporated into the Registry of Tumors of Lower Animals at the George Washington University Medical Center. Submissions to the Registry are compared with archived samples, thus providing a world-wide data base of marine parasites and pathogens and a mechanism whereby new discoveries can be evaluated in the light of existing information.

During his career as a government scientist and administrator, Aaron maintained unusually close ties with academic institutions. He has been a research associate at Georgetown University's Biology Department, an associate faculty member at the Johns Hopkins School of Public Health and Hygiene, and an adjunct professor at the University of Maryland, Center for Estuarine and Environmental Studies. He has been highly supportive of university researchers, enlisting funds from NOAA, the Army Corps of Engineers, the Environmental Protection Agency, the National Institutes of Health, the National Science Foundation, and other agencies for equipment, publications, and especially for meetings and workshops on special topics in marine pathology. Among the most important and influential of these were the "Shellfish Mortality Conferences," stimulated by the outbreaks of MSX disease in the late 1950s and early 1960s, which brought together most of the founding generation of molluscan pathologists in the US. Again in the 1980s, Aaron was instrumental in supporting a second series of these conferences, where a new generation of researchers, as well as many of the original participants, gathered to discuss the current status and future direction of molluscan pathology.

In addition to the Mortality Conferences, Aaron helped organize and support workshops in this country and abroad on pathology and *in vitro* biology for the American Fisheries Society, the Society for Invertebrate Pathology (of which Aaron was the permanent program chair), the American Institute for Biological Sciences, the National Shellfisheries Association, and the Society for *in Vitro* Biology. He helped found the Society for Invertebrate Pathology and was elected treasurer for 1983-1984. He has been an NSA member since 1962, becoming vice president in 1978 and president in 1979. Aaron has been very active on the international scene also, serving on oversight committees for collaborative programs in fisheries, aquaculture, and pathology between the US and Asia, including Japan, Indonesia, Taiwan, South Korea, and the People's Republic of China.

In recognition of his many achievements in government service, Aaron has received numerous awards from the US Department of the Interior and the National Oceanic and Atmospheric Administration. He was recognized by the Chesapeake Bay Foundation with an award for his conservation efforts, was awarded certificates of recognition by Maryland members of the US Congress, and received a certificate of achievement from the Governor of Maryland for his work on state resource issues. He was made an Honorary Life Member of the National Shellfisheries Association in 1991.

In 1987, when the Oxford Laboratory was turned over to the State of Maryland, Aaron took an Interagency Personnel Assignment at the University of Maryland's Center for Estuarine and Environmental Studies. There he continued his mission to organize workshops

and symposia, and to publish the resulting output. One of the most important of these was a session at the 1989 NSA meeting in Los Angeles on the subject of introduced species, which resulted in the seminal work "Dispersal of Living Organisms into Aquatic Ecosystems" (Maryland Sea Grant), published in 1992 with Aaron as senior editor. Aaron officially retired from NMFS in 1993, but he and Clarice continue to live in Easton, near the Oxford Laboratory (now the Sarbanes Cooperative Oxford Laboratory), where he can be found working in his office each day as a visiting senior scientist. In his "retirement," he has retained his global perspective, conceiving of and organizing symposia that attract participants from around the world. One of these, also held in conjunction with an NSA meeting (Orlando, FL in 1992), on the history and status of molluscan shellfisheries, resulted in a massive, three-volume set entitled "The History, Present Condition, and Future of the Molluscan Fisheries of North and Central America and Europe" (NOAA Technical Publications, 1997), of which Aaron was a co-editor. In 1996, he organized another major symposium, "The Blue Crab Fisheries of North America." Results of this symposium occupy the entire September 1998 issue of the *Journal of Shellfish Research*. Aaron is currently exploring the possibilities of organizing a similar meeting in conjunction with the Organization of American States on "Crustacean Fisheries in the Americas." Aaron's ever active mind is constantly coming up with new ideas, which are focusing more and more on helping promote and invigorate the Eastern Shore of Maryland. Among these ideas are the establishment of aquaria on routes traveled by vacationers to the Atlantic beaches, which would have both educational and heritage components and would encourage participation by watermen; a summer teacher's institute where local teachers could learn about biotechnology; and a natural products laboratory that would investigate potential uses of estuarine organisms. Aaron's mind is sure to be stimulated even more by the Internet, which he has recently discovered, and which astounds and delights him.

In a typically self-effacing manner, Aaron measures much of his achievements by those of the Oxford Laboratory. He cites its impressive qualitative and quantitative publication record and its contributions to the scientific community through joint participation in professional and fishery conservation activities. He is most proud of the Laboratory's outstanding contributions toward advancing the fields of marine fish health research and comparative pathobiology, both of which were barely recognized by most fishery scientists and resource managers as integral parts of the marine fisheries ecosystems, or marine science in general, before he joined the Laboratory.

Susan Ford  
Port Norris, New Jersey  
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## OVERVIEW AND BIBLIOGRAPHY OF RESEARCH ON THE GREENSHELL MUSSEL, *PERNA CANALICULUS*, FROM NEW ZEALAND WATERS

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**ABSTRACT** The greenshell mussel, *Perna canaliculus* (Gmelin 1791), from New Zealand, is the most intensively cultivated member of the genus *Perna* within the mussel family Mytilidae. The aquaculture production of this species has grown extremely rapidly over the past 20 years and is now facing some constraints on further expansion. Overcoming these constraints will require a more thorough understanding of the biology of this species. Although *Perna canaliculus* has been the subject of extensive research, much of this information remains obscured in unpublished literature. Therefore, an overview of the biology and research on the greenshell mussel, *Perna canaliculus* (Gmelin 1791) from New Zealand waters is presented along with a comprehensive bibliography of over 500 references.

**KEY WORDS:** *Perna canaliculus*, greenshell mussel, green-lipped mussel, bibliography, aquaculture

### INTRODUCTION

In terms of world aquaculture production, volumes of the Greenshell<sup>®</sup> mussel *Perna canaliculus* is second only to the blue mussel, *Mytilus edulis* (Linnaeus 1758) (FAO 1999). The farming of *M. edulis*, which began in Europe over 700 years ago, now takes place in over 15 countries worldwide, and the aquaculture of *P. canaliculus* is confined to New Zealand and only began around 20 years ago (Greenway 1969a, Vakily 1989). *Perna canaliculus* is now internationally recognized as a premium eating mussel and, consequently, the New Zealand mussel aquaculture industry has continued to expand rapidly, with production almost doubling in the last 4 years to reach 70,000 tonnes/yr<sup>-1</sup> (Fig. 1) (New Zealand Mussel Industry Council unpublished data). The continuing rapid expansion of this industry will increasingly rely on an improved understanding of the biology of this species, particularly in relation to other members of the family Mytilidae, which are also cultured on a large scale. Although a great deal of research has been done on *P. canaliculus*, much of it is in the form of unpublished reports, theses, and local publications. Therefore, the purpose of this paper is to draw together a bibliography of this information and provide a synthesis of the current state of our knowledge for this species and suggest suitable avenues for future research.

### TAXONOMY

*Perna canaliculus* is endemic to New Zealand, where it is commonly known as the green-lipped mussel because of a characteristic emerald green interior shell margin. It is also known by the tradename Greenshell<sup>®</sup> mussel.

This species was first described from New Zealand as *Mytilus canaliculus* by Martyn in 1784, but was not recognized until sometime later (Gmelin 1791, Siddall 1980). Other names such as *Mytilus latus* (Chemnitz, 1785) and *Mytilus smaragdinus* (Hutton 1873) have been proposed, but failed to be recognized.

Fleming (1959) proposed the incorporation of the species into the genus *Perna* (Retzius 1788) after the initial work of Soot-Ryen (1955), who used hinge and ligament structures and muscle scars as a basis for establishing taxonomic differences between *Perna* and *Mytilus*. The differentiation of *Perna* from *Mytilus* is determined by the presence of postmetamorphic lateral hinge teeth on the larval shells (Siddall 1980); whereas, adult *Perna* lack an an-

terior adductor muscle and have a discontinuous posterior retractor muscle.

Other members of the genus *Perna* are found in coastal South America and Africa (*Perna perna* Linnaeus 1758) and the Indo-Pacific (*Perna viridis* Linnaeus 1758). These species are distinguished by their geographic origin, coloration, soft part morphology, and most reliably, by differences in the patterns of shell muscle scars (Siddall 1980).

### ECOLOGY

Fossilised *P. canaliculus* is known from a number of shallow water marine deposits around New Zealand from the lower Pliocene, 13 million years ago (Powell 1979). Extant *P. canaliculus* is widely distributed throughout much of New Zealand, but is most common in central and northern New Zealand, where it frequently forms dense beds of up to 100 m<sup>-2</sup> (Fig. 2) (Stead 1971, Flaws 1975, Hickman 1991). It is found from the midlittoral to depths of over 50 m (Powell 1979, Buchanan 1994a). The physiological inability of small mussels to survive aerial exposure restricts the occurrence of these mussels on the upper shore (Paine 1971, Kennedy 1976, Marsden and Weatherhead 1998); whereas, lower depth limits are thought to be regulated by predation rather than competition (Paine 1971). Mussels in the littoral zone maintain lower condition indices than subtidal individuals, (Hickman and Illingworth 1980), because aerial exposure reduces the oxygen uptake by up to 87% (Weatherhead 1993); decreasing feeding time; and, therefore, the energy available to maintain basal metabolism and growth (Vakily 1989).

*Perna canaliculus* are found in a variety of habitats, attached to rock faces, wharf piles, and among algal holdfasts in the intertidal, and in deeper water they are often found living over mud or sand (Morton and Miller 1973). Consequently, *P. canaliculus* is known to tolerate a wide range of water salinities and temperatures. Normal coastal salinities for this mussel are in the range of 30 to 35 ppt, but laboratory experiments have shown that *P. canaliculus* can survive at 25 ppt and that lower salinities can also be tolerated for short periods (Flaws 1975). This mussel is known to inhabit waters with water temperatures as low as 5.3 °C in the south of New Zealand and 27 °C in northern areas (MacDonald 1963, Hickman 1991). Through genetic research, populations at either end of the country have been shown to be partially differentiated stocks.

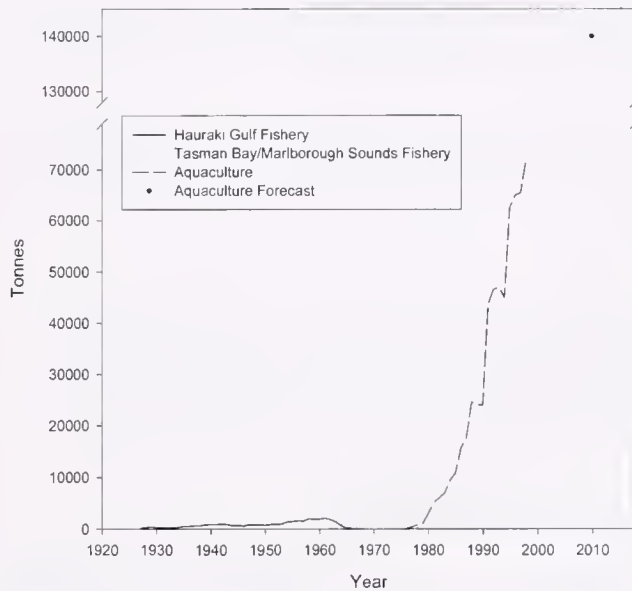


Figure 1. Graph showing past changes in the annual production of *P. canaliculus* from dredge fisheries and aquaculture in New Zealand (Greenway 1969h, Hickman 1979h, FAO 1996, FAO 1999, New Zealand Mussel Industry Council 1998).

These differences are thought to be attributable to the adjustment to local temperature regimes that are maintained by currents acting as barriers to larval mixing (Sin et al. 1990, Gardner et al. 1996a, Gardner et al. 1996b).

#### LIFE HISTORY CHARACTERISTICS

Some of the reproductive behavior of *P. canaliculus* has been well described, particularly in reference to determining a reliable larval supply for commercial use (Hickman 1987a, Hickman and Illingworth 1980, Tortell 1980, Hayden 1995, Fox 1996). As with most other mytilids, *P. canaliculus* is a dioecious broadcast spawner (Jenkins 1985). Gonadal development only occurs at temperatures above 11 °C and is also related to food availability (Jenkins 1985, Redfearn et al. 1986, James and Ross 1997). Ripe female gonads have a reddish-orange color, and male gonads remain creamy white (Hayden 1994a). Most spawning occurs from late spring to early autumn, although gametes can be shed throughout the year (Jenkins 1985, Hayden 1994a). Individual mussels can maintain spawning condition for several months (Flaws 1975, Tortell 1976c). Female *P. canaliculus* produce up to 100 million eggs per season (Jenkins 1985). Mature eggs are 56 to 62 µm in diameter, and viable sperm measure 54 µm in length (Redfearn et al. 1986). External fertilization of eggs is dependent on the proximity of adults and the length of time that gametes remain viable in the water column (Hayden 1994a).

A lecithotrophic trochophore larvae develops from the fertilized egg (Buchanan 1994a), followed by early veliger development at 24 to 48 hours from fertilization (Hayden 1994a). The veligers are in the plankton for a further 3 to 5 wk feeding on a range of microalgae and dissolved organic matter (Hayden 1995). During this time, the prodissoconch II forms and then grows to 100 to 250 µm in length. The larval shells of *P. canaliculus* are recognizable by a broadly rounded umbo, high angular shoulders, and the presence of 19 to 24 provincial teeth (Booth 1977, Redfearn et al. 1986). The veliger larvae are thought to be capable of being

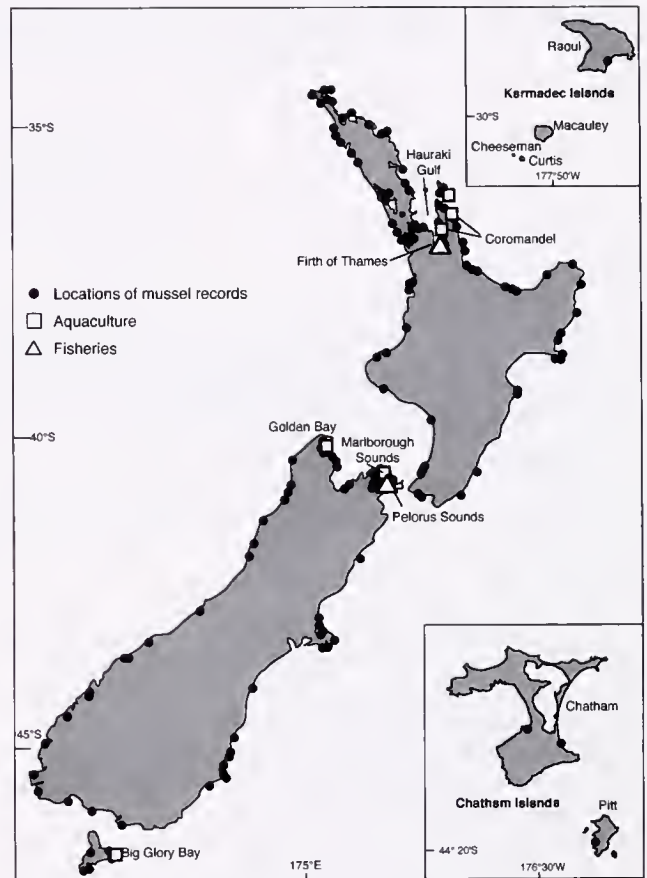


Figure 2. Maps of New Zealand showing the known locations of *P. canaliculus* compiled from the collections of the New Zealand Oceanographic Institute, Te Papa - National Museum of New Zealand and the Auckland Institute and Museum. The locations of fisheries and aquaculture activities for this species mentioned in this paper are also identified.

dispersed up to several hundred kilometers by near-shore currents (Hayden 1995).

Pediveligers develop at 4 to 6 weeks, depending upon nutrient availability, temperature, and salinity (Buchanan 1994a) and are from 220 to 350 µm in length (Booth 1977). The larvae prefer to settle on fine filamentous substrata, including hydroids and filamentous algae (Manning 1985a,b Buchanan 1994a). In the absence of suitable settlement substrata, pediveligers can remain planktonic for several weeks (Buchanan 1994a, Hayden 1995). Settlement of the pediveliger is completed with the attachment of a byssus thread, subsequent metamorphosis and growth of the dissoconch shell (Buchanan 1994a). Settlement of *P. canaliculus* is most intense from late winter to early summer (Luckens 1976), but settlement is highly variable on both spatial and temporal scales (Hayden and Kendrick 1992).

Primary settlement of *P. canaliculus* larvae into beds of adult mussels is uncommon (Buchanan 1994a,b). Secondary settlement, involving a form of byssopelagic migration, or mucus drifting, is thought to be the means by which juveniles recruit into mussel beds (Buchanan 1994a,b). To undertake postsettlement movement the plantigrade, or spat, first detach from the substrate by severing the byssus threads. Secreted mucus strands then enable the spat to swing, or drift, into new areas for attachment. Juvenile mussels may move in this manner numerous times before recruitment into

adult mussel beds. The ability for byssopelagic migration is lost once the spat reach 6 mm in length attributable to anatomical changes of the pedal glands (Buchanan and Babcock 1997).

Post-settlement growth rates of *P. canaliculus* are extremely variable among individuals, localities and years. This variability in growth is mostly attributable to differences in phytoplankton supply and water temperature (Hayden 1995). As a ciliary–mucoid filter feeder (Vakily 1989), large *Perna* are able to increase the rate of filtration with increased abundance of phytoplankton (James and Ross 1996). Filtration rates for individual adult mussels of 350 l d<sup>-1</sup> have been recorded (James and Ross 1996). Mussels in suspended culture typically grow from 10 to 75 mm in 6 mo to 110 to 115 mm in 1 y, to 140 mm in 1.5 y, and to 195 mm in 3.5 y (Flaws 1975). Sexual maturity is reached in the first year (Jenkins 1985) and adult *P. canaliculus* can grow to over 240 mm in shell height (Stead 1971).

### Fisheries

The significance of *P. canaliculus* in the diet of New Zealanders extends back to prehistoric times, with mussel shell remains present in Maori middens throughout the country (Hickman 1991). Harvesting of mussels for domestic use is still popular in many locations where mussels still persist. However, in many locations, the harvesting by hand-picking, snorkelling, and grabs has greatly diminished or removed wild populations (Paul 1966, Kilner and Akroyd 1982).

Commercial harvesting of *P. canaliculus* began with handpicking of intertidal beds in the last century and expanded in 1927 with the development of a dredge fishery of subtidal mussels in the Hauraki Gulf (Fig. 1) (Flaws 1975, Jenkins 1985, Fisher 1993). After a brief decline in catch rates from 1935 to 1945, mussel landings increased steadily to peak in 1961 at more than 2,000 tonnes (Greenway 1969b, Reid 1969, Flaws 1975). Overexploitation of the Hauraki Gulf mussel beds caused the fishery to close in 1966 (Hickman 1991). A second dredge fishery was developed in Tasman Bay and Kenepuru Sound in 1962; however, this fishery also began to decline within the following 5 years (Fig. 1) (Stead 1971a, 1971b). Intensive dredging of beds of *P. canaliculus* living on sand or mud removes both juveniles and shell matter leaving an unstable soft substrate no longer suitable for settlement and attachment of mussels (Stead 1971a, 1971 b).

### AQUACULTURE

The collapse of both commercial and domestic fisheries for *P. canaliculus* provided the impetus for the subsequent development of aquaculture methods for this species. The initial development of New Zealand's mussel aquaculture industry was hampered by public concerns over the social and economic acceptability of farms (Hickman 1989a–c). Early commercial cultivation attempts for *P. canaliculus* were based on Spanish raft techniques and were largely unsuccessful because of the instability of the rafts (Edmond 1986a,b). Subsequent adaptation of the Japanese mussel longline cultivation system produced the first significant harvest of 300 tonnes in 1977 (Hickman et al. 1991). The refinement and upscaling of this longline culture system combined with increased mechanization in the handling of cultured mussels has led to a dramatic increase in production of mussels over the last 20 years (Fig. 1) (Hickman 1989a–c).

Sites for farming *P. canaliculus* using the longline culture system require reasonable shelter from waves and wind, high water

quality, adequate tidal flow and depths of at least 5 m (Guard 1971). About 1,840 ha of mussel farming licences are in place in the Marlborough Sounds, with a further 414 ha of licences mainly in the eastern Hauraki Gulf (Fig. 2). A small number of farms are found in Golden Bay (104 ha) and Big Glory Bay (144 ha) (N.Z. Mussel Industry Council unpublished data). A typical mussel farm of 3 ha contains up to 10 longlines often running parallel to shore. Each longline consists of 30 to 40 large plastic buoys supporting two 110-m backbones that run side-by-side (Fig. 3). From the backbones are suspended culture ropes in loops, or “droppers,” that are 5- to 10-m in over-all length, depending upon water depth. The droppers are seeded with mussel spat collected from the wild in two ways.

About 80% of *P. canaliculus* spat for aquaculture come from large quantities that wash up intermittently on Ninety Mile Beach adhering to various species of detached macroalgae (Fig. 4) (Hickman 1982c, Fox 1998). The spat vary in density from 200 to 2 million kg<sup>-1</sup> of wet algae and can be from < 50 µm to 10 mm in shell height (Hickman 1982c, Pooley 1991c). The spat-covered algae are gathered from the beach and transported to mussel farms where they are transferred and held on the cultivation dropper ropes with a biodegradable stocking.

Wild mussel spat are also caught closer to aquaculture areas by using fibrous ropes as an artificial settlement surface. These are held at 15 to 20 m water depth for 4 to 8 weeks in areas with a history of high spat settlement (Meredyth-Young 1985d, Hayden 1994b). Spat catching by this method can be difficult because larval settlement is highly variable (Meredyth-Young and Jenkins 1980, Hayden and Kendrick 1992). In addition, there is high predation and mortality of newly settled mussels, as well as competition from fouling organisms (Hickman 1979b, Hayden 1984).

After the spat have grown to 10–20 mm on the seed droppers, they are stripped from the droppers and relaid onto grow-out droppers at optimal growing densities of 200–300 m<sup>-1</sup> (Hickman 1976, Hayden 1995). Mussels on-grown in this manner grow to 80 mm in length in about 13 mo, at which time they are harvested, with each longline typically yielding a crop of around 25 tonnes of mussels.

Less than 10% of the total mussel harvest is sold live, but the majority (70%) are heat shucked and then frozen on the half-shell. The remainder are processed into meat only products or medicinal powders.

Exports of frozen mussels from New Zealand began in 1978 and rapidly expanded to over 50 countries, with the largest market the United States (Hickman 1978, Hickman 1989c, New Zealand Seafood Industry Council Database 1999). About 33,100 tonnes, or 50%, of the annual *P. canaliculus* aquaculture production is

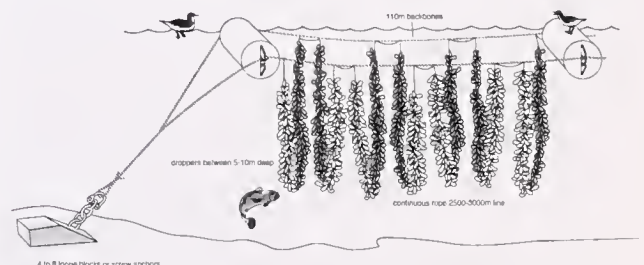
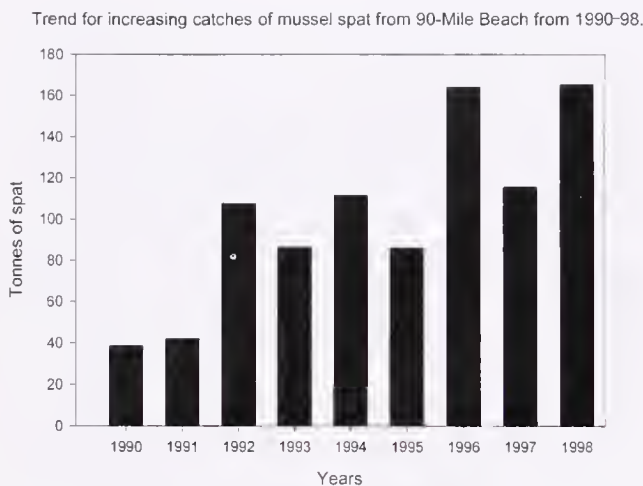


Figure 3. Figure showing the layout of the longline culture system now widely used for the aquaculture production of *P. canaliculus* in New Zealand.



**Figure 4.** Graph showing the annual supply of wild *P. canaliculus* spat material from Ninety Mile Beach to the aquaculture industry in New Zealand (compiled from catch log books). The graph indicates a trend for increasing reliance on the wild spat resource as a source of seed for aquaculture.

now exported, at a total value of NZ\$ 118 M in 1998 (New Zealand Seafood Industry Council Database 1999).

In addition to being a premium eating mussel, *P. canaliculus* is thought to possess unique remedial properties in rheumatoid and osteoarthritis (Couch et al. 1982, Weston 1983). Freeze-dried mussel extracts are sold as a health supplement (Croft 1980). The beneficial effects are consistent with nonsteroidal anti-inflammatory drugs (Miller and Ormrod 1980, Miller and Wu 1984). Lysolecithin, an anti-histaminic has also been isolated from *P. canaliculus* as well as Lyprinol, a compound preliminary research indicates may be effective in treating some cancers (Kosuge et al. 1986).

*Perna canaliculus* has several natural parasites, but infection rates are low, with none causing significant mortalities in cultivated mussels (Hickman 1978). These parasites include the larval digenean *Cercaria haswelli* (Linzey 1971, Jones 1975b), *Nematopsis* spp. (Jones 1975a), the pea crab *Pinnotheres novaezelandiae* (Jones 1975b, Jones 1977a, Jones 1977b, Stevens 1990), and the copepods *Pseudomyicola spinosus* and *Lichomolgus unicus* (Jones 1975). Of greater concern to the aquaculture industry are ongoing problems of high mortality of postsettlement mussels in which an RNA virus has been implicated (New Zealand Mussel Industry Council 1995, Jones et al. 1996).

Suspended cultivation eliminates problems of such benthic predators as octopus; however, predation by the fishes, sea bream (*Pagrus auratus*) (Bloch and Schneider 1801), spotty wrasse (*Notolabrus celidodus*) (Bloch and Schneider 1801), and triggerfish (*Parika scaber*) (Bloch and Schneider 1801) can result in losses of mussel stock of up to 100% (Bartram 1985d, Jenkins 1985, Meredith-Young 1985d, Hickman 1991). Fish predation can be reduced by establishing farms in areas of deep water or by keeping seed mussels well above the seafloor and by reducing the amount of fouling on mussel farm moorings (Carbines 1993).

Recently the concerns over the possible environmental impact and long-term sustainability of expanding mussel aquaculture have prompted a number of scientific investigations. The deposition of particulate organic material beneath mussel farms has been found to be related to the 30% reduction in the velocity of water currents approaching the farm (Gibbs et al. 1991). In addition, feces and

pseudofeces falling from the cultivated mussels enrich fine sediments beneath the farm causing a change in benthic feeders, reducing benthic species diversity, as well as increasing biological respiration and microbial activity (Kaspar et al. 1985, Croft 1995a, Hawkins et al. 1999). Up to 5% of farmed mussels drop to the seafloor from the cultivation lines, and these form epibiotic reefs under the mussel farm (Kaspar et al. 1985, Grange and Cole 1997). The impacts on the benthos caused by mussel farming are very localized, being restricted to only a few meters beyond the perimeter of the mussel farm (Gillespie 1989), and there has been no evidence of significant ecological imbalance (Croft 1995a,b).

The sustainability of an area for mussel cultivation has been shown to be largely determined by the primary productivity of phytoplankton, which in turn is related to nutrient levels and rates of tidal flushing of an area (Gibbs 1991, Gibbs et al. 1992, James and Ross 1996, Proctor and Hadfield 1996, Hadfield and Sutton 1996, Sutton and Hadfield 1997, Ross et al. 1998a,b).

#### FUTURE DIRECTIONS

The aquaculture production of greenshell mussels in New Zealand is expected to continue to increase at a rapid rate, trebling in the next decade (New Zealand Mussel Industry Council 1998). Some serious constraints on the future expansion of this industry have emerged in recent years.

##### *Environmental Sustainability*

The rapid expansion of mussel farming in some areas has raised concerns about the potential effects on the environment, how environmental parameters affect mussel condition, and the productivity of existing neighboring mussel farms. The New Zealand Mussel Industry Council has taken a lead role in coordinating further research to address these concerns (New Zealand Mussel Industry Council 1997a, New Zealand Mussel Industry Council 1997b). Mussel farm impacts have generally been found to be mostly confined to the immediate vicinity of the farm itself (Gillespie 1989, Croft 1995a,b, Cole and Grange 1996). Waste material from mussel processing is generally disposed of in land-based facilities, although there is the potential to develop alternative uses for this material (Dias 1984, New Zealand Mussel Industry Council 1995). The recent recording and modeling of phytoplankton production and consumption in embayments containing mussel farms is leading to the development of the ability to predict carrying capacity of mussel farms (Hadfield and Sutton 1996, James and Ross 1996, Proctor and Hadfield 1996, Ross et al. 1998a,b). This will allow for more efficient production from the available growing space and reduce the immediate need for developing new farming areas that have caused public concerns (James and Ross 1997). The productivity models derived from this research can also be used to begin to gauge the effects on the wider ecosystem of the loss of primary productivity to mussel farms (Grange and Cole 1997).

##### *Seed Mussels*

The mussel aquaculture industry is entirely dependent on the collection of wild mussel seed, which is extremely variable in its quality, quantity, and timing of arrival. The most important source of seed mussels for aquaculture is from Ninety Mile Beach, a resource that is coming under increasing pressure (Fig. 4). Surprisingly, the locations of the parental wild mussel populations giving rise to this vital spat source are unknown and, therefore, remain unprotected (Bartram 1983b,c). Likewise, the breeding cycles of



wild mussels, larval behavior and transport, and the origin of the macroalgal settlement substrate are also entirely unknown for this area. The collection of mussel spat on artificial settlement material in other areas is also plagued by unpredictable and unreliable arrival and retention of spat (Hayden 1995). The inability to predict the location and timing of spatfall events is largely caused by the lack of understanding of the biological and physical factors that control the variability observed in mussel spat settlement and subsequent survival.

Alternatives to the collection of wild spat have been hampered by a lack of suitable hatchery techniques for artificially raising commercial quantities of mussel spat (Tortell 1976c, Hayden 1995, Redfearn 1998). Although it may yet become feasible to rear

commercial quantities of spat, it is unlikely that it will be cost competitive when compared to the relatively low cost of wild spat sources. Regardless, a reliable supply of consistent quality mussel seed will be essential to the industry's future.

#### ACKNOWLEDGMENTS

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## THE REPRODUCTIVE CYCLE OF *LIMNOPERNA FORTUNEI* (DUNKER, 1857) (MYTILIDAE) FROM A NEOTROPICAL TEMPERATE LOCALITY

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**ABSTRACT** *Limnoperna fortunei* is a dioecious, freshwater mytilid native to Asia. The reproductive biology of this invasive species is analyzed for the first time in its neotropical habitat. About 35 specimens were processed monthly using standard histological techniques (sections ~ 6 µm thick). The size of mussels at sexual maturity varied seasonally. From June to October, sex differentiated at 5–6 mm shell length (SL), and from March to May at 7–10 mm SL. Adult males contained mature sperm throughout the year. Mature oocytes measured 70–100 µm. Three main spawning events occurred in May to July 1993, April to May 1994, and October 1994. Two partial spawnings were observed in September to October 1992 and December 1992 to January 1993. No spawnings were recorded from October 1993 to February 1994 and from June to October 1994. Oocyte proliferation was continuous and immature oocytes were recorded throughout the year. The population appears to have continuous reproduction with peaks of maturing activity related to temperature changes.

**KEY WORDS:** reproductive biology, bivalve, invasive species, freshwater, South America

### INTRODUCTION

*Limnoperna fortunei* (Dunker 1857) is a freshwater species, native of rivers and creeks of China and southeastern Asia (Morton 1977). It was first recorded in the Americas in Bagliardi Beach (lat 34°55'S, long 57°49'W), in the argentinian littoral zone of the Río de la Plata, in September 1991 (Pastorino et al. 1993), at a density of 4 to 5 specimens/m<sup>2</sup>. During 1993, maximal densities of about 80,000 specimens/m<sup>2</sup> were recorded in this same locality (Darrigran 1995). Its current density reaches 150,000 specimens/m<sup>2</sup> (Darrigran et al. 1998b), similar to the densities of populations of the rocky shore marine mytilid *Brachidontes rodriguezii* d' Orb. found in Mar del Plata (lat 38°00'S, long 57°33'W) (Penchaszadeh 1973). *L. fortunei* invaded the Hong Kong area in the late 1960s (Morton 1977), and Japan in the 1970s (Kimura 1994). In the Americas, this species is found currently in the rivers Río de la Plata, Paraná, and Paraguay. It causes an important impact in the human environment (the principal problems caused by larval invasion, settlement, and maturity of *L. fortunei*, into water distribution systems, are: reduction of pipe diameter, blockage of the pipeline, decreased water velocity caused by friction, accumulation of empty shells, contamination of water pipelines by mass mortality and filters occlusion) and natural environment (affecting the taxocenosis of autochthonous molluscs, favoring the settlement of other macroinvertebrate fauna not common in the environment; Darrigran et al. 1998b).

Up to now, the reproductive cycle of *L. fortunei* is only known in subtropical habitats of Hong Kong (Morton 1982). The Hong Kong population of *L. fortunei* is dioecious and semelparous, and no cases of hermaphroditism have been reported (Morton 1982, Morton 1991). In this study, a description of the reproductive cycle of a Río de la Plata population of *L. fortunei* is presented.

### METHODS

Collections of *Limnoperna fortunei* from Bagliardi Beach, Río de la Plata, Argentina, were made from the rocky coast, during low

tides (Table 1). This locality (lat 34°55'S, long 57°49'W) is characterized by temperate environmental conditions. Monthly mean air temperatures range between 7.8 °C (July) and 24.1 °C (January) (Fig. 1), and water temperatures between 14.0 °C (May) and 24.0 °C (February) (Guerrero et al. 1997).

The shell length (SL) of 638 specimens was measured. Specimens were then fixed in Zenker's fluid with added formalin (10%

TABLE 1.

Dates and number of specimens histologically processed per sample.

Dates	Total n	Size range (cm)	Undifferentiated n	male n	female n
11/07/92	29	0.5–1.6	6	11	12
29/09/92	30	0.5–1.7	3	13	14
19/10/92	24	0.5–1.9	3	14	7
13/12/92	41	0.3–1.9	12	17	12
26/01/93	23	0.6–1.4	0	12	11
27/02/93	36	0.3–1.8	9	11	16
28/03/93	35	0.4–2.1	13	11	11
16/05/93	35	0.3–1.0	9	9	17
14/08/93	28	0.5–1.5	5	13	10
7/10/93	25	0.6–2.2	1	12	12
3/01/94	28	0.9–1.5	3	10	15
31/01/94	31	1.0–1.9	0	19	12
28/02/94	27	1.1–1.8	0	13	14
27/03/94	33	0.5–2.1	4	15	14
25/04/94	29	0.7–2.3	7	15	7
30/05/94	37	0.6–1.9	9	13	15
24/06/94	42	0.6–2.0	0	22	20
28/09/94	37	0.6–1.8	1	20	16
25/10/94	38	0.5–1.6	4	23	11
25/11/94	30	0.5–2.4	4	16	10
TOTAL	638	0.3–2.4	93	289	256
	%		14.57	45.29	40.12

## RESULTS

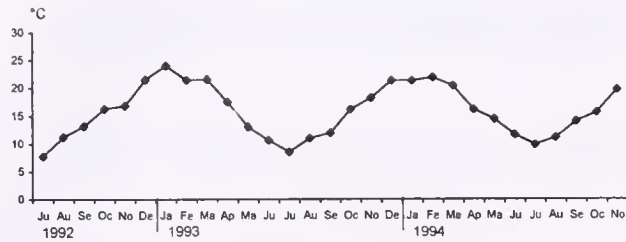


Figure 1. Temporal variation of air temperature.

in mixture volume) for 4 h, washed in tap water for 24 h, dehydrated 3 times in ethanol 96% (each wash of 2 h), and washed 4 times in N-butanol (each wash of 24 h). Finally, specimens were embedded in paraffin. Sections of ~ 6 µm thick were stained with Mayer's hematoxylin and eosin.

Oocytes with conspicuous nucleoli were measured (10 to 25 oocytes from each gonad, 7 to 16 females from each sample), including those found free in the follicular lumen, as well as those smaller ones attached to the wall.

The percentage of males with mature spermatozoa was calculated for each month's sample.

The percentage of follicular occupation of the mantle for each mussel was calculated (200×) in three different sections of the mantle (upper, middle and lower sections) by visual estimation of the fields of view. The periods of lysis and oocyte reabsorption were determined by microscopic analysis.

From a total of 638 specimens, 45% were males, 40% females, 15% undifferentiated. Figure 2 shows different morphological aspects that characterize the reproductive process of *L. fortunei*.

Female and male follicles differentiate at 5 mm (spring) to 9 mm (summer and fall).

The minimal size of sexual differentiation was found to be 5 mm SL, both for males and females, while gonadal maturity was attained at 6 mm SL, both in males and females. Size of sexual maturity varied through time.

Size-frequency histograms of oocytes showed (Fig. 3):

- Five spawning events occurring: September to October 1992, December 1992 to January 1993, May to July 1993, April to June 1994, and October to November 1994. The first two events were less apparent than the last three.
- No spawning events were recorded from October 1993 to February 1994, and from June to October 1994.
- Oocyte proliferation was continuous throughout the sampling period. But from December 1993 to March 1994, small oocytes (<30 µm) were less numerous or were absent.
- The total range of oocyte sizes was uniform in most of the samplings.

Figure 4a shows the temporal variation in the percentage of follicular occupation of the mantle for both sexes. There were periods of follicular repletion (June to August 1993; January to February 1993; December 1993 to January 1994), and periods of

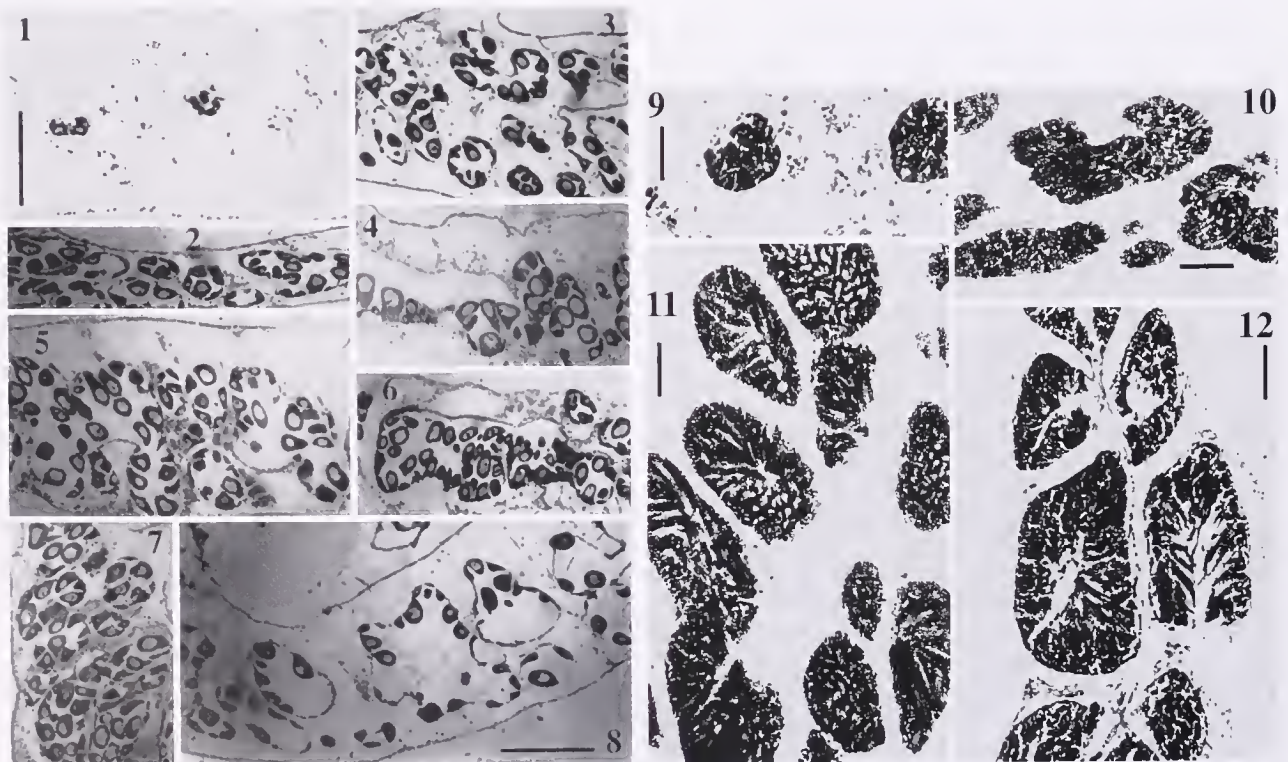


Figure 2. Light micrographs of the mantle of adult females (Fig. 2a) and adult males (Fig. 2b) of *Limnoperna fortunei*. Fig. 2a: (1) Early development of the ovary with small isolated follicles. (2 and 3) Follicles invading the space previously occupied by connective tissue, a stage of oocyte proliferation and growth. (4 and 5) enlarged oocytes with new growing ones. (6 and 7) Ripe ovaries ready to spawn. (8) Spawned ovary with remnant oocytes and few new growing cells. Fig. 2b: (9) early development of testicular follicles. (10) growing follicles invading the space previously occupied by connective tissue. (11 and 12) mature males; Scale: (1–8) scale bar 225 µm; (9) scale bar 100 µm; (10, 11, 12) scale bar 150 µm.

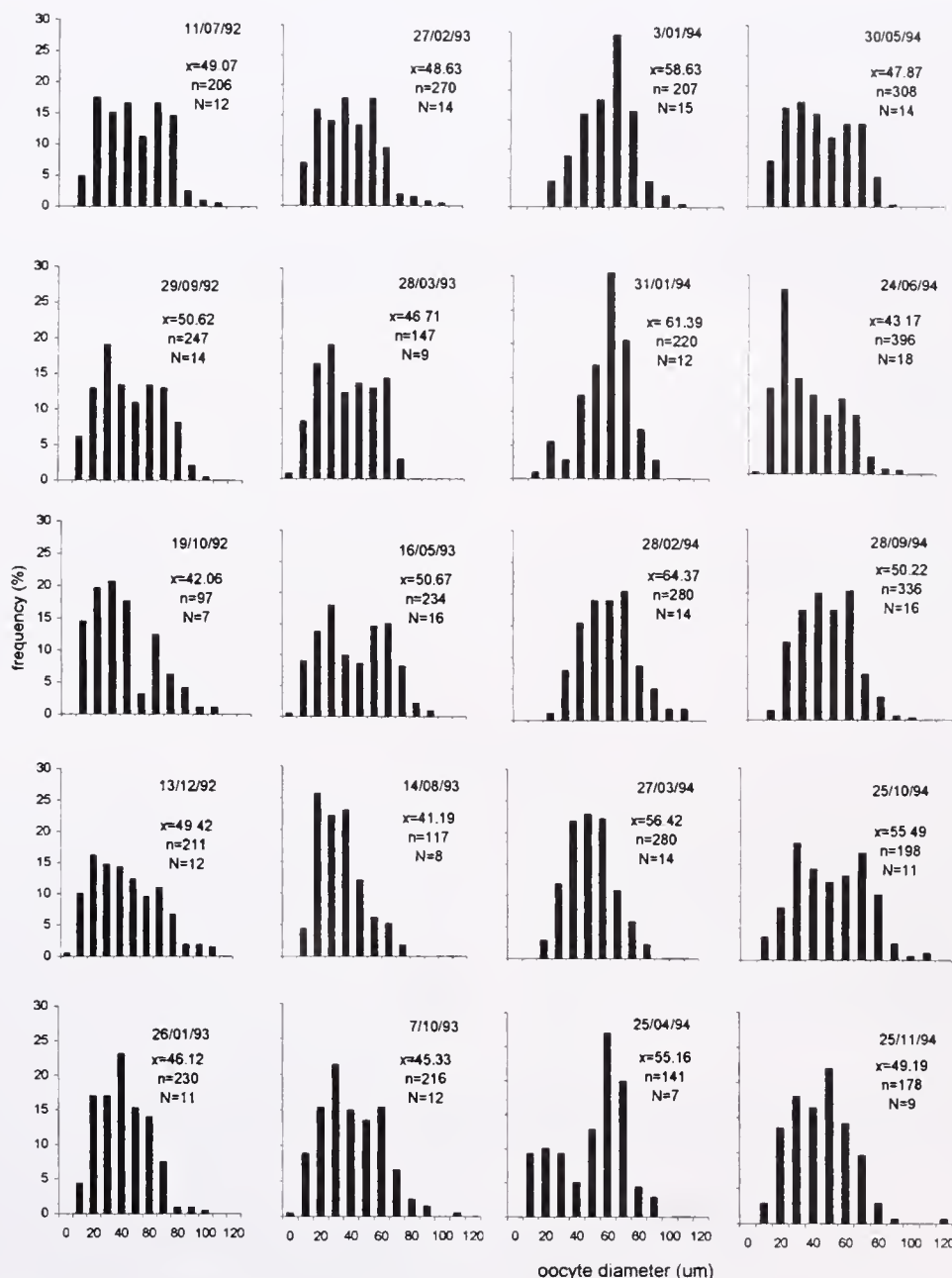


Figure 3. Frequency (%) of oocyte size (µm), x, mean oocyte size; n, number of oocytes; N, number of females.

maximal gonadal follicle occupation of the mantle (January 1993; May 1993; October to December 1993; November 1994).

The analysis of the percentage of immature oocytes showed three times of maximal proliferation (Fig. 4b): July to November 1992 with a maximum in October; March to November 1993 with a maximum in August; and April to July 1994 with a maximum in June.

Likewise, two outstanding periods were found when examining the immature oocytes: a continuous and high proliferation (December 1992 to May 1993), and a scarce oocyte proliferation (December 1993 to March 1994). Figure 4c shows the percentage of mature oocytes, showing 2 main evacuation processes and 4 secondary evacuations with a quick recovery.

The main evacuations occurred in May to July 1993 and April to June 1994 and the secondary evacuations in September to October 1992, December 1992 to January 1993, October to November 1994, and February to May 1994. No spawnings were recorded between August and December 1993.

August 1993 was mainly a proliferation period. Between October 1993 and May 1994, there was clear oocyte growth, and energetic losses in maturation or recovery of small oocytes did not occur (Fig. 4b and 4c).

Figure 4d shows a long period of sperm accumulation in the follicles that lasted from August 1993 to January 1994. Two main periods of evacuation and 3 secondary periods may also be observed.

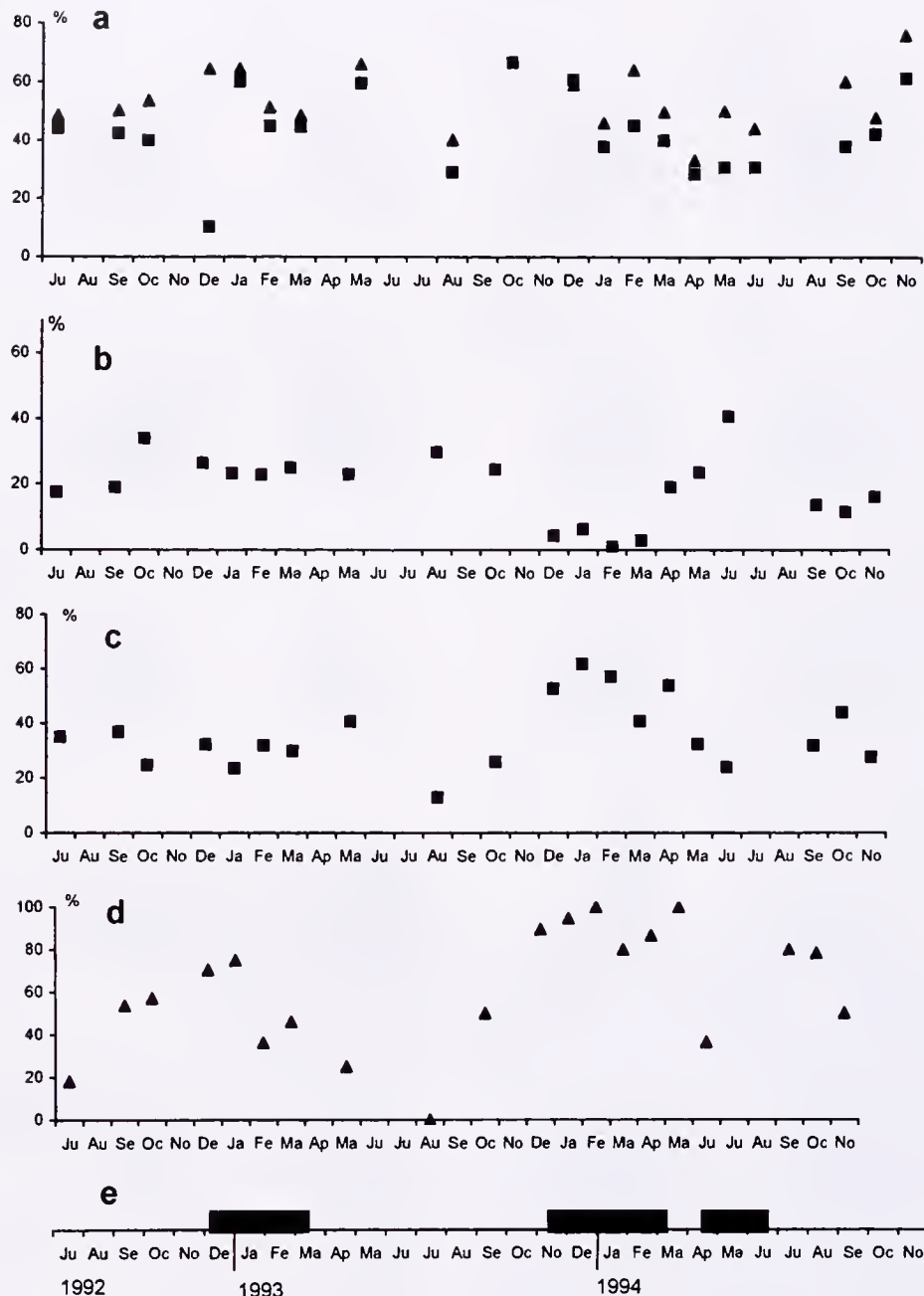


Figure 4. Temporal gonadal development. (a) percentage of occupation of the mantle in males (▲) and females (■). (b) percentage of oocytes smaller than 20  $\mu\text{m}$ . (c) percentage of oocytes larger than 60  $\mu\text{m}$ . (d) percentage of male follicles with sperm. (e) periods of oocyte lysis.

Lysis phenomena were recorded from December 1992 to March 1993, from November 1993 to March 1994, and from April to June 1994 (Fig. 4e).

#### DISCUSSION AND CONCLUSIONS

*Linnoperna fortunei* is a dioecious species. However hermaphrodite specimens occur frequently in other mytilid species (Lubet 1959), and unlike what Morton (1982) observed in Hong Kong, 0.55% of hermaphrodites (Darrigran et al. 1998a) were recorded.

Gametogenic cycles are generally ruled by external environmental factors that may trigger and synchronize the "timing" of the different stages (Lubet 1983). The synchronization of gonadal cycles of a population is probably also the result of some kind of exogenous regulation (Gallardo 1989).

The reproductive pattern of this population, examined during 1992 and early 1993, differs from the period recorded later. As *Linnoperna fortunei* is an invasive species introduced in 1991 in the Americas, and the study began in the middle of 1992, the observed reproductive pattern probably resulted from the lack of fitness and synchronization of the reproductive cycle of this population to the new environment.

Short evacuations of low intensity were recorded from September 1992 to January 1993 (3 events). These events were associated with a partially stable stage of the follicular occupation of the mantle. These short evacuations were preceded by a period of proliferation of oocytes smaller than 20  $\mu\text{m}$ . The proliferation was continuous and, in males, the amount of spermatozoa first increased and then decreased in January to February.

After February 1993, two main spawning events could be recognized in the reproductive process, in agreement with Morton (1982). The first event was May to July 1993, and the second April to May 1994. During May 1993, there was a high occupation of the mantle; and the abundance of oocytes larger than 60  $\mu\text{m}$  reached 40%. Sperm decreased sharply until no sperm was recorded in August 1993. From June to August 1993, oocytes smaller than 20  $\mu\text{m}$  increased and those of 60  $\mu\text{m}$  decreased. However, unlike what Morton (1982) recorded in Hong Kong, no clear periods of gonadal inactivity were recorded after either of the two evacuation processes.

After a long period of increasing oocyte size and scarce gonadal proliferation, two evacuation events were recorded: February to March 1994 and a larger one April to June 1994. During this latter event, there was an increase in oocytes smaller than 20  $\mu\text{m}$  coincident with a decrease in the sperm percentage. The percentage of occupation of the mantle shows a low coincidence with the first evacuation event (February to March), and a slight recovery during the following event. Thus, gametogenesis was continuous in the study population and major and minor spawning events occurred during the study period.

Iwasaki and Uryu (1998) found that *L. fortunei* from Kyoto reproduce only once a year, from June to September. Like the observations of Morton (1982) in Hong Kong, the larger spawn-

ings in the Río de la Plata develop during temperature changes, starting with both the maximums and the minimums.

The results of this study demonstrate a correlation between environmental temperatures and the reproductive cycle of *Limnoperna fortunei* in Bagliardi Beach. The processes of gametogenesis and spawning in bivalves are related to temperature changes (Lubet 1983). Temperature must be considered as the main factor responsible for the variations in the reproductive cycle (Kimura and Sekiguchi 1996). However, changes in water quality (pH, salinity, dissolved oxygen) may also affect this cycle. Consequently, multiple factors could trigger this biological process (Morton 1982).

The combination of early sexual maturity, high fecundity, semelparity, and wide environmental toleration permits this species to be transported and easily introduced into a new environment. Likewise, it rapidly colonizes the new environment, eventually becoming dominant (Morton 1989).

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## DISTRIBUTION, GENETIC STRUCTURE, AND MORPHOMETRY OF *MYTILUS EDULIS* AND *M. TROSSULUS* WITHIN A MIXED SPECIES ZONE

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**ABSTRACT** During 1994 to 1996, we sampled 25 wild mussel sites and 15 mussel farm sites distributed widely throughout coastal Newfoundland, Canada. Allele frequencies at four loci, *Mpi*, *Lap*, *Pgm*, and *Gpi* were determined, and several shell morphometric parameters were measured. Allelic variation at the *Mpi* locus was used as the discriminating criterion to distinguish between the two species, *Mytilus edulis* and *M. trossulus*. Both species are widely distributed throughout Newfoundland. Sites typically have mixtures of both species dominated by *M. edulis* but with *M. trossulus* ranging from a low of 0% to a high of 84% at individual sites. Commercial stocks on mussel farms tended to have significantly higher frequencies of *M. trossulus* as compared to wild mussel beds. Analysis of population structure using Wright's F statistics revealed mussel populations throughout Newfoundland are genetically highly differentiated. Sites in close proximity to each other were as genetically diverse and varied as much in species proportions as sites large distances apart without any apparent macrogeographic distributional pattern. Morphometrically, cultured *M. edulis* and *M. trossulus* differ significantly in shell width, depth, cavity volume, and shell weight (*M. edulis* > *M. trossulus* for all variables). Intraspecific variation in morphometric phenotype in both species was significantly related to variation in multilocus genotype. The impact of this morphometric variation on commercial mussel farm production in mixed *M. edulis/trossulus* areas is discussed.

**KEY WORDS:** allozymes, genetics; F statistics, morphometry, *Mytilus edulis*, *Mytilus trossulus*

### INTRODUCTION

Electrophoretic studies of allozymes in the marine mussel complex, *Mytilus* spp. (Koehn et al. 1984, Varvio et al. 1988, McDonald et al. 1991) have demonstrated the existence of two electrophoretically distinct species, *Mytilus edulis*, and *M. trossulus* in eastern North America. The allozyme-based separation of the two species has since been validated by nuclear and mtDNA marker techniques (Zouros et al. 1992, Heath et al. 1995). Allele frequencies at the *Mpi* locus were found particularly useful in separating individuals of the two species (McDonald and Koehn 1988, McDonald et al. 1991) and have been considered virtually diagnostic for *M. edulis* and *M. trossulus* in North American populations (Varvio et al. 1988).

Along the Atlantic coast of Canada, *Mytilus edulis* and *M. trossulus* are widely distributed, extending throughout the Gulf of St. Lawrence, the Quebec north shore, the Gaspé Peninsula, New Brunswick, Cape Breton Island south through Nova Scotia (Mallet and Carver 1992; McDonald et al. 1991). Bates and Innes (1995) reported both species to be widespread along a section of the northeastern coast of Newfoundland. In Atlantic Canada, *M. edulis* is typically the numerically dominant species (McDonald et al. 1991, Mallet and Carver 1992, Bates and Innes 1995), although some populations (e.g., in the Bras D'Or Lakes of Cape Breton Island and northern Gulf of St. Lawrence) are predominately *M. trossulus*. This distributional pattern overlaps the major growth centers of the developing Atlantic Canadian commercial mussel aquaculture industry.

*Mytilus* spp. in wild populations have long been recognized for their considerable morphological diversity (see Gosling 1992 for review). Using samples from wild populations representing the very extensive geographic range of the various species, McDonald et al. (1991) described the interspecific morphological relationships between allopatric populations of wild *M. edulis* and *M. trossulus*, and developed a discriminant function that accurately classified the two species based on a suite of morphometric and morphological characters. In Europe, interspecific morphological

differences between *M. edulis* and *M. galloprovincialis*, which are evident in allopatric populations, become eroded in areas where hybridization between the two species occurs (Gardner 1996). Whether similar morphological erosion occurs in areas of contact between *M. edulis* and *M. trossulus* has not been determined. However, because mixed species stocks of *M. edulis* and *M. trossulus* are the norm rather than the exception within the distributional area encompassed by the developing Canadian mussel culture industry, inter- and intraspecific relationships among commercially important morphological, morphometric, or physiologic parameters and stock genetics may have considerable commercial impact.

The distribution and allozyme frequencies of *Mytilus* spp. from the northeast coast of Newfoundland have previously been reported by Bates and Innes (1995). This paper extends knowledge concerning the distribution and population structure of *Mytilus* spp. to include the entire coast of Newfoundland, an area including nearly 10,000 km of coastline. Inter- and intraspecific relationships among morphometric and allelic variation are explored within several mixed species cultured stocks. Evidence is presented supporting the theory that morphometric variation in shell characters is significantly associated with genotypic variation in *Mytilus* spp.

### METHODS

#### *Electrophoresis*

During the period 1994 to 96, a total of 25 samples from wild mussel beds and 15 samples of rope-cultured mussels from mussel farms were collected. Wild samples were taken from beaches, with the exception of the samples from Shag Rocks, Comfort Cove, and Stock Cove, which were collected by diver at depths of 3-10 meters. Rope-cultured samples were collected by stripping commercial mussel socks directly on site or at the processing plant before grading. Samples were returned to the laboratory and held in tanks at ambient water temperatures until used. Individuals used for subsequent analysis ranged from 46-88 mm (mean = 65 mm)

in shell length. Hepatopancreas tissue was excised, lyophilized, and stored at 5 °C for later analysis. A small amount of freeze-dried material was chopped to a fine powder and ground with Tris HCL pH 8.0 buffer with 20% glycerol. Four polymorphic loci were investigated: mannose phosphate isomerase (*Mpi*, EC 5.3.1.8), aminopeptidase-I (*Lap*, EC 3.4.11.-), phosphoglucosylase (*Pgm*, EC 2.7.5.1), and glucose-6-phosphate isomerase (*Gpi*, EC 5.3.1.9). Cellulose acetate electrophoresis and staining were carried out according to procedures described by Hebert and Beaton (1989), with the single modification that *Lap* was run with Tris glycine pH 8.6 buffer. We attempted a fifth locus, esterase-D (EST, EC 3.1.1.1), but were unable to obtain consistent band patterns with freeze-dried tissue on the cellulose acetate system. Allele nomenclature is similar to that employed by previous authors (Koehn et al. 1984, McDonald and Koehn 1988).

### Statistical Analyses

Analysis of allele frequencies, Wright's F statistics with jack-knifed estimators (Weir and Cockerham 1984) and associated probability testing were carried out using Fstat version 1.2 (Goudet 1995). Heterozygote deficiencies ( $1-H_o/H_e$ ) and associated  $\chi^2$  significance testing were calculated using G-Stat, version 3.1 (Siegismund 1995) with genetic distance output files processed into UPGMA cluster dendrograms using PHYLIP, version 3.57c (Felsenstein 1995). A multilocus probabilistic estimation function, the Campden and Utter (CU) Index (Campden and Utter 1985) was used to consolidate multilocus allelic variation into a single linear vector for use in further data analyses. In general terms, the CU index uses multilocus allelic frequency data to estimate and compare the probability that an individual's multilocus genotype belongs to one or the other of two reference groups, given a set of allelic frequency data for both reference groups. Thus, all resulting CU scores range from 0 to 1, with individuals with multilocus allelic frequencies most similar to one or the other of the reference groups having calculated CU scores approaching 0 or 1, respectively, and individuals with intermediate allelic frequencies approaching 0.5. Campden and Utter (1985), Gardner (1996), and others have used these scores to infer the existence of hybrids among mixed species populations.

When applied to a set of multilocus frequency data for two species with allelic frequencies at one or more loci being diagnostic (mutually exclusive frequencies), the resulting CU scores become bimodal and nonoverlapping between species. Individuals of either species, but possessing alleles that are more typical of the opposite species at one or more loci, will have CU scores approaching 0.5. Individuals with CU scores within the range generated by the respective reference population may be considered "pure" individuals of that species (if the reference samples represent pure, allopatric populations); whereas, individuals outside that range and approaching 0.5 may be considered atypical of the reference population (species) and intermediate between it and the opposite species. Thus, composite genotypic allelic groupings may be created in a manner similar in concept to the derivation of compound genotypic allele classes by Skibinski (1983) for European mixed populations of *M. galloprovincialis*, *M. edulis*, and their hybrids.

A site on Prince Edward Island and another in Bras D'Or Lake were the geographically nearest known allopatric populations of *M. edulis* and *M. trossulus*, respectively (Mallet and Carver 1992). Selection of allopatric populations as reference standards for com-

putation of the CU index is consistent with similar recent usage on other *Mytilus* spp. populations (see Gardner 1996). We re-sampled these populations and obtained the same result, confirming Mallet and Carver's (1995) findings. The Prince Edward Island *M. edulis* and Bras D'Or Lake *M. trossulus* populations yielded CU scores > 0.63 and < 0.32, respectively. In the present analysis, allele frequencies derived from the re-sampling of these allopatric populations were used as reference standards to classify individuals in the Newfoundland population samples into four discrete composite allelic groups, denoted as E/E, ("pure" *M. edulis*, CU scores within the same range as the allopatric *M. edulis*; CU score > 0.63); E/T, (*M. edulis*, CU scores 0.5 < E/T < 0.63); T/E, (*M. trossulus*, CU scores 0.5 > T/E > 0.32); and T/T, ("pure" *M. trossulus*, CU scores within the same range as the allopatric *M. trossulus*; CU score < 0.32). Because no hybrid *Mpi* genotypes between the two species were observed, the two composite genotypic classes, E/T and T/E, contain individuals of *M. edulis* or *M. trossulus*, respectively, but with allelic frequencies at one or more of the *Lap*, *Pgm*, or *Gpi* loci, which are probabilistically, according to their CU scores, more typically found in individuals of the alternate species.

The derived CU index scores were used in two ways: (1) as a single linear continuous vector to consolidate multilocus allelic variation for use as a covariate in regression analyses; and (b) to construct composite-allele genotypic classes consolidating multilocus allelic variation for use in analysis of variance tests for relationships between multilocus allelic variation and morphometric variation among *Mytilus* spp. All associated statistical analyses were accomplished by SAS statistical procedures (SAS Institute, Inc., Cary, North Carolina 1985).

## RESULTS

### Species Identity

Classification of each individual to either taxon was inferred from its *Mpi* genotype in accordance with interspecific genotypic patterns previously reported for North American *Mytilus* spp. (McDonald and Koehn 1988, Varvio et al. 1988, McDonald et al. 1991). The genotypes  $Mpi^{100,100}$ ,  $Mp^{90,90}$ , or  $Mpi^{100,90}$  distinguish *M. edulis* from *M. trossulus*, which, in turn, is represented by genotypes  $Mpi^{94,94}$ ,  $Mpi^{84,84}$ , or  $Mpi^{94,84}$ . Comparative allele frequencies of both species at the remaining three partially diagnostic loci (Table 1) are consistent with those reported previously using other allozyme-based or multivariate statistical protocols (McDonald and Koehn 1988, Varvio et al. 1988; McDonald et al. 1991, Bates and Innes 1995). This consistency with previous published reports is taken as corroborative evidence of the validity of our *Mpi*-based classification of species at Newfoundland sites. Allele frequency distributions at the *Lap*, *Pgm*, and *Gpi* loci are significantly different ( $\chi^2$ ,  $P < .001$ ) but overlapping between the two mytilid species. With the possible exception of the very rare alleles  $Lap^{102}$ ,  $Gpi^{79}$ , and  $Gpi^{75}$ , which are too rare to be practically useful, no species-specific diagnostic alleles were found at these three loci. The alleles  $Lap^{98}$ , and  $Pgm^{100}$  are strongly associated with *M. edulis*; whereas,  $Lap^{94}$  and  $Pgm^{111}$  are associated with *M. trossulus*. No genotypic combinations between the four diagnostic *Mpi* alleles, which would have indicated the presence of  $F_1$  hybrids, were observed. However, several instances of dilocus combinations of  $Mpi^{100}$  with the partially diagnostic  $Pgm^{111}$  ( $n = 5.8\%$ ), or  $Mpi^{94}$  with  $Pgm^{100}$  ( $n = 7.7\%$ ) were noted. These frequencies are considerably greater than those observed in the two

TABLE 1.

Comparative allele frequencies of *Mytilus edulis* and *M. trossulus* in Newfoundland, using the *Mpi* locus (McDonald and Koehn 1988, Varvio et al. 1988; McDonald et al. 1991) as the distinguishing criterion, with  $\chi^2$  test for interspecific differences in frequency distributions (n = 2031).

Locus	Allele	<i>M. edulis</i>	<i>M. trossulus</i>	$\chi^2$
<i>Mpi</i>	100	0.915	—	
	94	—	0.976	
	90	0.085	—	
	84	—	0.024	
<i>Lap</i>	100 <sup>a</sup>	0.016	0.007	221.9** (df = 5)
	98	0.536	0.176	
	96	0.351	0.398	
	94	0.088	0.324	
	92	0.008	0.081	
	90 <sup>b</sup>	0.001	0.014	
<i>Pgm</i>	118	0.004	0.011	435.5** (df = 5)
	114	0.037	0.096	
	111	0.049	0.464	
	106	0.159	0.220	
	100	0.670	0.186	
<i>Gpi</i>	93 <sup>c</sup>	0.081	0.022	67.8** (df = 7)
	110	0.013	0.009	
	107	0.123	0.025	
	102	0.151	0.058	
	100	0.213	0.147	
	98	0.206	0.359	
	96	0.134	0.232	
	93	0.088	0.094	
	89	0.055	0.060	
86	0.012	0.011		
83 <sup>d</sup>	0.005	0.004		

<sup>a,b</sup> Includes rare *Lap*<sup>102</sup> and *Lap*<sup>88</sup> alleles, respectively.

<sup>c,d</sup> Includes rare *Pgm*<sup>89</sup>, *Gpi*<sup>79</sup>, and *Gpi*<sup>75</sup> alleles respectively.

\*\* Denotes  $P < .01$ .

Where necessary, frequencies of rare alleles were pooled to ensure statistical validity.

allopatric Prince Edward Island *M. edulis* and Bras D'Or Lake *M. trossulus* populations (3.0 and 1.9%, respectively).

#### Species Distribution

All populations, except at one site, Shag Rocks (site #27), were found to be mixtures of the two species (Fig.1). At Shag Rocks, only *M. edulis* was found. Their relative frequencies varied considerably between individual sites. *M. edulis* was, typically, the

dominant species in terms of relative abundance, but *M. trossulus* was found at relatively higher frequency at four sites, reaching its greatest relative frequency (84%) at Prince Edward Bay (site #2). Sites with high proportions of *M. trossulus* were widely dispersed, often in close proximity to other sites with high proportions of *M. edulis*. However, relatively low numbers of *M. trossulus* were found all along the southwest and west coasts (site # 1, 31, 34–39). This area is also the least heavily indented section (perhaps coincidentally) of the island's coastline, with a high proportion of sites relatively unsheltered from macroscale wind and wave action. Samples from cultured populations had higher relative frequencies of *M. trossulus* as compared to wild beds ( $R \times C \chi^2$  test of association,  $P < .01$ ). As a group, the mean relative proportion of *M. trossulus* in cultured samples was 26% versus 14% at wild beds.

#### Genetic Structure

Within and among site variation in genetic structure was explored using Wright's F statistics and  $\chi^2$  analysis. Significant heterozygote deficiencies ( $\chi^2$ ,  $P < .05$ ) at individual loci were found to be common in both species. For both *M. edulis* and *M. trossulus*, heterozygote deficiencies ( $1-H_e/H_o$ ) occurred at one or more loci at more than 70% of all sites. Analysis of within site and intersite allelic variation using jack-knifed estimation of Wright's F statistics (Weir and Cockerham 1984) revealed significant  $F_{IS}$ ,  $F_{IT}$ , and  $F_{ST}$  values for most species  $\times$  locus combinations ( $P < .01$ ) (Table 2). Estimators of the means pooled over all four loci were also significant for both species, indicating the existence of significant intraspecific genetic heterogeneity among sample sites. For *M. trossulus*, significant intersite genetic heterogeneity was evident at only the *Pgm* locus.

To explore the cause or causes of this heterogeneity, we calculated matrices of pairwise  $F_{ST}$  values for each site  $\times$  site combination separately for both species. We then determined whether these were associated with between-site species structure, geographic distance, and sample type (e.g., cultured or wild) differences. For *M. edulis*, pairwise  $F_{ST}$  values were significantly correlated with the magnitude of intersite difference in species frequencies (analysis of variance [ANOVA,  $P < .0001$ ]). This indicates intersite variation in genetic structure within *M. edulis* is significantly associated with intersite variation in the presence of *M. trossulus*. However, the reverse was not true for intersite genetic variation in *M. trossulus* (ANOVA,  $P > .05$ ). Intersite variation in genetic structure was graphically illustrated by clustering dendrograms (UPGMA method) using Nei's genetic distance matrices (Fig. 2). For *M. edulis* (Fig. 2a), the first three clusters from the root include five sites with relatively high *M. trossulus* frequencies

TABLE 2.

Summary of Wright's F statistics for individual loci and jack-knifed estimators of the means over all loci for *M. edulis* and *M. trossulus*.

Locus	<i>M. edulis</i>			<i>M. trossulus</i>		
	$F_{IS}$	$F_{IT}$	$F_{ST}$	$F_{IS}$	$F_{IT}$	$F_{ST}$
<i>Mpi</i>	0.180**	0.196**	0.019**	0.170**	0.164	-0.008
<i>Lap</i>	0.081**	0.085**	0.004	0.166**	0.180**	0.017
<i>Pgm</i>	0.145**	0.167**	0.026**	0.115**	0.137**	0.026**
<i>Gpi</i>	0.138**	0.145**	0.008**	0.203**	0.217**	0.017
Mean	0.124**	0.134**	0.011**	0.165**	0.181**	0.019**

\*\*  $P < .01$ , Bonferroni adjusted.

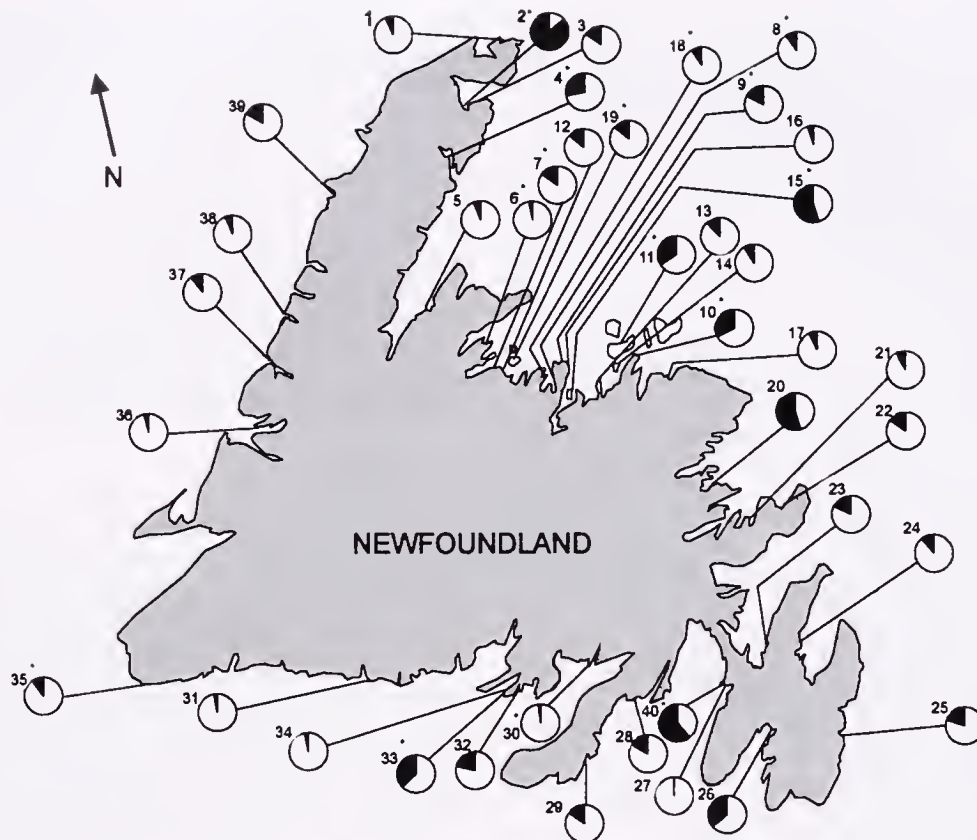


Figure 1. Proportions of *Mytilus edulis* (white) and *M. trossulus* (black) in both wild and cultured populations in Newfoundland. Cultured populations are denoted by an asterisk.

(sites 2, 4, 15, 33, 40). However, no identifiable clustering patterns are found for *M. trossulus* (Fig. 2b). This pattern of relationship between genetic distance and occurrence of the alternate species is consistent with the expectation based on the analysis of variance test results above. Geographic distance between sites was not a significant factor in explaining the variation in pairwise  $F_{st}$  values for either species (ANOVA,  $P > .05$ ). Likewise, sample type (wild or cultured) was not a significant contributing factor to variation in intersite  $F_{st}$  values for either species (ANOVA,  $P > .05$ ).

#### Genetic Identity and Distribution of Composite Genotypic Classes

Differences in allele frequencies among the four composite genotypic classes are summarized in Figure 3. E/E composite genotype mussels had high loadings of  $Mpi^{100}$ ,  $Pgm^{100}$ ,  $Lap^{98}$ , and to a lesser degree,  $Gpi^{102}$  and  $Gpi^{107}$ . The conspecific E/T genotype mussels had relatively higher loadings of  $Mpi^{90}$ ,  $Pgm^{111}$ ,  $Pgm^{114}$ ,  $Lap^{94}$ ,  $Lap^{92}$ , and  $Gpi^{98}$ . T/T composite genotype mussels had high loadings on  $Mpi^{94}$ ,  $Pgm^{111}$ ,  $Lap^{96}$ ,  $Lap^{94}$ ,  $Gpi^{98}$ ,  $Gpi^{96}$  as compared to its conspecific T/E genotype mussels, which had high loadings of  $Mpi^{84}$ ,  $Pgm^{100}$ ,  $Lap^{98}$ ,  $Gpi^{100}$ ,  $Gpi^{102}$ , and  $Gpi^{107}$ . When allele frequencies at individual loci for E/T and T/E composite genotype individuals are compared to their conspecific E/E and T/T genotypes, the allele frequencies for both species at all loci are significantly different ( $\chi^2$ ,  $P < .001$ ), with the exception of the  $Mpi$  locus for *M. trossulus* (Fig. 3). The allele frequency loadings at  $Lap$ ,  $Pgm$ , and  $Gpi$  in composite genotypes E/T and T/E mussels demonstrate the intermediacy of the multilocus allele frequencies in these individuals between those of "pure" individuals of either species.

Individuals with composite genotypes E/T and T/E were distributed widely throughout Newfoundland. E/T genotype mussels were found at 26 out of 40 sampled locations, ranging in frequency from 3–22% of the *M. edulis* population. T/E genotype mussels occurred at 35 of 40 sites, ranging from 9–85% of the *M. trossulus* population. Proportionally, E/T genotypes were present, on average, much less frequently than T/E genotypes (mean = 7% versus 32% over-all populations combined;  $P < .01$ ). Intrinsically, the relative frequencies of E/T and T/E genotypes varied with sample type. Wild samples of *M. trossulus* contained T/E genotype mussels at significantly higher frequencies than did cultured samples ( $\chi^2$ ,  $P < .001$ ; 53% vs. 18%). Conversely, *M. edulis* E/T genotype mussels were more abundant in cultured populations (10% vs. 4%) than in wild populations, but the difference in frequencies was not significant ( $\chi^2$ ,  $P > .05$ ).

#### Morphometric Relationships in Cultured Mussels

Relationships among shell width, shell depth, cavity volume, and shell weight, with shell length and genotype were determined by analysis of covariance (ANCOVA) analyses. Individuals of both species were pooled over all sites for these analyses. Using shell length as a covariate, significant relationships were found with CU score for shell width, shell depth, and shell weight (Table 3). Using composite genotype classes to group CU scores, ANCOVAs were done to compute least-square means (LSMs) of shell width, shell depth, cavity volume, and shell weight for each of the four genotype classes (Table 4). For all four morphometric characters, LSMs were greater in *M. edulis* than in *M. trossulus*. The

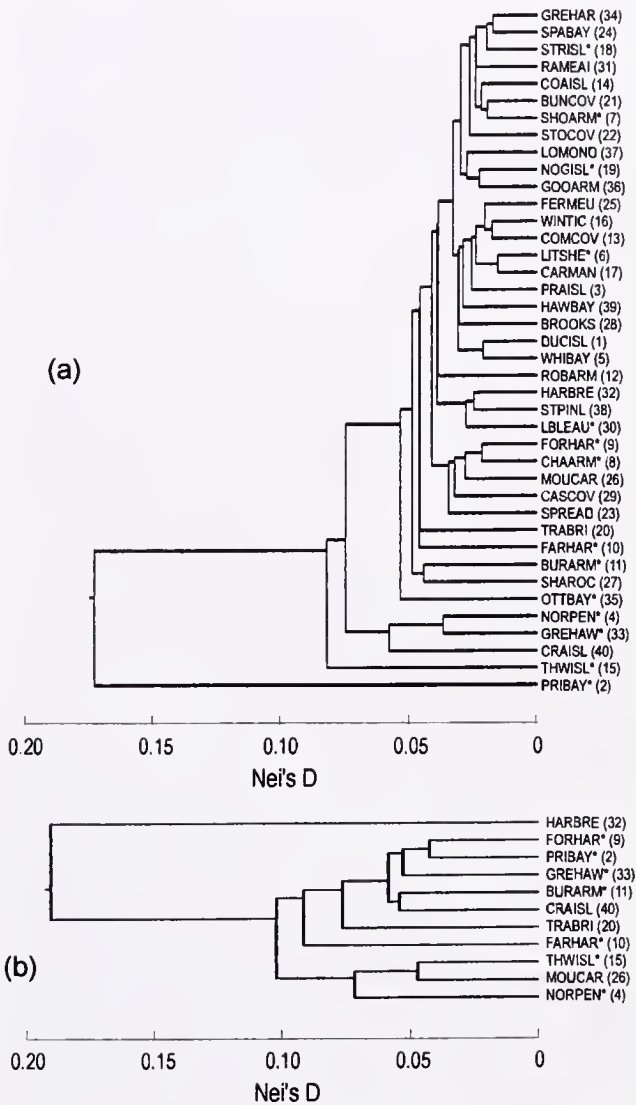


Figure 2. UPGMA clustering dendrograms of the 40 sample sites in Newfoundland for (a) *M. edulis*, and (b) *M. trossulus*. Cultured sample sites are denoted by an asterisk. Numbers in brackets refer to site numbers in Figure 2.

composite genotype classes, E/T and T/E, also had LSMs for shell width and shell weight intermediate between those of the other two genotype classes.

Summarized for a typical harvest size, cultured mussel (55–65 mm shell length), the differences in shell width and weight translate into approximately 8–9% wider shell aspect and a 25–27% heavier shell weight for E/E genotype *M. edulis* as compared to T/T genotype *M. trossulus*. E/T genotype *M. edulis* mussels were approximately 3–4% narrower in shell aspect and 12–14% lighter in shell weight than a comparable E/E genotype *M. edulis*. Conversely, T/E genotype *M. trossulus* mussels were about 10% wider in shell aspect and had about 11–12% heavier shells than comparable T/T genotype *M. trossulus* mussels. LSMs for shell depth and cavity volume were not intraspecifically significantly different between genotypes, although the interspecific differences in LSMs between the two species were significant.

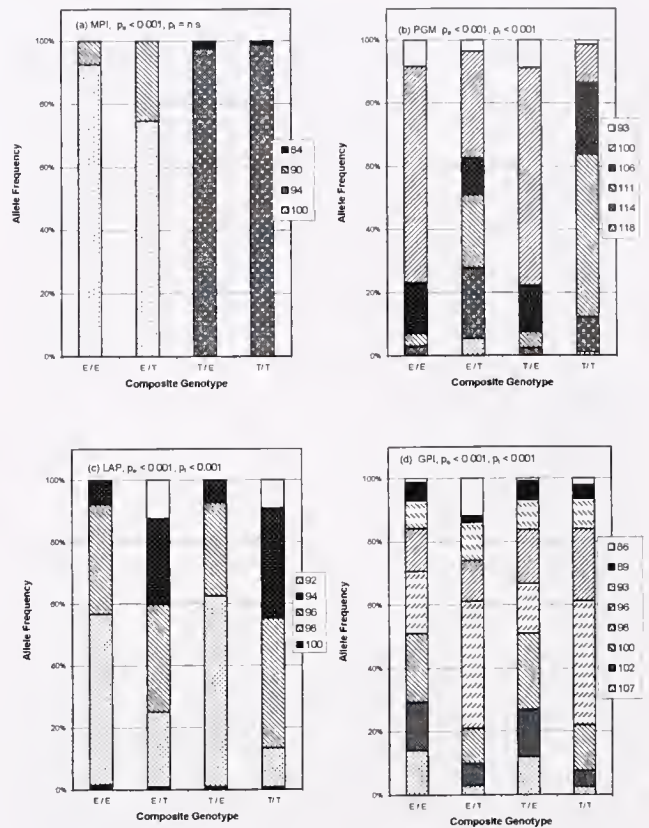


Figure 3. Comparison of allele frequencies among the four composite genotype classes at each of four loci (a) *Mpi*, (b) *Pgm*, (c) *Lap*, and (d) *Gpi*. Vertical bars denote individual alleles numbered as per the side legend. Alleles at very low frequency are pooled with the nearest adjacent allele at each locus. Probability values,  $P_e$  and  $P_t$ , are for  $\chi^2$  tests for intraspecific differences between allele frequencies ( $P_e = E/E$  vs.  $E/T$ ;  $P_t = T/T$  vs.  $T/E$ ).

DISCUSSION

The present study uses allozyme variation at the *Mpi* locus as the distinguishing criterion to separate individuals of *M. edulis* from *M. trossulus* in Newfoundland. This technique results in reported allele frequencies at the *Pgm*, *Lap*, and *Gpi* loci consistent with those determined previously for the two species using other allozyme protocols (McDonald et al. 1991, Bates and Innes 1995). Before the present work, Koehn et al. (1984) and Bates and Innes (1995) reported the presence of *M. trossulus* from several sites on the northeast coast of Newfoundland. Bates and Innes (1995) used a statistical approach based on observed allelic variation at the *Est* and *Pgm* loci to distinguish between species. Our study has confirmed the presence of both species in Newfoundland using the diagnostic *Mpi* locus (Varvio et al. 1988). We extended both species' respective known ranges to include the entire coast of Newfoundland, an area in excess of 10,000 km of coastline. All but one site contained mixtures of both species with *M. edulis* typically predominating in relative frequency, a finding consistent with Bates and Innes (1995) study of the northeast coast area. The seemingly haphazard nature of the two species intermingling throughout the island lends weight to the idea the overall population structure of *Mytilus* spp. in Newfoundland may be termed a species "mosaic."

TABLE 3.

Statistical results of the covariance analyses on shell morphometrics.

Model (R <sup>2</sup> )	Mean Square	Prob. >F
Shell width (0.73)		
CU score	102.2	0.0001
Shell length	2457.1	0.0001
CU score × shell length	16.3	n.s.
Shell depth (0.66)		
CU score	40.1	0.01
Shell length	1696.0	0.0001
CU score × shell length	47.5	0.01
Log cavity volume (0.88)		
CU score	0.1	n.s.
Shell length	21.3	0.0001
CU score × log cavity volume	0.1	n.s.
Log shell weight (0.59)		
CU score	1.73	0.005
Shell length	45.8	0.0001
CU score × shell length	1.1	0.01

Within each species, analyses of Wright's F statistics found significant intersite geographic heterogeneity in species' genetic structure. This heterogeneity can best be described as 'patchy,' because no identifiable macrogeographic pattern was evident. However, site × site pairwise  $F_{st}$  values for *M. edulis* were greater between sites with the largest differences in relative proportion of *M. trossulus*. Sites in close proximity to each other displayed genetic variation on a scale comparable to sites large distances apart. Heterozygote deficiencies at one or more loci were common. Raymond (1997) proposed that the significant heterozygote deficiencies observed in mussel populations in such areas as Newfoundland, where two species co-exist sympatrically, may be explained simply by the Wahlund effect. However, the contribution of other such factors, as genotype-dependent variation in survival related to environmental or other ecological or physiological factors cannot be ruled out. Such environmental influences on population allele frequencies at the *Lap* locus, in particular, have been well documented in *Mytilus* spp. (Koehn et al. 1980, Hilbish and Koehn 1984, Gardner and Palmer 1998). In sympatric European populations of *M. edulis* and *M. galloprovincialis*, genotype-dependent mortality patterns are known to occur (Gardner and Skibinski

TABLE 4.

Least-square means (LSMs) of composite genotype classes for shell morphometric characters in *M. edulis* and *M. trossulus*.

Genotype	Shell Width	Shell Depth	Cavity Volume	Shell Weight
<i>M. edulis</i>				
E/E	31.55 <sup>a</sup>	23.25 <sup>a</sup>	2.73 <sup>a</sup>	1.95 <sup>a</sup>
E/T	30.59 <sup>b</sup>	23.70 <sup>a</sup>	2.75 <sup>a</sup>	1.86 <sup>b</sup>
<i>M. trossulus</i>				
T/E	30.55 <sup>b</sup>	22.46 <sup>b</sup>	2.65 <sup>b</sup>	1.85 <sup>b</sup>
T/T	27.85 <sup>c</sup>	22.48 <sup>b</sup>	2.64 <sup>b</sup>	1.66 <sup>c</sup>

Values with different column superscripts are significant at  $P < .05$ . Values for cavity and shell weight are log transformed.

1991, Gardner et al., 1993, Wilhelm and Hilbish 1998). Further studies on this aspect in sympatric *M. edulis* and *M. trossulus* populations are in progress in Newfoundland.

We have determined that the shell morphometry of cultured *M. edulis* differs significantly from that of *M. trossulus*. At harvest size, *M. edulis* are larger in shell width, depth, cavity volume, and weight as compared to *M. trossulus*. Furthermore, we have shown that intraspecific variation in shell morphometry was not random. Intraspecific morphometric variation was significantly associated with variation in multilocus allele frequencies, represented by CU index scores and related composite genotype classes. Morphometric phenotype (shell width and weight) of individuals with intermediate composite genotypes (E/T and T/E genotypes) was also intermediate between those with composite E/E and T/T genotypes. This is consistent with previously reported significant relationships between commercially relevant production characteristics and variation at various enzyme loci in other bivalve species (Gaffney and Scott 1984, Diehl and Koehn 1985, Krause and Bricelj 1995).

Instances of erosion in interspecific phenotypic differences, comparable to that found in our work, have been noted in wild populations of *M. galloprovincialis*, where they overlap distributionally and hybridize readily with either of *M. edulis* (Gardner 1996) or *M. trossulus* (Sarver and Foltz 1993). However, such linkages between morphometric phenotype and genotype have not previously been reported between *M. edulis* and *M. trossulus*. In the case of Sarver and Foltz (1993) and Gardner (1996), the pattern of erosion of interspecific differences in morphometry were interpreted as evidence of introgressive hybridization between *M. galloprovincialis* and *M. trossulus* or *M. edulis*, respectively. Gardner (1996) theorized that *M. edulis* and *M. galloprovincialis* maintain their genetic identities in areas of geographic overlap, despite high dispersal potential, widespread hybridization, and high levels of introgression, as a result of adaptation to different environments. Localized genotype-dependent adaptation of indigenous mussel stocks to environmental conditions may be a contributory cause of the extensive intersite variation in  $F_{st}$  values found among the Newfoundland sites.

The observed pattern of genotype-linked erosion in interspecific phenotypic characters (shell morphometry) between sympatric *M. edulis* and *M. trossulus* in Newfoundland is comparable to that reported by Sarver and Foltz (1993) and Gardner (1996) for *M. galloprovincialis* and its congeners. In England, the extensive hybridization between sympatric *M. edulis* and *M. galloprovincialis* has resulted in a continuum of individuals that are most *M. edulis*-like to individuals that are most *M. galloprovincialis*-like in genetic structure (Skibinski 1983, Gardner and Skibinski, 1988). These hybridized populations also exhibit genotype-dependent breakdown of species-specific morphometric differences that are evident between allopatric populations of the two species (Gardner 1996). Mallet and Carver (1992) reported hybrid *Mpi* genotypes between *M. edulis* and *M. trossulus* at frequencies ranging from 2.5 to 19.5% in Nova Scotia and New Brunswick. However, we found no *Mpi* genotypes expected of  $F_1$  hybrids in Newfoundland; nor did Bates and Innes (1995). Therefore, our data do not support the conclusion the linkage we observed between shell morphometry and genotype in mixed *edulis/trossulus* populations in Newfoundland results from hybridization.

However, the possibility individuals with E/T and T/E composite genotypes could be backcross hybrids of mixed ancestry

cannot be ruled out. This possibility is worth noting, because we know from DNA marker research that true interspecific hybrids do exist in Newfoundland (Comesaña et al. 1999), although  $F_1$  hybrids are apparently quite rare. From samples taken at two sites in Trinity Bay, Newfoundland, Comesaña, et al. (1999) noted < 2% of all individuals present could be classified as  $F_1$  hybrids either by allozymes alone or in combination with nuclear DNA markers. Backcrossed hybrids were much more abundant (26% overall) but concentrated in smaller size classes < 35 mm in shell length (80% of all backcross hybrids). This implies that *edulis/trossulus* hybrids, particularly  $F_1$  individuals, may suffer relatively high mortality rates as compared to nonhybrids. If this were indeed the case, it offers an explanation as to how the geographically widespread E/T and T/E genotype individuals could be back-cross hybrids of mixed ancestry, although  $F_1$  *Mpi* genotypes were not found. It also may explain the apparent disparity concerning the occurrence of hybrids detected by Comesaña et al. (1999) with DNA markers and that of allozyme-based studies (this study, Koehn et al. 1984, Bates and Innes 1995), because these allozyme studies also used adult specimens. Further research on this aspect is clearly required to confirm the correct taxonomic identity of E/T and T/E genotype mussels from Newfoundland and to define the basis for the observed genotype-dependent erosion of interspecific morphometric characters. Such research may also elucidate whether the considerable intersite genetic heterogeneity observed in Newfoundland mussel populations is indicative of environmentally induced localized adaptive responses or simply to intersite variability in rates of interspecific hybridization.

In Nova Scotia, mussel farms using indigenous *M. trossulus* stocks are considered to be at some economic disadvantage as compared to others using *M. edulis* stocks (Mallet and Carver 1995). Using cultured mussel data from a site in Lunenburg Bay in Nova Scotia, Mallet and Carver (1995) estimated, based on comparative weight and survival data, and commercial grading trials during product processing, that the over-all economic value of *M. edulis* was 1.7 times higher than *M. trossulus*. However, whether these findings are valid for other areas outside Mallet and Carver's study area remain to be convincingly demonstrated. Because most cultured stocks are likely to be mixtures of the two taxa, what proportion of *M. trossulus* within a farm stock is commercially significant in terms of any realized production or marketing disadvantage becomes of critical importance. Moreover, the economic implications of *M. trossulus* culture reported by Mallet and Carver (1995) need to be re-assessed to account for the influence of culturing stocks with intermediate morphometric characteristics due to presence of E/T and T/E genotype mussels.

Differences in shell shape are of commercial concern to farmers because shape differences indirectly influence buyer perception of over-all product quality. Such perceptions of quality differences

may limit a farmer's ability to market product successfully in a competitive industry. Further research needs to be done to determine whether observed shell shape and cavity volume differences among mussels of different genotypes translate into differences in meat weight. Differences in weight have a more direct impact on farm profitability, because they directly affect economic returns from production. The difference in shell weight between E/E genotype *M. edulis* and T/T genotype *M. trossulus* is commercially highly relevant, on the order of 25% loss of weight for *M. trossulus*. Other genotypes produce intermediate values of shell weight. Because intermediate genotypic mussels are both widespread and, at some sites, locally abundant, this has obvious important implications for commercial industry.

The findings of the present work suggest that cultured stock attributes of shape and production weight may be substantially improved at some sites by substitution of the indigenous stocks with another derived from an external source with higher over-all proportions of E/E genotype *M. edulis*. However, in recognition of the possibility that indigenous stocks may be better adapted to local environmental conditions, such stock substitutions should be approached on a pilot scale initially and carefully monitored. In consideration of the widespread occurrence of E/T and T/E genotypes throughout Newfoundland, potential substitution candidate stocks should be carefully screened genetically. The best alternative may be to import stock from outside the Newfoundland area. Such work should be a priority for further applied research efforts.

Another approach to improve stock performance may be spat grading. Coincidentally or not, the Prince Edward Island stock used in the present work as the reference E/E genotype *M. edulis* population was a stock subjected to grading at the spat stage before commercial stocking. Spat grading has not normally been applied on mussel farms in Newfoundland. The possibility that interspecific differences in shell shape found here for market-size mussels also extends to spat-size individuals (~10 mm) should be determined. If it does, then the possibility for rigorous spat grading to decrease the frequency of T/T and T/E genotype *M. trossulus* and E/T genotype *M. edulis* among cultured stocks may exist. Further work on this subject and the related question of whether such stocks also exhibit genotype-dependent growth and survival are in progress.

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## SALINITY AND SEDIMENT-MEDIATED BYSSAL THREAD PRODUCTION BY *MYTILUS EDULIS* LINNAEUS AND *GEUKENSIA DEMISSA* DILLWYN FROM NEW JERSEY SALT MARSHES

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**ABSTRACT** Sediment grain size and salinity influenced byssal thread production by epibyssate *Mytilus edulis* and endobyssate *Geukensia demissa* in week-long experiments in closed aquaria. Experimental substrata included sieved sediment ranging from fine sand (0.13 mm) to gravel (3 mm) in seawater with a salinity of 30 ppt. Experimental salinities over a constant, coarse-grained substratum ranged from 15 to 45 ppt in increments of 5 ppt. Mean byssal thread production by the much larger *Geukensia demissa* significantly (ANOVA) exceeds that of *Mytilus edulis* on fine gravel over the entire experimental range of salinities. However, mass-normalized mean thread production for *M. edulis* exceeds that of *G. demissa* at the optimum salinity (30 ppt.) water, even though the ribbed mussels experienced a slightly higher mean temperature during the experiments (23.5 vs. 21.5 °C). This disparity in thread production in favor of the blue mussel reflects its adaptation for secure attachment against acceleration forces on its shell surface area exposed in *Spartina*-free low intertidal habitats. Acceleration forces against shells of endobyssate adult ribbed mussels are reduced by minimum exposure of the valve posterior, coupled with current baffling by surrounding grasses. Mean thread production at progressively lower salinities decreases at a greater rate for *M. edulis* than for *G. demissa*. This distinction reflects increased sensitivity to osmotic stress by the lower intertidal blue mussels in comparison to ribbed mussels acclimatized to perched tide pools wherein occur seasonal dilutions through snow melt and elevation through summertime evaporation. Mass-normalized and unnormalized mean thread production by *M. edulis* increases substantially from medium sand to gravel at 30 ppt at 15–16 °C, a contrast with insignificant changes by *G. demissa* over the same range of substrata in water 17–20 °C. Substratum texture may have more influence on thread production by *M. edulis* than *G. demissa* possibly because critical masses of thread-bound, agglomerated sediment grains provide the anchor for epibyssate blue mussel against dislodgment. Ribbed mussels mostly buried in cohesive mud rarely have to cope with erosional forces destabilizing their substrata.

**KEY WORDS:** *Mytilus edulis*, *Geukensia demissa*, byssus, salinity, substrata

### INTRODUCTION

Byssal thread production by the blue mussel *Mytilus edulis* Linnaeus and ribbed mussel *Geukensia demissa* Dillwyn are affected by several abiotic factors. Thread production by *M. edulis* decreases above or below optimum temperature (Allen et al. 1976, Glaus 1968) and salinity (Glaus 1968, Van Winkle 1970, Allen et al. 1976). Water velocity (Maheo 1970, Van Winkle 1970, Price 1981), agitation (Young 1985), and oxygen tension (Widdows and Bayne 1971), in addition to circadian and tidal rhythms (Martella 1974), significant affect secretion of byssal threads. Thread production by *M. edulis* is also influenced by sediment grain size, increasing from very fine sand to gravel (Meadows and Shand 1989). However, documented influences on thread production by *G. demissa* (Van Winkle 1970, Shumway et al. 1987) do not evaluate substrata.

Blue and ribbed mussels occupy overlapping distributions in New Jersey salt marshes, where two of the above-mentioned abiotic variables, salinity and substrata, vary appreciably from peaty, gravelly embankments abutting tidal flats to microbial mat-carpeted, perched tide pools in the high salt marsh along the southern New Jersey inlets and bays. Salinities near the blue mussel-veneered embankment bathed by open ocean waters is uniformly 33 ppt throughout the year, but may reach 45 ppt during the summer in evaporative, perched tide pools in the high salt marsh inhabited exclusively by *G. demissa* (Alexander, pers. comm.). In the winter, occasional ice melt temporarily dilutes salinities to 25 ppt in perched tide pools in the high marsh (McAloon and Browne 1998). Salinity may vary by as much as eight ppt during one tidal cycle in evaporated high marsh tide pools in late summer, while

concurrently lower salt marsh tide pools, which are disconnected from the open ocean for a short portion of the tidal cycle, fluctuate by less than 3 ppt. The extent to which this naturally occurring magnitude of salinity fluctuations may differentiate byssal thread production between these two mussels is central to this investigation.

Epibyssate blue mussels and endobyssate ribbed mussels (Fig. 1) are commonly attached to a wide variety of substrata that includes conspecific shells, valves of other clams, quartz pebbles and coarse sand, stranded drift wood, roots of the marsh grass *Spartina alterniflora*, and fucoid algal holdfasts. Byssal attachment of *G. demissa* to substrata need not be as secure as that for *M. edulis* according to Stanley (1970, Stanley 1972) because adult ribbed mussels (Fig. 1) are often semi-encased in cohesive muddy sediments that require higher current velocities to erode than sand (Hjulstrom 1939). Acceleration forces against their minimally protruding shell posterior, which intercepts little of the flow, may be reduced further by flanking stalks of *Spartina alterniflora* that baffle the current (Smith and Frey 1985). In contrast, *M. edulis* (Fig. 1) has the entire surface of one valve, or cross-sectional area of both valves, exposed to acceleration forces of waves and currents (Bell and Gosline 1997, Denny 1995), necessitating a secure byssal attachment against dislodgment (Stanley 1970), particularly at the *Spartina*-free embankments, where wave shock is greatest. Accordingly, substrata composition, texture, and mass are predicted to influence significantly byssal thread production to agglomerate a stable anchor for epibyssate blue mussels. Conversely, thread production by ribbed mussels sheltered in marsh grass patches should be less influenced by the substrata in which they are mostly buried.

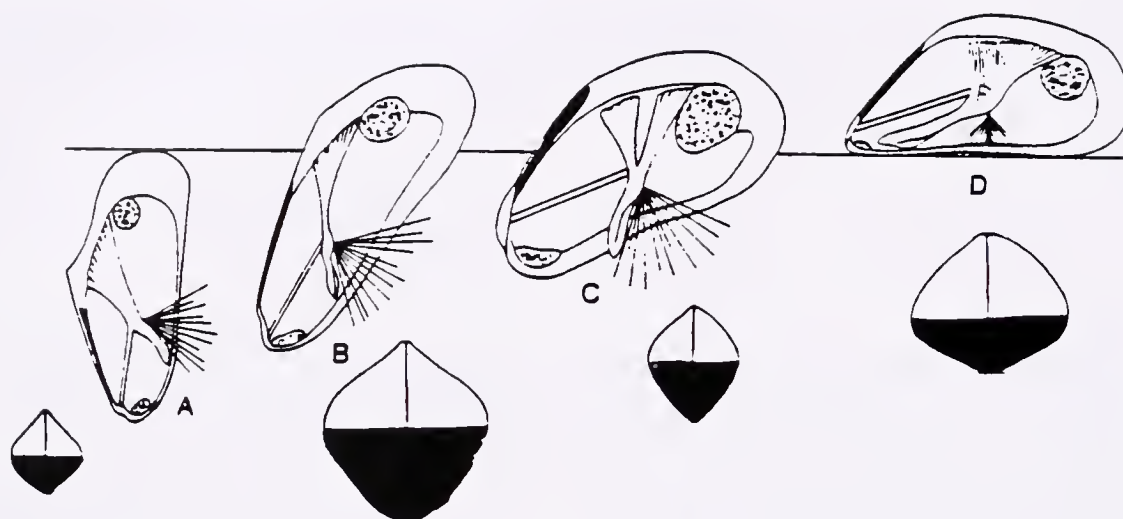


Figure 1. Mytiloid adductor (stippled), byssal, and pedal retractor musculature, usual life position relative to the sediment surface (represented by horizontal line) and cross-sectional shape (shaded to show position of maximum width). Specimens not to scale. A, *Brachidontes citrinus*; B, *Geukensia demissa*; C, *Modiolus modiolus*; D, *Mytilus edulis* (modified from Stanley, 1972).

A series of laboratory experiments were designed to test the influence of sediment substrata and salinities on thread production by each mussel species. Unlike the pattern of increasing thread production by *M. edulis* from fine to coarse sand (Meadows and Shand 1989), we predicted *a priori* that thread production by endobysate *G. demissa* should not vary substantially with increasing sediment grain size. Similarly, we predicted that thread production by *M. edulis*, restricted to more stenohaline lower intertidal habitats, should decrease significantly in salinities progressively lesser or greater than the optimum (Glaus 1968); whereas, thread production by *G. demissa*, acclimatized to seasonal salinity fluctuations, should not decrease appreciably in salinities divergent from the optimum.

#### MATERIALS AND METHODS

Ribbed mussels were collected from tide pools above the midtide line in salt marshes at Tuckerton, NJ during January through March of 1996. Sampled tide pools were monitored for

diurnal variation in temperature, salinity, dissolved oxygen, pH, turbidity, and CO<sub>2</sub> during each season. Mussels were submerged in their native seawater in an aerated, 55 gallon holding aquaria for one day while seawater equilibrated with air temperature in the experimental room. Foot-probing by mussels was monitored to select the most active specimens for experimentation. Selected specimens had their byssus trimmed even with the ventral shell margin, without stimulating expulsion of the byssal stem (Price 1981), the same procedure utilized by Van Winkle (1970), Meadows and Shand (1989) and Lee et al. (1990) on blue and horse mussels. Number of threads in the severed byssus were counted as a base line for comparison with experimental production. Wet mass of tissue plus shell of individuals was recorded. Specimens were apportioned among seven, hooded, aerated, 15 gallon aquaria, each filled with eight gallons of seawater from native tide pools wherein salinity was 29 ppt (Table 1). Equal numbers of small, intermediate, and large size individuals were distributed to each tank. Experiments were repeated in successive weeks.

TABLE 1.

Range of values for temperature, dissolved oxygen, and current velocity in aquaria experiments and sampled tide pools from Tuckerton, NJ

Tested Species	Tested Variable	Duration	Mean/Range of Daily Temperature (°C)	Concurrent Maximum Diurnal Range in Native Tide Pools	Range of Daily Dissolved Oxygen Values (ppm)	Concurrent Maximum Diurnal Range in Native Tide Pools
<i>G. demissa</i>	Substratum	2/7-2/16/97	17/13-19	5-16	7.2-7.8	5.0-10.0
<i>G. demissa</i>	Substratum	2/22-2/29/96	20/17-21	5-16	7.0-7.5	5.0-10.0
<i>G. demissa</i>	Substratum	1/3-7/3/96	19/19-20	5-16	7.1-7.5	5.0-10.0
<i>G. demissa</i>	Substratum	3/25-4/1/96	20/19-21	5-16	7.0-7.5	5.0-10.0
<i>M. edulis</i>	Substratum	1/8-1/15/97	15/15-16	5-16	7.3-8.3	5.0-10.0
<i>M. edulis</i>	Substratum	1/15-1/22/97	16/15-17	5-16	6.0-8.3	5.0-10.0
<i>G. demissa</i>	Salinity	6/26-7/3/96	23/22-25	20-29	6.0-8.0	4.1-8.9
<i>G. demissa</i>	Salinity	7/10-7/18/96	24/21-26	20-29	6.0-8.6	4.1-8.9
<i>G. demissa</i>	Salinity	7/18-7/25/96	24/22-26	20-29	5.9-8.2	4.1-8.9
<i>M. edulis</i>	Salinity	9/17-9/24/96	21/19-22	18-23	5.3-7.0	4.9-6.6
<i>M. edulis</i>	Salinity	9/26-10/3/96	22/21-23	18-23	5.6-7.0	4.9-6.6

Source for diurnal fluctuations, Browne and McAloon 1998 and personal measurements.

Specimens were placed ventral side down on sediment surfaces in plastic trays each filled with a different mean grain size. Sieved, well-sorted sediments included: (1) very fine sand (0.06 to 0.125 mm); (2) fine sand (0.125 mm–0.25 mm); medium sand (0.25 mm–0.50 mm); coarse sand (0.50–1.00 mm); very coarse sand (1.00–2.00 mm), and granules or gravel (2.00–4.00 mm), a range of grain sizes used by Meadows and Shand (1989) on *Mytilus edulis* and *Modiolus modiolus*. Trays were filled to a minimum depth of five cm, which prevented a fully extended, probing mussel foot from accessing the plastic bottom of any tray. Specimens were positioned so that the foot could not access the tray sides without "crawling" across the sediment surface, a capability displayed mostly by juveniles. Although specimens were not fed, each tank was monitored daily for foot-probing activity by all specimen. After 1 week, specimens were removed, and the number of byssal threads attached to sediment grains were counted. Specimens that did not secrete threads, but actively probed sediment throughout the experiment, were recorded as a zero in the production tally. However, specimens that showed no foot-probing activity during the experiment were deleted from the tally.

Temperature, dissolved oxygen, and pH were recorded daily for each tank (Table 1). These variables fluctuated slightly throughout the week in closed aquaria, although all tanks fluctuated in unison. Variations in these factors were within ranges of diurnal fluctuations experienced in native tide pools during the same time frame. Thread production has been shown to be surface area related (Lee et al. 1990), which may be approximated by the square of the anterior–posterior length. Specimen mass (g) is highly correlated with length ( $r = 0.92$  and  $0.91$  for *M. edulis* and *G. demissa*, respectively, with  $n = 100$ ), and this readily obtainable measurement is used to normalize thread production for different size specimens (Table 1). Freshly acquired seawater and mussel specimens from the previously sampled tide pools were used in each succeeding series of experiments. After experiments on *G. demissa* were completed in January–March 1996, the previously described procedure was repeated for *M. edulis* in January–March 97, except that blue mussels were collected from below the midtide line near the embankment. Diurnal range of abiotic variables in sampled tide pools were comparable for both years, although winter 1997 aquaria experiments with *M. edulis* experienced slightly cooler mean temperature (15–16 °C) than winter 1996 trials with *G. demissa* (17–20 °C) (Table 1).

Following completion of experiments with different sediment grain sizes on *G. demissa*, samples of ribbed mussels and blue mussels were again collected from the same tide pools/embankment area in June–July, 1996 and August–September, 1996, respectively. Samples were subjected to the same protocol as previously described, except that in these summertime experiments, flattened plastic beads 3-mm diameter, filled each tray to ensure uniformity in particle roundness, texture, and size. Meadows and Shand (1989) indicated that fine gravel size particles are optimum for thread production by blue mussels. Distilled water or instant ocean was added to the initial 8 gallons of tide pool seawater, with a salinity of 29 ppt in each aquarium to produce tanks of different salinities ranging from 15 to 45 ppt in increments of 5 ppt. Subsequently, water was withdrawn to ensure equal volumes in each tank. Salinities ranging from 21–40 ppts were recorded for perched, high marsh tide pools during successive seasons (McAlloon and Browne 1998), a spectrum that influenced delimitation of the range of experimental salinities.

Ten specimens were placed in each aquarium during successive

weekly experiments. No foot-probing activity was observed for some specimens at extreme salinities. Consequently, slightly more observations are recorded for intermediate salinities than extremes. Evaporation from hooded tanks did require that the salinity in each tank be reduced during the experimental period, but never by more than 1 ppt. on any given day (Table 1). All tanks experienced the same range of dissolved oxygen, temperature, and pH for the week (Table 1), which were, again, within diurnal ranges in native tide pool for the same time period. Number of byssal threads per specimen were mass-normalized. Experiments were repeated with fresh seawater and mussels.

Both mass-normalized and unnormalized thread production values were regressed separately on sediment grain size and salinity for each species. Because a range of size (mass) classes of mussels were utilized for each species, thread production was regressed on specimen mass for each species in each salinity and on each sediment grain size to determine if ontogenetic (growth) stage of the mussel influenced thread production. Both linear regression and second-order polynomial regression (curvilinear) were executed to determine the best fit line or curve for the distribution of thread production by mussels of each species across the spectrum of experimental salinities or sediment grain size. The second-order polynomial invariably produced the better fit, or the greater correlation value ( $r$  referred to as the eta coefficient for curvilinearly related, ratio-scale data). Subsequently, analysis of variance (ANOVA) was performed on data for each species for each abiotic variable to determine if mean thread production for each species varied significantly across the spectrum of sediment grain sizes or salinities. In addition, a pair of two-way ANOVA tests were executed that compared thread production between species (also different temperature regimes) for varying salinities and between species (temperature regimes) on varying substrata.

## RESULTS AND DISCUSSION

Mean number of threads in the pre-experimental byssus trimmed off of a sample of blue and ribbed mussels was 45 ( $n = 23$ , avg. mass = 4.8 g) and 295, respectively ( $n = 12$ ; avg. mass = 14.3 g), respectively. Some individuals produced as many byssal threads in 1 week at optimum salinity (25–30 ppt.) or on optimum grain size (fine gravel) (see Figs. 3, 5, 7, 9) as the mean cited above for their respective species. However, the predicted effect of ontogeny (growth stage) was not apparent in all experiments (Fig. 2). Only *G. demissa* in the salinity experiments displayed the predictable, significant increase in thread production with increasing specimen size (Fig. 2A). This pattern was reversed for *M. edulis* in salinity experiments, however, with smaller specimens producing more threads than larger specimens, resulting in an inverse correlation. (Fig. 2B). Neither species displayed a statistically verified ontogenetic effect in experiments dealing with sediment grain sizes (Fig. 2C, D).

Not surprisingly, larger ribbed mussels produced a greater mean number of threads than smaller blue mussels bathed in slightly cooler water than ribbed mussels (Table 1) at each experimental salinity and sediment grain size (Table 2; see Fig. 3 vs. 5; and Fig. 7 vs. 9). However, when thread production is mass-normalized, distinction between species disappears despite the temperature difference in the experiments (Table 1). Indeed, blue mussels produced more threads, normalized for specimen mass, in slighter cooler mean water temperature (15–16 °C) than ribbed mussels in slightly warmer water temperature (17–20 °C) experi-

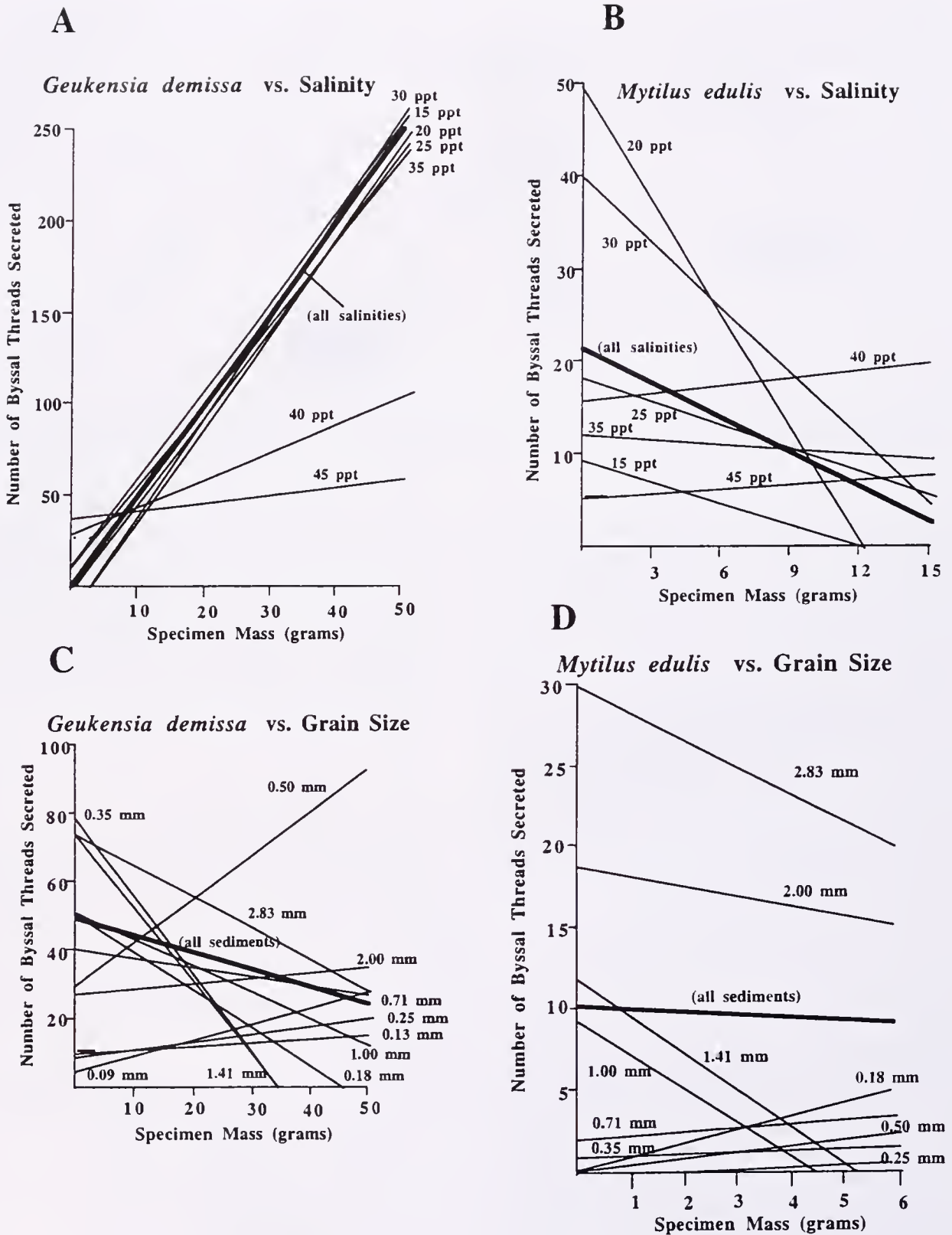


Figure 2. Regression lines for number of byssal threads secreted regressed on specimen mass at various experimental salinities (A, B) and sediment grain sizes (C, D) for each mussel species. Significant ( $P < .05$ )  $r$  values for *G. demissa* (A) at combined salinities,  $r = 0.654$  ( $n = 118$ ); for 35 ppt,  $r = 0.871$  ( $n = 16$ ); for 30 ppt.,  $r = 0.76$  ( $n = 14$ ); for 25 ppt.,  $r = 0.695$  ( $n = 16$ ); for 20 ppt.,  $r = 0.720$  ( $n = 15$ ); for 15 ppt.,  $r = 0.783$  ( $n = 15$ ). Significant  $r$  values for *M. edulis* (B) at combined salinities  $r = 0.244$  ( $n = 135$ ); for 30 ppt.,  $r = 0.546$  ( $n = 20$ ); for 20 ppt.,  $r = 0.52$  ( $n = 20$ ); for *G. demissa* (C) and for *M. edulis* (D) with sediments combined,  $r = 0.140$  ( $n = 117$ ) and  $r = 0.053$  ( $n = 165$ ), respectively. Both  $r$  values statistically insignificant, as are all  $r$  values for data on each substrata.

TABLE 2.

Mean number of byssal threads secreted by blue (at lower temperature, see Table 1) and ribbed mussels (at higher temperature, see Table 1) on each sediment grain size and in each salinity during week-long aquaria experiments. Data analyzed by two-way (species, temperature treatment) ANOVA below.

Sediment Grain Size (mm) (Salinity Constant at 30 ppt.)									
Species	0.18 mm	0.25	0.35	0.50	0.71	1.00	1.41	2.00	2.83
<i>G. demissa</i>	32 threads	21	13	63	42	44	25	44	30
<i>M. edulis</i>	4 threads	10	7	6	7	13	6	4	9
Salinity (ppt.)									
Species	15 ppt.	20	25	30	35	40	45		
<i>G. demissa</i>	70 threads	57	69	72	83	39	38		
<i>M. edulis</i>	5 threads	26	14	26	15	18	4		
Variable	df			F-ratio			P-value		
Salinity									
Mussel species/temp (A)	1			88.31			.001		
Salinity (B)	6			2.71			.015		
Factor interaction (AB)	6			1.75			.073		
Sediment texture									
Mussel species/temp. (A)	1			63.57			.001		
Sediment texture (B)	8			2.43			.015		
Factor Interaction (AB)	8			2.45			.014		

ments on coarse sands and gravel (see Fig. 4 vs. 6). Mean thread production by *M. edulis* was maximum on medium sand (13 threads/week; Fig. 3) and decreased significantly on progressively finer grained sediments (Table 2). In fact, blue mussels did not attach threads to very fine sand grains (<0.18 mm) but instead attached threads to themselves after the foot probed the sediment. This significantly correlated relationship between thread production and grain size ( $r = 0.625$ ; Fig. 3) persisted even after thread production was mass-normalized ( $r = 0.525$ ; Fig. 4; Table 3).

Similar to *M. edulis*, specimens of *G. demissa* also produced the highest average of threads (44) on coarse sand (Table 2, Fig. 5), but unnormalized thread production by the ribbed mussel was not significantly correlated with sediment grain size ( $r = 0.198$ ; Table 3), and mean thread production values were not significantly different from fine- to coarse-grained sediments (Table 3). However, correlation between thread production and sediment grain size was significant (Table 3) when thread production values were mass-normalized for ribbed mussels ( $r = 0.316$ ; Fig. 6). Likewise, mean thread production by ribbed mussels on each sediment grain size were significantly different when these values are mass-normalized (Table 3). Statistical comparison of correlation coefficients for thread production regressed on sediment grain size for *M. edulis* ( $r = 0.627$ ) versus *G. demissa* ( $r = 0.198$ ) were significantly different (Z value of 3.52,  $P < .002$ ).

Thread production by individuals of *M. edulis* regressed on salinities from 15 to 45 ppt. showed a significantly correlated ( $r = 0.298$ ; Table 3) hyperbolic (inverted U-shaped) distribution of mean values across the salinity spectrum (Fig. 7). This pattern indicates that production decreased significantly (Table 3) in salinities below and above the optimum salinity interval, of 25–30 ppt. The hyperbolic relationship of thread production regressed on salinity persists (Fig. 8), and significant correlation between salinity and thread production remained ( $r = 0.297$ ; Table 3), when values were mass-normalized. Interestingly, the greatest mass-normalized mean value, 22 threads/g/week, occurred at 30 ppt. (Fig. 8), which is also the salinity in the tide pools to which the

blue mussels are native. Although blue mussels in waters of 22–23 °C; Table 1) secreted fewer mean number of threads than ribbed mussels in waters of 25–26 °C (Table 1) at every salinity (Table 2), mass normalized values for *M. edulis* exceeded those for *G. demissa* at intermediate (20–35 ppt.) salinities (Fig. 8 vs. 10).

In contrast to *M. edulis*, thread production of *G. demissa* was not significantly correlated with salinity ( $r = 0.183$ ; Table 3), despite the hyperbolic pattern of thread production values regressed on salinity values (Fig. 9). The greatest mean value, 83 threads/week (Table 2), occurred at a salinity of 35 ppt., but this mean was not significantly different than the mean value at 15 ppt., namely, 70 threads/week (Table 3). Furthermore, mass normalization of the data (Fig. 10) did not significantly increase either the correlation between mean thread production and salinity ( $r = 0.127$ ), or the differences in mean thread production values from lowest to highest tested salinities (Table 3). Comparable maximum values, 12 threads/g/week, occurred at salinities of 15 and 35 ppt.

DISCUSSION

Admittedly, dynamics of tide pool setting of ribbed and blue mussels cannot be reconstructed in closed aquaria experiments. Laboratory experiments on byssal thread production did not demonstrate possible synergistic effect of the combined influence of substratum and salinity concurrently, because each variable, salinity and substrata, was treated separately in different seasons. Comparisons of thread production between mussel species must be restricted to same-season tests of the same abiotic variable because of the disparity in the temperature regimes under which the substrata versus salinity experiments were conducted (Table 1). Furthermore, other potentially very influential variables, such as current velocity or agitation (Van Winkle 1970, Young 1985), cannot be evaluated in a closed aquaria system. Current velocities, however, in *Spartina*-fringed, microbial mat-veneered high marsh tide pools at Tuckerton are between 5 and 10 cm/s for most of the tidal cycle, exceeding 10 cm/s value for only 15 min of the 12-h tidal

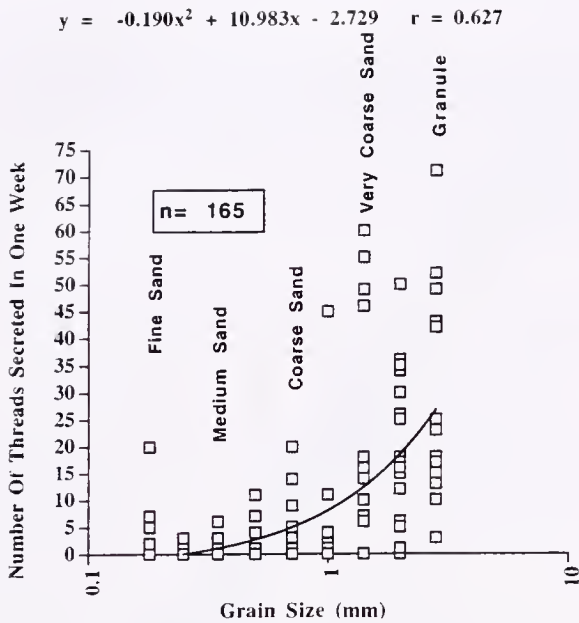


Figure 3. Byssal thread production by *Mytilus edulis* in aquaria during 1 week on very well sorted sieved sediment, fine sand to fine gravel, at 30 ppt. Successive experiments conducted Jan. 8 to Jan. 22, 1997. Range of mean daily temperature for successive tests 15–16 °C; Range for daily dissolved oxygen 6.00 to 8.3 ppm. Significance of correlation coefficient and differences in successive means on each sediment indicated in Table 3. Significance of correlation coefficients and differences in successive means on each sediment indicated in Table 2. Best fit curve is second order polynomial.

cycle (Browne, pers. comm.). Current velocities in isolated perched tide pools is negligible for much of the tidal cycle when pools are disconnected. In addition, daily temperature ranges and dissolved oxygen readings in individual tide pools for any one season are comparable to the range of temperature and DO values experienced in the aquaria over a week during the same season (Browne and McAloon 1998) (Table 1). A potentially important factor precluded in the aquarium experiments is diurnal tidal submergence and emergence. However, submergence–emergence of blue mussels and the tidal cycle fluctuation have been reported to show little or no effect on byssus production (Martella 1974, Price 1982, Young 1983). Bubbling aerators in the half-filled 15-gallon aquaria provided as much water agitation as mussels experienced in emergent, isolated tide pools. Nevertheless, fluctuations in the aforementioned abiotic factors in the aquaria experiments did not differ appreciably from fluctuations occurring concurrently in tide pools, except for variables that were deliberately controlled; namely substratum and salinity (Table 1).

Despite certain debatable limitations, closed system experiments facilitated evaluation of the influence of incremental increases in salinity on thread production, as per other experimental investigations (Glaus 1968, Van Winkle 1970, Allen et al. 1976). Results of this investigation on *M. edulis* are congruent with those of Glaus (1968), who showed that thread production was optimum for the blue mussel at 31 ppt and decreased substantially in salinities of 15 and 46 ppt, because of increasing osmotic stress for mussels in ambient hyposaline or hypersaline water. Results of experiments on *G. demissa* paralleled results of Van Winkle (1970); greater thread production occurred in ribbed and horse mussels species at 30 and 32 ppt, respectively, versus 15 and 16

ppt, respectively. Similar to published results, decreasing thread production is attributed to increasing stress associated with a hyposaline medium bathing ribbed and horse mussels.

Results on the influence of substratum are also congruent with other investigations that compared thread production of the epibyssate blue mussel with an endobyssate mussel; namely, *Modiolus modiolus* (Meadows and Shand 1989). The horse mussel produced more byssal threads per unit time at all experimental sediment grain sizes, 0.05 mm to 16 mm, versus the blue mussel. Similarly, the endobyssate *G. demissa* produced more threads on all sediment grain sizes, 0.12 to 2.0 mm, and at slightly higher temperatures (17–20 vs. 15–16 °C) relative to *M. edulis* (Table 2). Although thread production by *M. edulis* increased progressively from finer to coarser textured sediments in both experiments (this investigation and Meadows and Shand 1989), the substratum that induced production of the most threads among either endobyssate clam *G. demissa* (this investigation) or *M. modiolus* (Meadows and Shand 1989) was not the coarsest used in either experiment. Maximum thread production by *M. modiolus* and *G. demissa* occurred on coarse sand (Meadows and Shand 1989) and very coarse sand (this investigation), respectively, but declined on gravel in both experiments.

Ribbed mussels (Figs. 5, 9) secreted more threads in unit time relative to the blue mussel (Table 2; Figs. 3 vs. 5, and 7 vs. 9), an observation consistent with the temperature-dependent experiments of Van Winkle (1970) on *M. edulis* versus *G. demissa*. Part of this difference in thread production between the two mussel species may reflect the slightly elevated water temperatures for the substratum experiments on *G. demissa* (17–20 °C) versus *M. edu-*

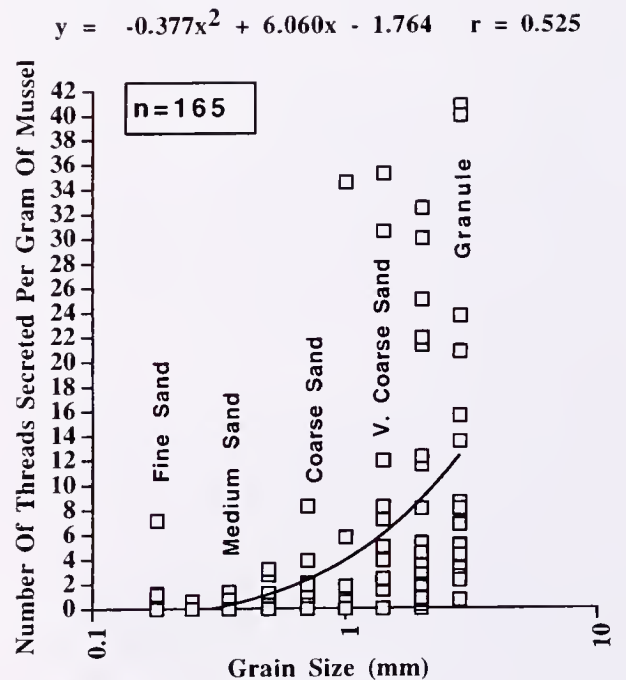


Figure 4. Byssal thread production by *Mytilus edulis*, normalized for (divided by) mussel mass (g), in aquaria during 1 week on very well sorted sieved sediment, fine sand to fine gravel, at 30 ppt. Successive experiments conducted Jan. 8 to Jan. 22, 1997. Range of mean daily temperature for successive tests 15–16 °C; Range for daily dissolved oxygen values 6.00 to 8.3 ppm. Significance of correlation coefficient and differences in successive means on each sediment indicated in Table 3. Best fit curve is second-order polynomial.

TABLE 3.

Statistical significance of eta correlation coefficients,  $r$ , and compared means for unnormalized and mass-normalized thread production by *Geukensia demissa* and *Mytilus edulis* versus salinity (15–45 ppt.) and sediment grain size (0.09 to 2.8 mm).

Compared or Regressed Variables	Calculated Value	Probability and Decision
Regression: <i>G. demissa</i> Thread production vs. salinity	2nd-order polynomial $r = 0.183$	$P = 0.0905$ ; accept null hypothesis of no correlation
Regression: <i>G. demissa</i> Threads/gram of mussel vs. salinity	2nd-order polynomial $r = 0.126$	$P = 0.4306$ ; accept null hypothesis of no correlation
Regression: <i>G. demissa</i> Thread production vs. grain size	2nd-order polynomial $r = 0.198$	$P = 0.1018$ ; accept null hypothesis of no correlation
Regression: <i>G. demissa</i> Threads/gram of mussel vs. grain size	2nd-order polynomial $r = 0.316$	$P = 0.0025$ ; reject null hypothesis of no correlation
Regression: <i>M. edulis</i> Thread production vs. salinity	2nd-order polynomial $r = 0.298$	$P = 0.0006$ ; reject null hypothesis of no correlation
Regression: <i>M. edulis</i> Threads/gram of mussel vs. salinity	2nd-order polynomial $r = 0.267$	$P = 0.00076$ ; reject null hypothesis of no correlation
Regression: <i>M. edulis</i> Thread production vs. grain size	2nd-order polynomial $r = 0.627$	$P = 0.001$ ; reject null hypothesis of no correlation
Regression: <i>M. edulis</i> Threads/gram of mussel vs. grain size	2nd-order polynomial $r = 0.525$	$P = 0.001$ ; reject null hypothesis of no correlation
ANOVA: <i>G. demissa</i> Thread production vs. salinity	F ratio = 1.37 with 6/134 df	$P = 0.231$ ; accept null hypothesis that means are similar
ANOVA: <i>G. demissa</i> Threads/gram of mussel vs. salinity	F ratio = 0.60 with 6/101 df	$P = 0.731$ ; accept null hypothesis that means are similar
ANOVA: <i>G. demissa</i> Threads vs. grain size	F ratio = 1.90 with 10/106 df	$P = 0.052$ ; accept null hypothesis that means are similar
ANOVA: <i>G. demissa</i> Threads/gram of mussel vs. grain size	F ratio = 2.06 with 10/106 df	$P = 0.034$ ; reject null hypothesis that means are similar
ANOVA: <i>M. edulis</i> Thread production vs. salinity	F ratio = 5.09 with 6/156 df	$P = 0.0001$ ; reject null hypothesis that means are similar
ANOVA: <i>M. edulis</i> Threads/gram of mussel vs. salinity	F ratio = 4.27 with 6/156 df	$P = 0.0006$ ; reject null hypothesis that means are similar
ANOVA: <i>M. edulis</i> Thread production vs. grain size	F ratio = 8.16 with 8/90 df	$P = .0001$ ; reject null hypothesis that means are similar
ANOVA: <i>M. edulis</i> Threads/gram of mussel vs. grain size	F ratio = 7.38 with 8/89 df	$P = .0001$ ; reject null hypothesis that means are similar

lis (15–16 °C) (Table 1). Thread productivity increases with increasing temperature (Allen et al. 1976, Glaus 1968). Nevertheless, Meadows and Shand (1989) demonstrated that *M. modiolus* secreted more threads than *M. edulis* on a spectrum of sediment grains sizes under the same temperature regime, further substantiating the size dependency of thread production (Lee et al. 1990).

When thread production was normalized for mass, however, the epibyssate *M. edulis* shows greater thread production per gram of mussel than for *G. demissa* (Table 2), even given the slightly lower aquaria temperatures for experiments on the blue mussels (Table 1). This greater mass-normalized value for blue mussels may reflect the need to counter the greater acceleration forces experienced by the greater amount of shell surface area of *M. edulis* intercepting the current relative to *G. demissa*. Attachment strength is directly related to number of byssal threads secreted, and dislodgment force of blue mussels is directly proportional to shell area (Lee et al. 1990, Witman and Suchanek 1984) or shell height (Willis and Skibinski 1992). Both measurements are proportional to the shell cross-sectional area intercepting the current. Whereas mostly buried adult specimens of *G. demissa* may expose less than a one cm squared surface area to *Spartina* grass-baffled currents, the smaller *M. edulis* often exposes several square cm of shell surface area to intercept the current. Even under the stress of suboptimum salinities, blue mussels produce more threads per

specimen gram than ribbed mussels (Table 2). Threads of *M. edulis* are also thicker than those of *G. demissa*. Thicker and longer threads may have greater tensile strength (Price 1982). *Mytilus californianus* has greater adherence “tenacity” than *M. trossulus* because of among other variables, greater thread thickness (Bell and Gosline 1997). Although it secreted fewer threads, attachment strength of *M. edulis* may be greater than *G. demissa* given the greater collective strength of individually thicker threads.

The absence of the expected ontogenetic effect of increased thread production with increasing size (mass) of blue mussels for varying salinities (Fig. 2B) may indicate a possible inhibitory effect on thread production induced by suboptimum salinities. Indeed, the inverse relationship between thread production and specimen size suggests that thread secretion by smaller blue mussels is less stressed and inhibited by the associated with suboptimum salinities. The predicted direct relationship between thread production and specimen mass (Lee et al. 1990) did materialize for *G. demissa* for all experimental salinities (Fig. 2), an indication that any size-dependency of thread production by ribbed mussels was not obfuscated by osmotic stress. This mussel occupies perched tide pools where salinities fluctuate by as much as 20 ppt between seasons. The lack of correlation between thread production and mussel specimen mass on each tested sediment’s texture (Fig. 2) is enigmatic, but may indicate that a threshold of current

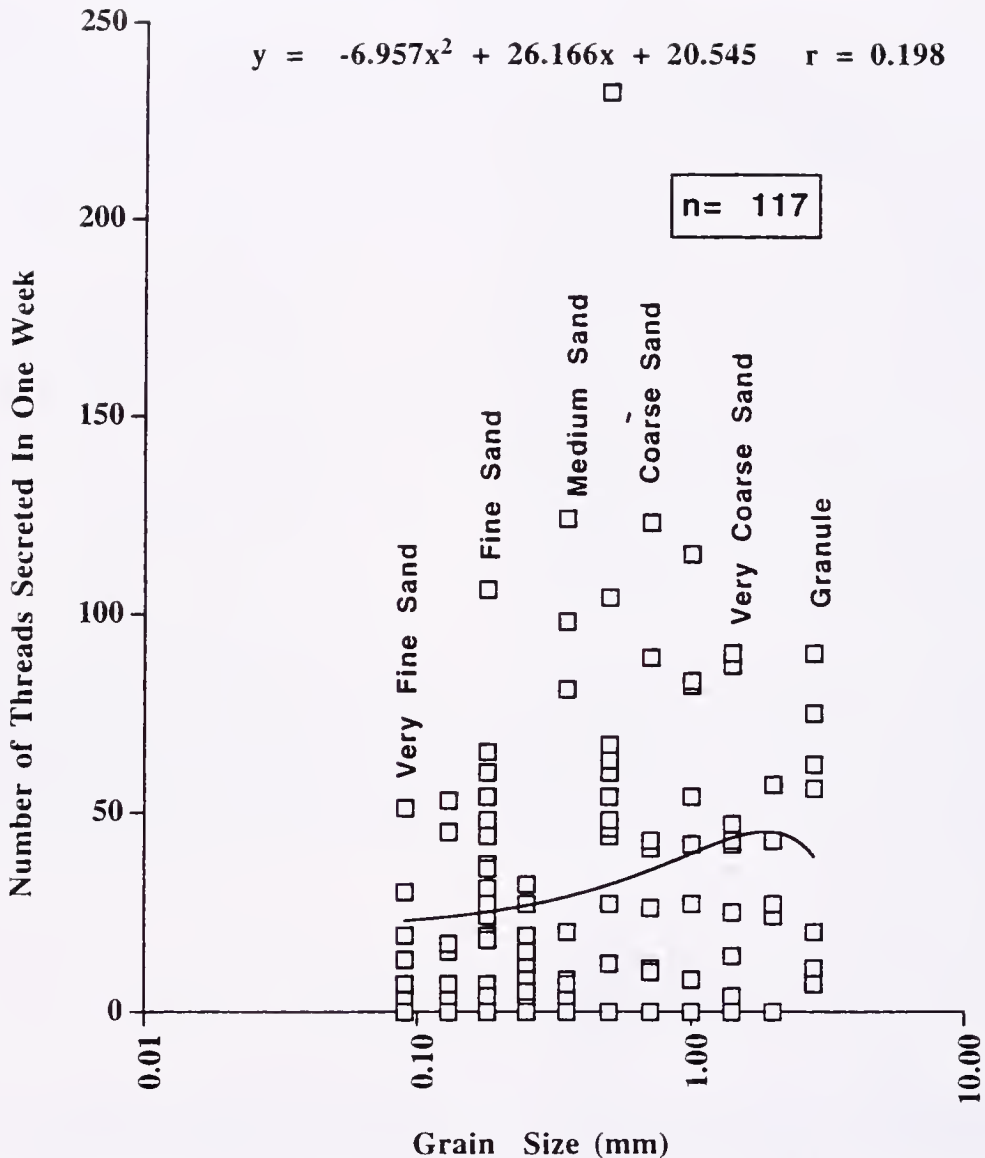


Figure 5. Byssal thread production by *Geukensia demissa* in aquaria during 1 week on very well sorted sieved sediment, fine sand to fine gravel, at 30 ppt. Successive experiments conducted Feb. 7 to April 1, 1996. Range of mean daily temperature for successive tests: 17–20 °C. Range for daily dissolved oxygen values, 7.0–7.8 ppm. Significance of correlation coefficient and differences in successive means on each sediment indicated in Table 3. Best fit curve is second-order polynomial.

agitation is needed to stimulate thread production commensurate with specimen size, as noted by Lee et al. (1990). Current agitation may merely reinforce the existing pattern established in less agitated closed aquaria experiments wherein blue mussels secrete more threads per gram than ribbed mussels.

Comparison of correlation coefficients for mass-normalized thread production regressed on sediment grain size for *M. edulis* ( $r = 0.627$ ) versus *G. demissa* ( $r = 0.198$ ) were significantly different ( $Z$  value of 3.52,  $P < .002$ ), a disparity that suggests greater sensitivity to substrata texture on the part of blue mussels. Speculation on how the substratum may mediate thread production focuses on tactile cueing of the probing foot. The tapered end of the probing foot may possibly sense the diameter of the sediment particle. Sites to which threads are attached are first cleaned before secretion of the thread and the adhesive disk by a “plungered” foot (Amato 1981). If particle sizes are sensed as too small in diameter,

the foot may continue probing for alternative, larger surfaces. Failing to locate suitable sized grains, many mussels placed on fine sand (0.13 mm) secreted threads attached to their own shell exclusively. Whatever the stimulus, thread production by blue mussels did vary significantly on different grain size sediments in contrast to ribbed mussels. Agglomeration of a critical mass of sediment particles would be essential to stabilize the epibyssate blue mussel in place at wave-affected marsh embankment.

#### CONCLUSIONS

Mean byssal thread production per week by the larger *Geukensia demissa* exceeded that of the smaller *Mytilus edulis* on a fine gravel-sized substratum over the entire experimental range of salinities. However, mass-normalized mean thread production for *M. edulis* exceeded that of *G. demissa* at the optimum salinity; namely, 30 ppt. Furthermore, mean thread production at progres-



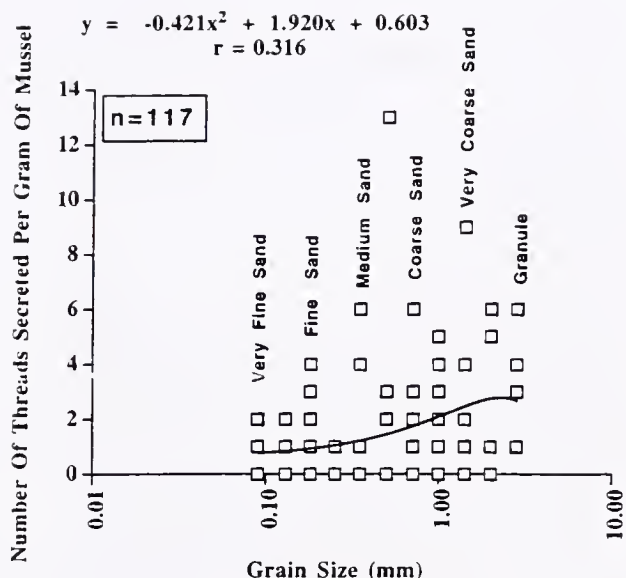


Figure 6. Byssal thread production by *Geukensia demissa*, normalized for (divided by) mussel mass (g), in aquaria during 1 week on very well sorted sieved sediment, fine sand to fine gravel, at 30 ppt. Successive experiments conducted Feb. 7 to April 1, 1996. Range of mean daily temperature for successive tests: 17–20 °C. Range for daily dissolved oxygen values, 7.0–7.8 ppm. Significance of correlation coefficient and differences in successive means on each sediment indicated in Table 3. Best fit curve is second-order polynomial.

sively lower salinities decreases at a greater rate for *M. edulis* than for *G. demissa*. In addition, smaller blue mussels secreted more threads than larger specimens at all salinities; whereas, ribbed mussels displayed the expected increase in thread production with increasing specimen size (mass). All of these results indicate that

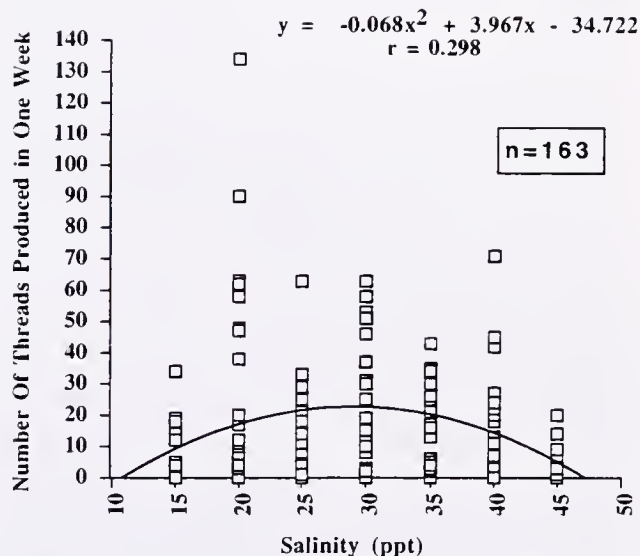


Figure 7. Byssal thread production by *Mytilus edulis* in aquaria during 1 week on 3-mm diameter plastic beads in varying salinities, 15 to 45 ppt. Successive experiments conducted Sept. 17 to Oct. 3, 1997. Range of mean daily temperature for successive tests, 21–22 °C. Range for daily dissolved oxygen values, 5.6–7.0 ppm. Significance of correlation coefficient and differences in successive means on each sediment indicated in Table 3. Best fit curve is second-order polynomial.

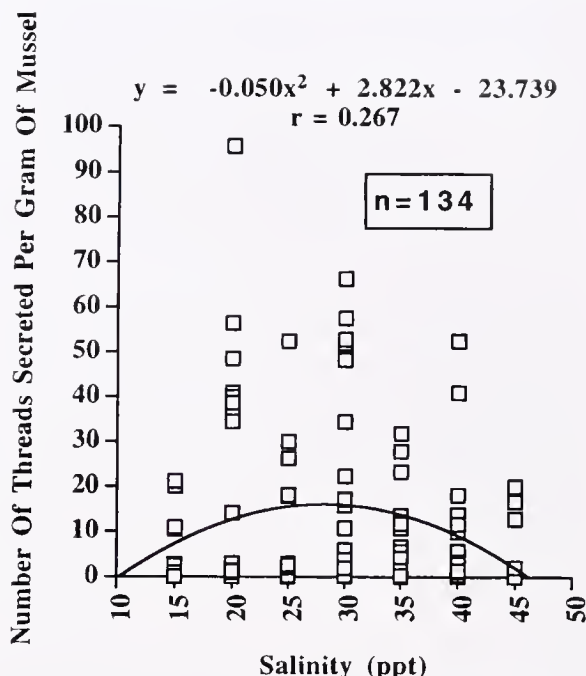


Figure 8. Byssal thread production by *Mytilus edulis*, normalized for (divided by) mussel mass (g), in aquaria during 1 week on 3-mm diameter plastic beads in varying salinities, 15 to 45 ppt. Successive experiments conducted Sept. 17 to Oct. 3, 1997. Range of mean daily temperature for successive tests: 21–22 °C. Range for daily dissolved oxygen values, 5.6–7.0 ppm. Significance of correlation coefficient and differences in successive means on each sediment indicated in Table 3. Best fit curve is second-order polynomial.

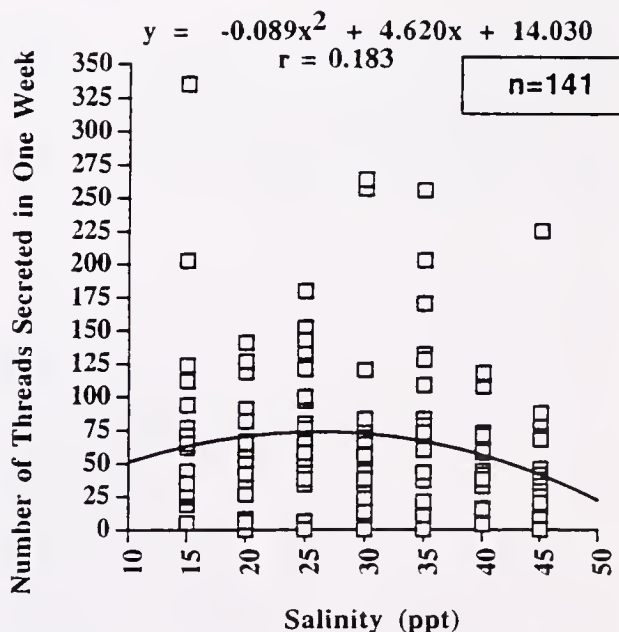


Figure 9. Byssal thread production by *Geukensia demissa* in aquaria during 1 week on 3-mm diameter plastic beads in varying salinities, 15 to 45 ppt. Successive experiments conducted June 26 to July 25, 1996. Range of mean daily temperature for successive tests, 23–24 °C. Range for daily dissolved oxygen values, 5.6–8.5 ppm. Significance of correlation coefficient and differences in successive means on each sediment indicated in Table 3. Best fit curve is second-order polynomial.

blue mussels, which live in the lower intertidal where salinity fluctuations are less pronounced, are more sensitive to stresses induced by salinity changes. Thread production by the higher intertidal *G. demissa*, a mussel acclimated to a wide range of salinity fluctuations in perched tide pools, is less affected by stress induced by salinity changes.

Similar to salinity-mediated production of byssal threads, mean thread production by *G. demissa* is greater than that for *M. edulis* on very fine sand to gravel at 30 ppt. Whereas mass-normalized and unnormalized mean thread production by *M. edulis* increased substantially from fine sand to gravel at 30 ppt, mean thread production by *G. demissa* increases insignificantly across the same spectrum of sediment grain sizes and salinity. Mass-normalized thread production by *M. edulis* exceeded that of *G. demissa* on coarse sands to gravel. Substratum texture has more influence on thread production by the epibyssate *M. edulis*, a mussel subjected to the acceleration forces of flow that may dislodge the individual, than by the mud-bound endobyssate *G. demissa* in salt marshes. Thread-bound agglomerated sediment particles constitute the anchor or "holdfast" or epibyssate blue mussels whose shell surface area intercept the flow. In comparison, only the posterior valve margin of ribbed mussels protrude above cohesive muds to intercept the flow, which is baffled by surrounding *Spartina* grass. Consequently, thread production by the mostly buried ribbed mussel is less sensitive to, or less selective for, differences in substrata texture.

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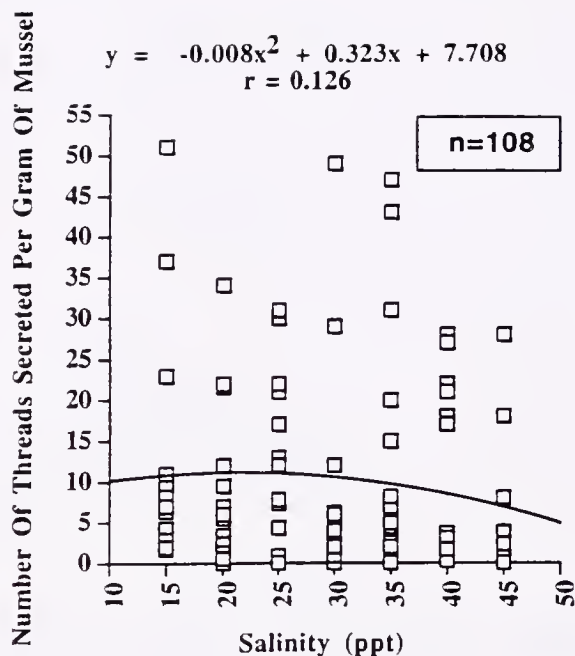


Figure 10. Byssal thread production by *Geukensia demissa*, normalized for (divided by) mussel mass (g), in aquaria during 1 week on 3-mm diameter plastic beads in varying salinities, 15 to 45 ppt. Successive experiments conducted June 26 to July 25, 1996. Range of mean daily temperature for successive tests, 23-24 °C. Range for daily dissolved oxygen values, 5.6-8.5 ppm. Significance of correlation coefficient and differences in successive means on each sediment indicated in Table 3. Best fit curve is second-order polynomial.

## OPTIMUM TEMPERATURE FOR GROWTH IN THE CATARINA SCALLOP (*ARGOPECTEN VENTRICOSUS-CIRCULARIS*, SOWERBY II, 1842)

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**ABSTRACT** In an attempt to determine temperature optimum for growth in juvenile (10.0–11.8 mm mean shell height) catarina scallop *Argopecten ventricosus-circularis*, scope for activity, ingestion and clearance rates, irrigation efficiency, and growth were investigated between 12 and 28 °C. The animals were acclimated at the same temperature as the assays. Results indicate that scope for activity (the arithmetic difference between active and standard respiration rates) increased from 12 °C to a maximum at 19 °C, and then declined to 28 °C. Mean ingestion rates (IR) were higher at 19 and 22 °C and lower at 16, 25, and 28 °C. Clearance rate (CR) was also higher at 19 and 22 °C, and lower at 16, 25, and 28 °C. No statistical difference ( $P > .01$ ) of IR and CR was obtained between 19 and 22 °C. Irrigation efficiency (liters of water filtered per ml O<sub>2</sub> consumed) followed the same trend as the scope for activity, with a maximum at 19 °C. The highest growth rate was at 19 °C in a 54-day experiment. All these results indicate the temperature optimum for the juvenile catarina scallop population studied was between 19 and 22 °C.

**KEY WORDS:** *Argopecten ventricosus-circularis*, catarina scallop, clearance rate, ingestion rate, irrigation efficiency, scope for activity, temperature optimum

### INTRODUCTION

Catarina scallop (*Argopecten ventricosus-circularis*) has been cultured commercially in Bahía Magdalena, Mexico (Maeda-Martínez et al. unpubl. data) using a bottom-culture technology (Maeda-Martínez and Ormart-Castro, 1995). The average temperature there is several degrees colder than in other places where this scallop has been exploited, such as Bahía de La Paz and Bahía Concepción in Mexico, or elsewhere in Panama and Ecuador. This lower temperature is caused by the cold waters of the California Current (Alvarez-Borrego et al. 1975; Lynn and Sympton 1987). From these experiences, differences in growth between growout zones were related to temperature, harvesting larger scallops in zones with lower temperature. There were no differences in growth and survival of wild spat versus hatchery-produced spat, making the hatchery an alternative for spat production when spat collection is insufficient or out of season. To determine the possibility of expanding the culture techniques to other areas and to optimize hatchery production by culturing at or close to optimum temperature, we needed to know the effect of temperature on the growth of this species.

Temperature is considered the most important determinant of the level of activity in poikilotherms (Bayne 1976). Temperature affects, directly or indirectly, larval and adult survival, and its effects on reproduction (i.e., gonad maturation, spawning) and development, larval life span, and settlement are known (Kinne 1970). The temperature optimum is commonly recognized as that temperature at which the organism has the highest energy for maintenance, growth, reproduction, movement, and so forth. Fry (1947) proposed scope for activity as a concept to determine temperature optimum in animals, defined as the arithmetic difference between active and standard rates of oxygen uptake. Thompson and Bayne (1972), Bayne (1973), Bayne et al. (1973), and Widdows (1973) have considered the scope for activity as a practical way to find the energy available to the organism for growth, reproduction, and so forth. Active O<sub>2</sub> uptake rate is obtained from an animal that has been fed continuously to satiation; whereas, the standard rate comes from an animal kept under inanition for a long

period (Bayne 1976). Here the O<sub>2</sub> uptake rate declines to an stable minimum value for several days before death.

Ingestion (the amount of particles cleared from a volume of water per unit time) and clearance (the volume of water cleared from particles by the animal per unit time) rates have also been used to measure the influence of environmental variables on bivalve mollusks (Widdows 1973, Gonzalez et al. 1990, Iglesias and Navarro 1991, Albentosa et al. 1994, Espina and Bückle-Ramírez 1994, Navarro and Iglesias 1995, Urrutia et al. 1996). These variables vary with age, size, and reproductive stage, but also are affected by such environmental factors as particle concentration, salinity, O<sub>2</sub> concentration, and, most importantly, temperature (Bayne 1976, Shumway 1991). Thus, ingestion and filtration rates could be used as temperature optimum indicators.

Irrigation efficiency (the amount of water pumped by the animal per ml of O<sub>2</sub> consumed) has been measured in several mollusks (Jørgensen 1960, Vahl 1972, McLusky 1973, Bayne 1976, Newell et al. 1977), but this has not been used to find the temperature optimum.

In the present work, the temperature optimum of a catarina scallop population has been investigated, studying the scope for activity, ingestion and clearance rates, and the irrigation efficiency between 12 and 28 °C. The results from these studies were contrasted with a growth experiment at the same temperatures.

### MATERIALS AND METHODS

#### Experimental Animals

Juvenile catarina scallop were collected in onion bags suspended from a longline in the tidal channel at Rancho Bueno, B.C.S., Mexico (Fig. 1). Spat were detached from collectors and transported to the laboratory in La Paz during a 3-hour trip in a plastic container receiving constant aeration. The spat were placed in 40-l plastic tanks containing well seawater at 19 °C, 30‰ salinity, and constant aeration. Well seawater was filtered through 5- $\mu$ m filter cartridges and then sterilized with UV radiation. The juveniles were fed  $1.2 \times 10^8$  cells/scallop/day of a mixture of 6:3:1 *Isochrysis galbana*, *Monochrysis lutheri*, and *Chaetoceros gracil-*

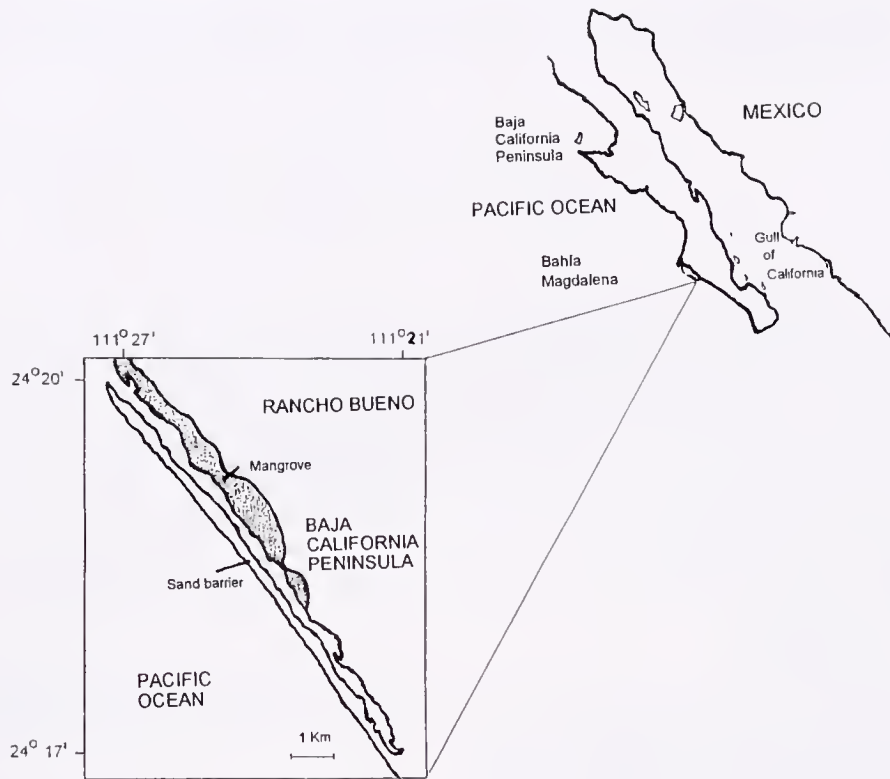


Figure 1. Spat collection site for catarina scallop (*Argopecten ventricosus-circularis*).

lis. Half of the water volume was changed every day, and the microalgae concentration was then re-established.

#### Dry Tissue Weights

In the present work, results were referred to the dry tissue weight of the animals. Nearly 50 scallops between 6 and 57 mm shell height were dried for 36 h in an oven at 65 °C. Constant weights of the animals were obtained. The tissues were then removed from the shells with a dissection needle and were weighed on an electronic microbalance with 0.1 mg resolution. With these data, regression analyses were made, looking for the best fit to describe the dry tissue weight–shell height relation.

#### Scope for Activity Experiments

Twelve groups of 30 juveniles (10 mm shell height) were placed in 19-L buckets containing 15 L of seawater from a coastal well at 19 °C and 30‰ salinity. The temperature of each bucket (with one replicate), was gradually set at six experimental temperatures (12, 16, 19, 22, 25, and 28 °C), varying  $1 \pm 0.5$  °C every 3 days, by using calibrated immersion heaters. In treatments lower than ambient temperature (25 °C), the buckets were dipped into a 1,100 l water bath at 12 °C, equipped with a 1.5 HP water chiller. Under these circumstances, all the animals were at their various temperature treatments in 27 days. The scallops were maintained at constant temperature for 10 days before starting the experiments. One group of each treatment was used for active rate and the other for the standard rate. The six active-rate groups were fed three times in a day with  $2.85 \times 10^8$  cells/scallop/day of a mixture of 6:3:1 *Isochrysis galbana*, *Monochrysis lutheri*, and *Chaetoceros gracillius*. The six standard-rate groups were kept in filtered (3 µm) well seawater with no food additions.

In this work, the rates of oxygen uptake ( $VO_2$ ) were measured periodically over 45 days with a microWinkler method (Maeda-Martinez 1985). Ten scallops of each group were incubated for 1 h in BOD bottles of 300 ml, containing  $O_2$ -saturated well seawater at 30‰ and at the experimental temperatures. Over this incubation time,  $VO_2$  was not affected by the drop in  $O_2$  concentration ( $PO_2$ ) in the bottles (Sicard 1999). The water was previously filtered through a GF/F (0.75 µm) membrane. One bottle without scallops served as a blank.

After incubation, the water of each bottle was siphoned into three ground-neck borosilicate tubes of approximately 7 ml. The  $PO_2$  in the tubes was measured with a miniaturized version (Maeda-Martinez 1985) (Fig. 2) of a titrator developed by Bryan et al. (1976).  $VO_2$  was calculated by sum of the  $PO_2$  in the blank bottle minus that in the animal bottles.

#### Ingestion and Clearance Rates

Ingestion (IR) and clearance (CR) rates were studied following the principle of the method by Winter (1973), which consists in keeping the algal concentration stable in the incubation chamber. Thus, the number of algae added to the chamber is equivalent to the number of algae consumed. For this work a microprocessor-controlled system (Fig. 3) was constructed to determine IR and CR, based on the design of Gallager and Mann (1980). Ten juvenile scallops ( $11.8 \pm 0.6$  mm shell height), acclimated to the experimental temperatures, were transferred to 1-L glass beakers containing GF/F-filtered well seawater with  $1.5 \times 10^5$  cells/ml of *Isochrysis galbana* at 30‰ salinity and at the experimental temperatures. The experiment was done in triplicate. A closed system was made to detect the cell concentration in the chambers. The

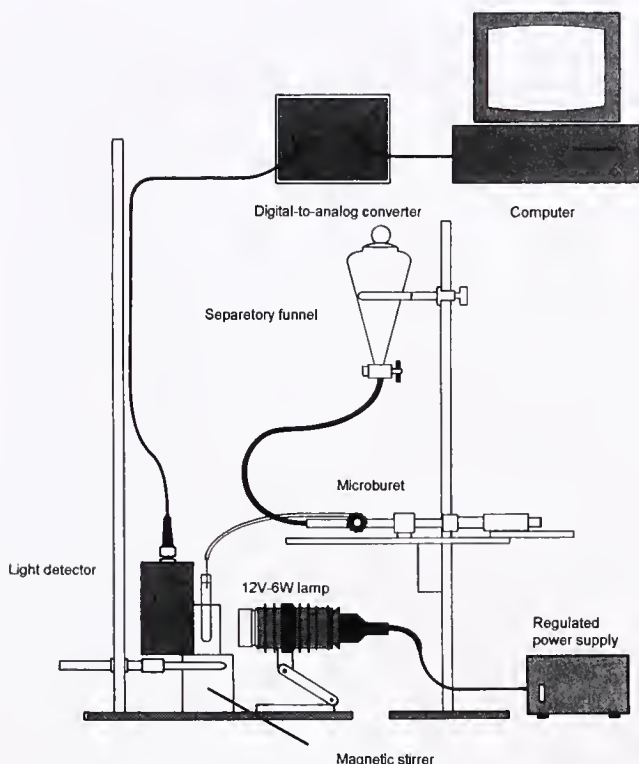


Figure 2. Minaturized version of the titrator employed for oxygen uptake determinations (after Maeda-Martinez 1985).

water in the beakers was continuously pumped with a peristaltic pump to a Turner 112 fluorometer at 87 mL/min, and the chlorophyll concentration in the water was read. The fluorometer was equipped with a flow-through cell and a 47B filter specific for chlorophyll *a* detection at 420–500 nm. The effluent of the cell was then returned to the beaker. A analog-to-digital converter digitalized the signal from the fluorometer, which was compared with the desired value stored in the memory of the computer (Fig. 4). If the value was equal or higher than the stored reading, the computer waited for 2 min before taking a second reading. However, when the value was lower, the system operated a second peristaltic pump (1.025 mL/min) to replace the algae consumed by the animals from a known algal suspension kept in a conical flask. The flask re-

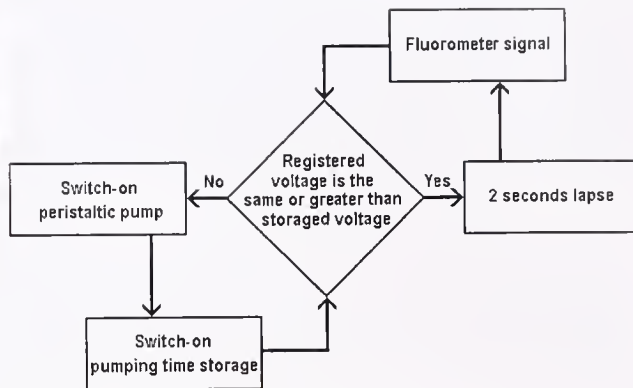


Figure 4. Flow diagram of the computer program used to control the ingestion and clearance rate apparatus.

ceived constant aeration to maintain the cells in suspension and was covered with aluminum foil to avoid algal reproduction during the experiment. The experiments were run for 3 h and in triplicate. A detailed description of the system is given by Sicard (1999). IR was calculated by dividing the number of cells replaced (consumed) per hour by the dry tissue weight (cells/g/h). IR was also calculated in terms of ingested biomass (mg/g/h) assuming 11.4 pg dry weight/cell (Walne 1970). CR (l/g/h) was calculated by dividing IR by the cell concentration in the incubation chamber.

*Irrigation Efficiency*

The irrigation efficiency (l/ml O<sub>2</sub>) at different temperatures was calculated by dividing CR by the active VO<sub>2</sub> from the previous experiments.

*Growth Experiment*

Five groups of 50 scallops of 10 mm shell height were placed in 40-L plastic tanks with well seawater at 30‰ salinity and at the same experimental temperatures as above. The scallops were fed

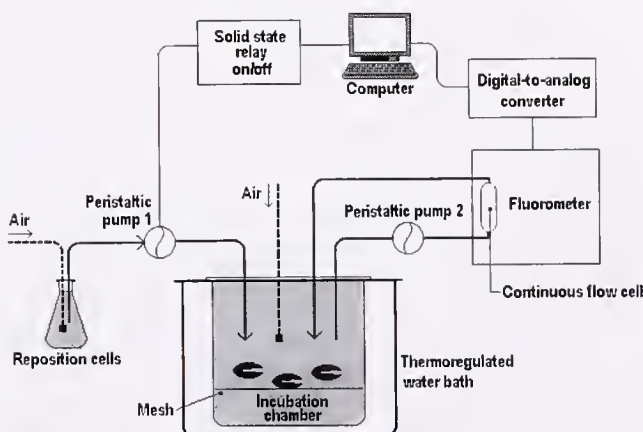


Figure 3. Apparatus developed for measuring ingestion and clearance rates on juvenile catarina scallop (*Argopecten ventricosus-circularis*).

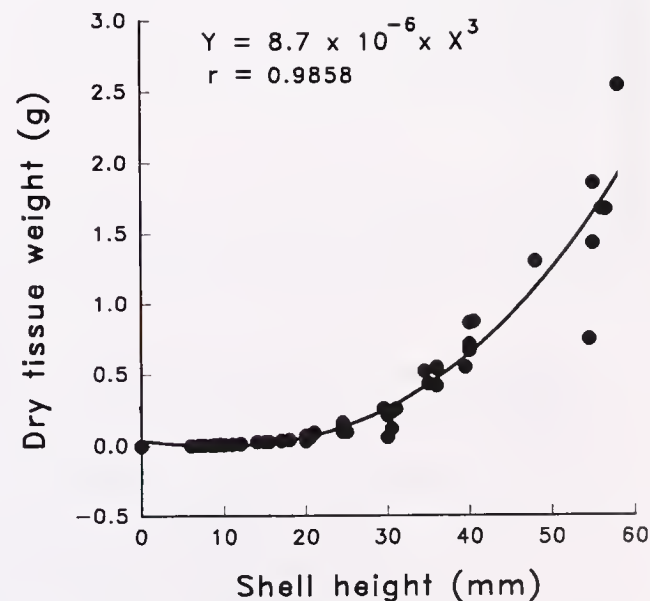


Figure 5. Relation between shell height and dry tissue weight in the catarina scallop (*Argopecten ventricosus-circularis*).

$2.4 \times 10^8$  cells/day of a mixture of 6:3:1 *Isochrysis galbana*, *Monochrysis lutheri*, and *Chaetoceros gracillis*. A 50% water exchange was done everyday, replacing the algae consumed. The shell height of the scallops from all treatments was measured periodically, with a plastic caliper, during the 54-day duration of the experiment.

RESULTS

Dry Tissue Weight

The relation between dry tissue weight (DTW) and shell height (SH) (Fig. 5) was closely described ( $r = 0.98$ ;  $n = 48$ ) by the equation:

$$DTW = 8.7 \times 10^{-6} \times SH^3$$

Scope for Activity

The  $VO_2$  of fed and starved juvenile catarina scallops at different temperatures during 45 days is shown in Figure 6. From this, the  $VO_2$  of fed animals reflecting the active rate remained steady during the 45 days, except in treatment at 12 °C, where a decline from 1 to 0.7 ml  $O_2/g/h$  was measured. In contrast, the  $VO_2$  of

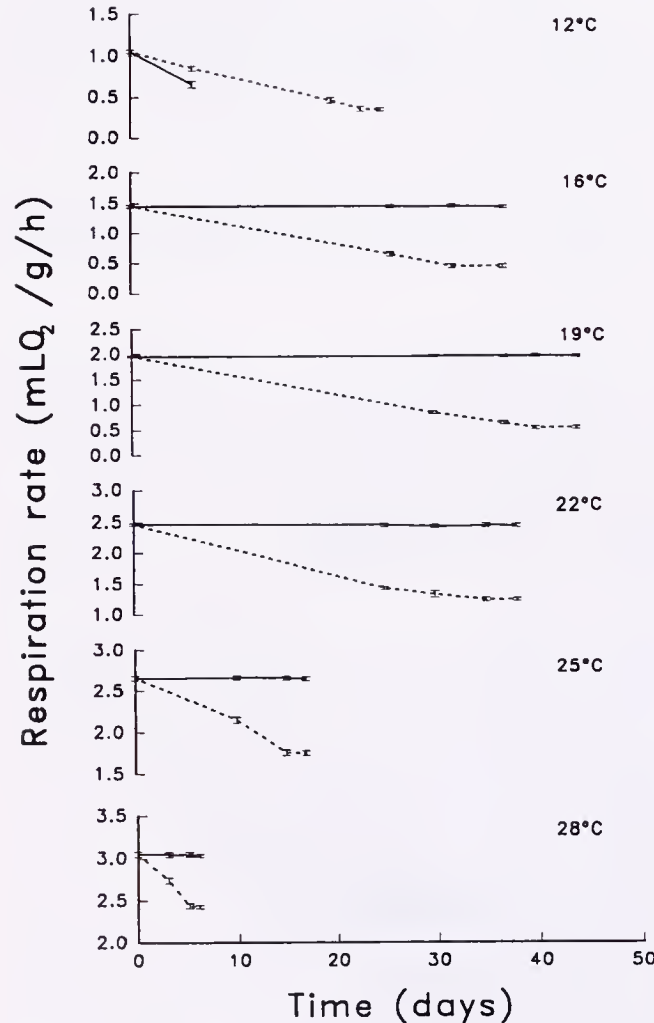


Figure 6. Respiration rate of fed (-) and starved (- -) juveniles of catarina scallop (*Argopecten ventricosus-circularis*) during a 45-day experiment at different temperatures. Values are the mean  $\pm$  s of the mean ( $n = 9$ ).

TABLE 1.

Active and standard respiration rates in juvenile catarina scallop (*Argopecten ventricosus-circularis*) at different temperatures ( $n = 9$ ).

Temperature (°C)	Active Respiration Rate (mL $O_2$ /g/h)		Standard Respiration Rate (mL $O_2$ /g/h)	
	$\bar{x}$	s	$\bar{x}$	s
12	1.05	0.02	0.34	0.02
16	1.45	0.02	0.44	0.03
19	1.95	0.02	0.55	0.02
22	2.46	0.01	1.24	0.02
25	2.65	0.02	1.75	0.03
28	3.05	0.03	2.43	0.02

starved animals declined in all treatments until a minimum value was reached, reflecting the standard rate. The standard rates were reached at 23, 32, 40, 35, 15, and 6 days from the beginning of the experiment at 12, 16, 19, 22, 25, and 28 °C, respectively.

The active and standard rates are in Table 1 and Figure 7a. From these, the scope for activity is shown in Figure 7b, with the highest (1.4 ml  $O_2/g/h$ ) at 19 °C.

Ingestion and Clearance Rates

Variations of IR and CR in juvenile catarina scallops at different temperatures are shown in Figures 8a, 8b, and 9. In Figure 8a, ingestion of *I. galbana* was highest at 22 °C, removing  $2.8 \times 10^9$  cells/g/h, with  $2.6 \times 10^9$  cells/g/h removed at 19 °C. At 25, 28, and 15 °C, IR declined to 1.9, 1.7, and  $1.3 \times 10^9$  cells/g/h. These values in terms of algal biomass fluctuated between 15 and 32 mg/g/h

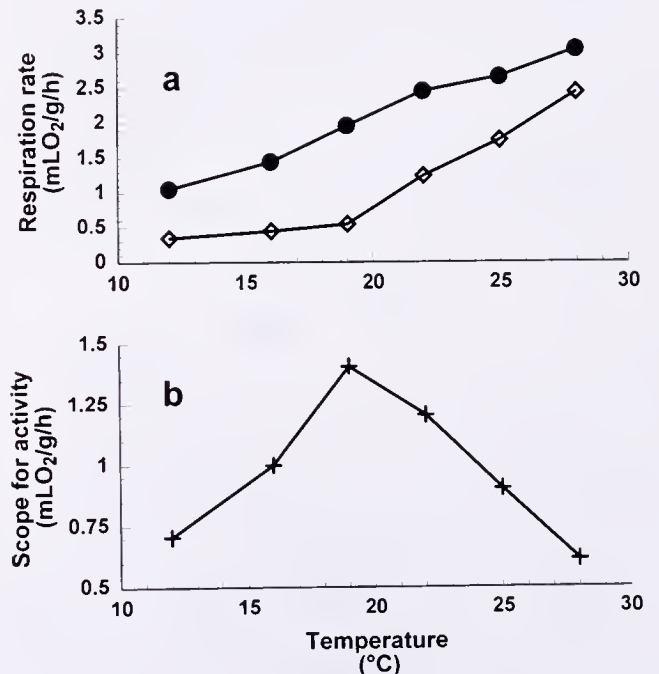


Figure 7. Active (●) and standard (◇) respiration rates (a), and the scope for activity (b) of juvenile catarina scallop (*Argopecten ventricosus-circularis*).

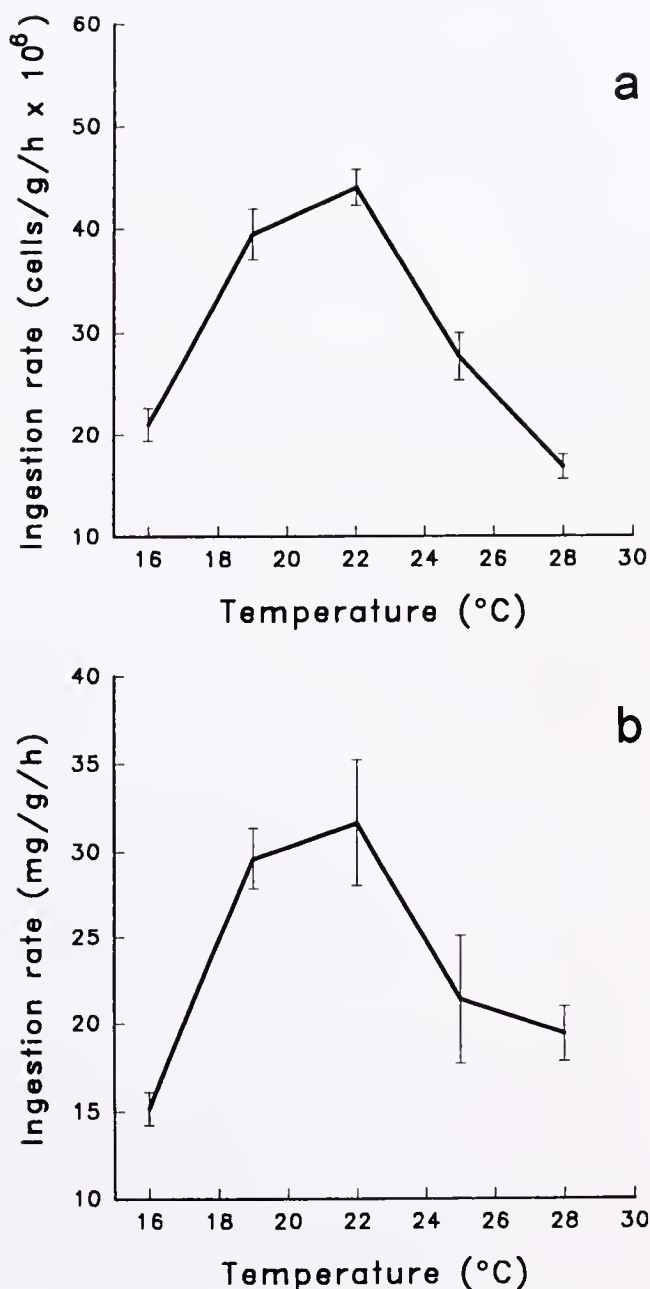


Figure 8. Ingestion rates of microalgae cells (a) and biomass (b) at different temperatures of catarina scallop (*Argopecten ventricosus-circularis*) juveniles. Values are the mean  $\pm$  s ( $n=3$ ).

(Fig. 8b). No pseudofeces were produced by the animals during the experiments. An analysis of variance (ANOVA) indicated a significant difference between treatments at  $P > .01$ . However, a Tukey's multiple range analysis (Table 2) showed that similar IRs were found among 16, 25, and 28 °C treatments and among 19, 22, and 25 °C treatments.

Because CR was calculated from IR data, the same trend was obtained (Fig. 9). Values varied between 8.7 and 17.8 l/g/h. An ANOVA indicated that differences between treatments were significant at  $P > .01$ . The Tukey's multiple range analysis (Table 3) showed similarities among 16, 25, and 28 °C treatments, for 22 and 25 °C, and for 19 and 22 °C.

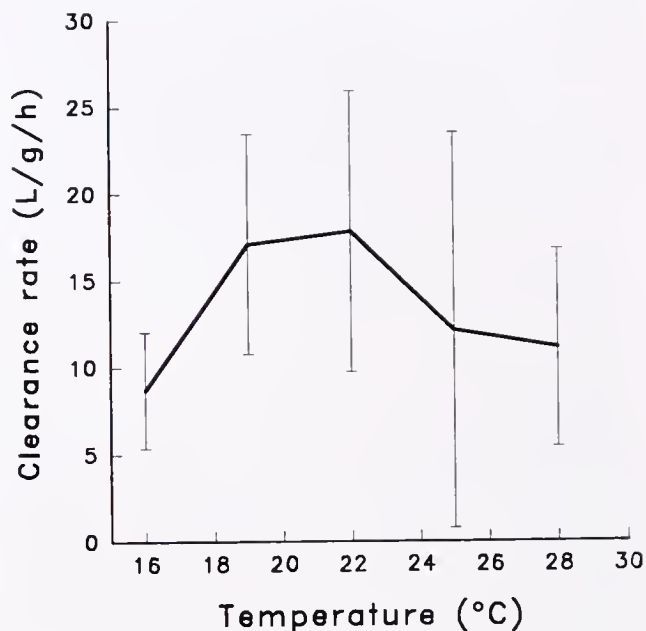


Figure 9. Clearance rates of catarina scallop (*Argopecten ventricosus-circularis*) juveniles at different temperatures. Values are the mean  $\pm$  s ( $n=3$ ).

#### Irrigation Efficiency

The ratio between liters of water cleared from particles against mL of oxygen consumed ( $L/mL O_2$ ) at different temperatures (Fig. 10) varied in the same manner as the scope for activity. Irrigation efficiency increased from 6  $L/mL O_2$  at 16 °C to 8.75  $L/mL O_2$  at 19 °C. Then this gradually declined to the minimum 3.65  $L/mL O_2$  at 28 °C.

#### Scallop Growth

Growth in scallop juveniles at different temperatures is shown in Figure 11. Growth was at a maximum at 19 °C, followed by 22, 16, 25, and 28 °C. A covariance analysis (Table 4) indicates there were significant differences between treatments at  $P > .01$ . However, the Tukey's multiple range analysis (Table 5) showed similarities for treatments at 19 and 22 °C and among 16, 25, and 28 °C treatments.

#### DISCUSSION

The scope for activity in catarina scallop juveniles showed the optimum temperature was between 19 and 22 °C. The concept of scope for activity introduced by Fry (1947) allowed us to deter-

TABLE 2.  
Tukey's multiple range test for ingestion rates in juveniles of catarina scallop (*Argopecten ventricosus-circularis*) at different temperatures.

Temperature	<i>n</i>	$\bar{x}$	Homogeneous Groups	
16	40	15.17	X	
19	39	29.61		X
22	16	31.63		X
25	29	21.37	X	X
28	39	19.38	X	

TABLE 3.

Tukey's multiple range test for clearance rates in juveniles of catarina scallop (*Argopecten ventricosus-circularis*) at different temperatures.

Temperature	n	$\bar{x}$	Homogeneous Groups		
16	40	8.71	X		
19	39	17.05	X		
22	16	17.84	X	X	
25	29	12.14	X	X	
28	39	11.14	X		

mine the temperature at which the scallops had the highest energy available for growth, independent from the energy needed for vital functions. Although the scope for activity provides valuable information, there are only a few reports in which this has been used in aquatic animals. This information is useful in the hatchery, where the production stages (i.e., maturation and larval and juvenile rearing) need to be done in as short a time as possible, for economic reasons. In addition, this information, together with thermotolerance data, is useful for site selection in aquaculture ventures. To get reliable data for scope for activity studies, a precise  $VO_2$  method is needed. In the present work, we used a miniaturized version (Maeda-Martínez 1985) of the Bryan et al. (1976) titrator. From unpublished data, the method has a resolution of  $2 \times 10^{-4}$  mL  $O_2/L$  and a coefficient of variation of 0.51 when using 7-mL ground neck borosilicate tubes. This microtitrator is currently under automatization at CIBNOR.

The method of using closed incubation chambers, as used in the present work (BOD bottle), gives fast and reliable results as in other work (Van Dam 1954, Read 1962, Iglesias and Navarro 1991, Navarro et al. 1991, Espina and Bückle-Ramírez, 1994). However, the closed chambers have several disadvantages, such as the decline in  $PO_2$  produced by the the respiration of the incubated organism (Bayne 1971, 1973; Silva-Loera 1986). To avoid incor-

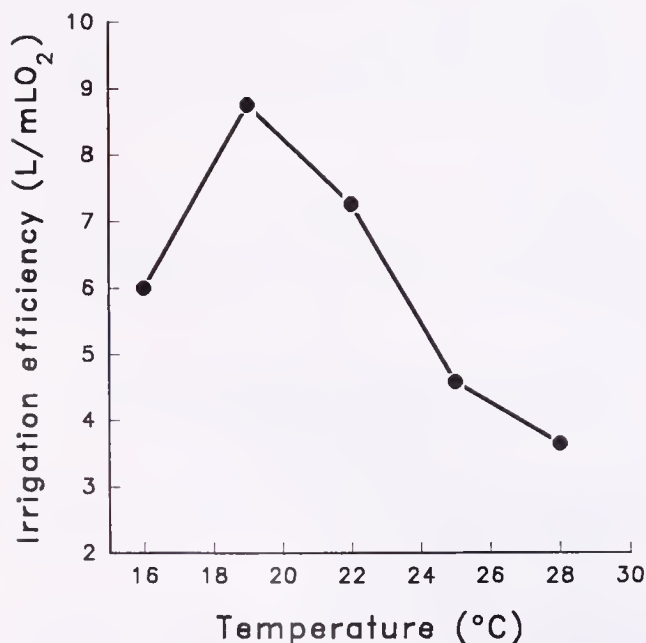


Figure 10. Irrigation efficiency of catarina scallop (*Argopecten ventricosus-circularis*) juveniles at different temperatures.

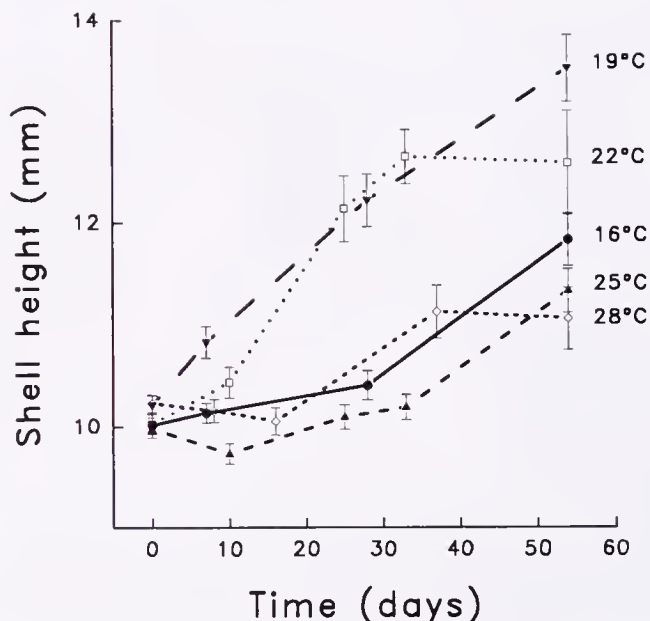


Figure 11. Growth of catarina scallop (*Argopecten ventricosus-circularis*) juveniles during 55-day experiment at different temperatures. Values are the mean  $\pm$  s (n = 50).

rect measurements, we need to know the  $VO_2$ - $PO_2$  relation in this species at different temperatures. Tang (1933), Mangum and VanWinkle (1973), and Sassaman and Mangum (1972) have proposed hyperbolic, quadratic, and semilogarithmic equations to describe the  $VO_2$ - $PO_2$  relation applicable to all aquatic organisms. Prosser and Brown (1961) defined the critical tension as the inflexion point of  $VO_2$  in relation to  $PO_2$  at which the organism loses its regulatory capacity to remain independent from  $PO_2$ . Critical tensions are known in several molluscan species (Van Dam 1938, Garder and Eliassen 1954, Van Dam 1954, Rothauwe 1958, Brand and Roberts 1973, Shumway 1983, Shumway and Scott 1983). Mangum and VanWinkle (1973) showed the difference between oxyconformer and oxyregulator species are only the extremes of a continuum. In our work, corrections for a drop in  $PO_2$  were not necessary, because  $VO_2$  was measured 1 hour after the start of incubation, before the critical tension was reached. In the catarina scallop, the critical tension was 76%  $O_2$  saturation (Sicard 1999), which is similar to the critical tension (75%) reported in *Mytilus edulis* (Bayne 1976).

The effect of temperature on IR and CR of catarina scallop juveniles was similar to the scope for activity, increasing from 16 to 19, and 22 °C, and decreasing to 25 and 28 °C. This indicates that IR and CR also serve as physiological indicators for ecophysiology studies. However, in *A. irradians*, CR remains independent

TABLE 4.

Covariance analysis between growth and temperature of juvenile catarina scallop (*Argopecten ventricosus-circularis*) with time as the covariate factor.

Source of Variation	Sum of Squares	D.F.	Mean Square	f	p
Time	592.1	1	592.1	281.3	0.000
Temp	332.6	4	83.0	39.4	0.000
Residual	2109.1	1002	2.1		



TABLE 5.

Tukey's multiple range test for growth of juvenile catarina scallop (*Argopecten ventricosus-circularis*) at different temperatures.

Temperature	n	$\bar{x}$	Homogeneous Groups
16	166	10.50	X
19	39	11.65	X
22	16	11.52	X
25	29	10.23	X
28	39	10.44	X

of temperature between 10 and 26 °C and is reduced considerably at 5 °C (Kirby-Smith 1970). No explanation could be drawn from the differences of CR versus temperature between these closely related species.

In the present work, CR was calculated assuming 100% retention efficiency. In filter feeders, the amount of food available to the organism varies directly with the volume of water pumped through the mantle cavity and the retention efficiency by the gill (Bricelj and Shumway 1991). The majority of filter-feeding bivalves are able to retain particles of 3–4 µm diameter or larger with 100% efficiency. This declines at lower particle size (25–90% for 2-µm particles) (Möhlenberg and Riisgard 1978, Riisgard 1988). In five members of the Pectinidae family, 100% retention efficiency has only been obtained when scallops were fed larger particles (5–7 µm). In the present work, IR and CR were studied using *I. galbana* cells of 3–4 µm diameter and 46–74 µm<sup>3</sup> volume (Enright et al. 1986). It would be convenient to determine retention efficiency versus particle size in the catarina scallop.

Another physiological indicator used in the present work was the irrigation efficiency (IE), also called convection requirement. In the catarina scallop, IE was a maximum at 19 °C and varied

between 3.7 and 8.8 l/ml O<sub>2</sub> within the range of temperatures studied. This is lower than that reported for other bivalve species. Mollusks living in coastal waters normally filter 15 l or more water per ml O<sub>2</sub> equivalent (Jørgensen 1975). A mean irrigation efficiency of 17 (8–25) was reported in *Pecten latiauratus* (Jørgensen 1960) and between 15 (at 5 °C) and 39 (at 20 °C) in 0.1 g dry tissue weight (DTW) *Chlamys opercularis* (McLusky 1973). The low IE values found in the catarina scallop could be caused by the small size of the juveniles employed (0.0087 mg DTW), because irrigation efficiency varies inversely proportional to the weight of the organism (McLusky 1973). In his work, irrigation efficiency of a 1.0 g DTW scallop was 11.5 l/ml O<sub>2</sub> as compared to 7.5 l/ml O<sub>2</sub> in a 0.1 g animal. The higher IE at 19 °C found in the catarina scallop indicates a maximum physiological efficiency at this temperature.

The results of scope for activity, ingestion rate (IR), clearance rate (CR), and IE were confirmed with the growth experiment. In this work, growth was a maximum at 19 and 22 °C and lower at lower (16 °C) and higher temperatures (25–28 °C). These results are in agreement with those of Monsalvo-Spencer (1998) in the same species, who reported a growth rate of 70 µm/day at 20 °C and 44 µm/day at 28 °C in 2.2 mm shell height juveniles. A similar correlation between growth rate and scope for activity has been reported in adult *Corbicula fluminea* (Foe and Knight 1986), *Crasostrea gigas* (Le-Gall and Raillard 1988), *Concholepa concholepa* (González et al. 1990), and in juvenile *Venerupis pullastra* (Albentosa et al. 1994) and *Ostrea edulis* (Beiras et al. 1994).

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## LEVELS OF RECRUITMENT AND ADULT ABUNDANCE IN A COLLAPSED POPULATION OF BAY SCALLOPS (*ARGOPECTEN IRRADIANS*) IN FLORIDA

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**ABSTRACT** We monitored the recruitment and density of adult bay scallops in a Gulf Coast of Florida population that had collapsed and not recovered. Recruitment and adult stock density were not closely related, and, although interannual variations in the levels of both were observed, no significant populational responses, including recovery of the population, were observed. Such natural stochastic events as toxic algal blooms, severe weather, and freshwater discharge, as well as human influences, act to depress populations. At low densities, recruitment failure because of low fertilization success is likely, and recovery of a population may be constrained by depensatory effects when stock density in a local population is low. Scallops were distributed patchily, and aggregative spawning as well as self fertilization may enhance spawning events generated from low adult densities. Natural recovery of populations may take a decade or more, and stock enhancement efforts may be necessary to restore harvestable stocks more quickly.

**KEY WORDS:** bay scallop, *Argopecten*, recruitment, stock–recruitment relationships

### INTRODUCTION

Fisheries biologists and ecologists are interested in the relationship between adult abundance and recruitment in broadcast-spawning invertebrates, albeit for different reasons. Biologists and managers concerned with invertebrate fisheries resources are interested in achieving some desirable and quantifiable goal, such as increasing the number of target organisms caught or preventing the overharvest of finite fisheries resources (Dredge 1988, Fogarty 1989, Murawski and Serchuk 1989, Quinn et al. 1993, Shepherd and Partington 1995). In some cases, managers need to make short-term predictions of the status of fisheries stocks and adjust allowable harvests based on those predictions. Fisheries models based on estimates of recruitment, growth, and mortality are desirable but problematic for short-lived invertebrates (Stearns 1976). The Australian abalone fishery is one example of how invertebrate fisheries can be managed based on stock assessment estimates (Prince and Shepherd 1992).

Ecological researchers have attempted to quantify relationships between stock and recruits to understand the population dynamics of broadcast-spawning invertebrates more completely. Although this information also would be useful to fisheries managers, the efforts at quantification have largely been unsuccessful for a variety of reasons (Loosanoff 1964, Loosanoff 1966, Hancock 1973, Muus 1973, Wolff 1988, Caddy 1989, Peterson and Summerson 1992, Rodríguez et al. 1993, Ólafsson et al. 1994, McShane 1995). Much of the basic research necessary to discern these relationships is beyond the scope or mission of fisheries scientists and begs for interdisciplinary cooperation with ecologists.

We have begun to focus on stock–recruitment relationships in Floridian populations of the bay scallop, *Argopecten irradians* (Lamarck 1819). Many bay scallop populations in Florida have experienced severe reductions in abundance during the past three decades (Arnold 1990), ostensibly because of overharvest and human-induced deterioration of water quality and scallop habitat. Concern has been expressed by citizens and biologists (Arnold et al. 1995) about the failure of many local scallop populations to recover following major declines. Stock densities in some populations may be so low that recruitment failure resulting from low

fertilization rates is acting against recovery. Research on fertilization dynamics in echinoderms suggests that this is a major concern (Pennington 1985, Levitan et al. 1992, Babcock et al. 1994, Levitan 1995).

We examined and describe here the relationship between adult abundance and recruitment in a local (*sensu* Hanski and Gilpin 1991) Floridian population of *A. irradians* that was very abundant before 1990 but has since declined and failed to recover. We discuss possible management strategies to enhance local bay scallop populations that rely on basic ecological research. We also discuss the dynamics of the local scallop population and speculate on why the population has failed to recover.

### MATERIALS AND METHODS

We began surveying adult scallop populations in June 1992; recruitment monitoring began during the late summer of 1992. We selected Homosassa Bay (Fig. 1) as a study site because of the relatively high abundance of bay scallops there. Unfortunately, the scallop population there declined substantially before we began the monitoring effort (Arnold et al. 1993).

Initially, we surveyed scallops within an area bounded by the latitudes 28°50'N and 28°40'N and depth contours from 1.2 m (4 ft.) to 3.7 m (12 ft.) below mean lower low water (MLLW). The latitudinal boundaries generally represent the geographic limits of Homosassa Bay, and the vertical (depth) boundaries were chosen based upon bay scallop distribution data gathered by Blake et al. (1991). We realigned the study area in 1993 to focus on habitats between 0.6 m (2 ft.) and 1.8 m (6 ft.) below MLLW, and we extended the northern latitudinal boundary to approximately 28°53'N. These adjustments were made to include what we had determined to be the limits of the majority of the historical Homosassa population.

#### Adult Survey

We surveyed the Homosassa Bay scallop population each June from 1992 through 1997. In 1992, we sampled 37 randomly located survey sites within the study area. At each site a 300-m.

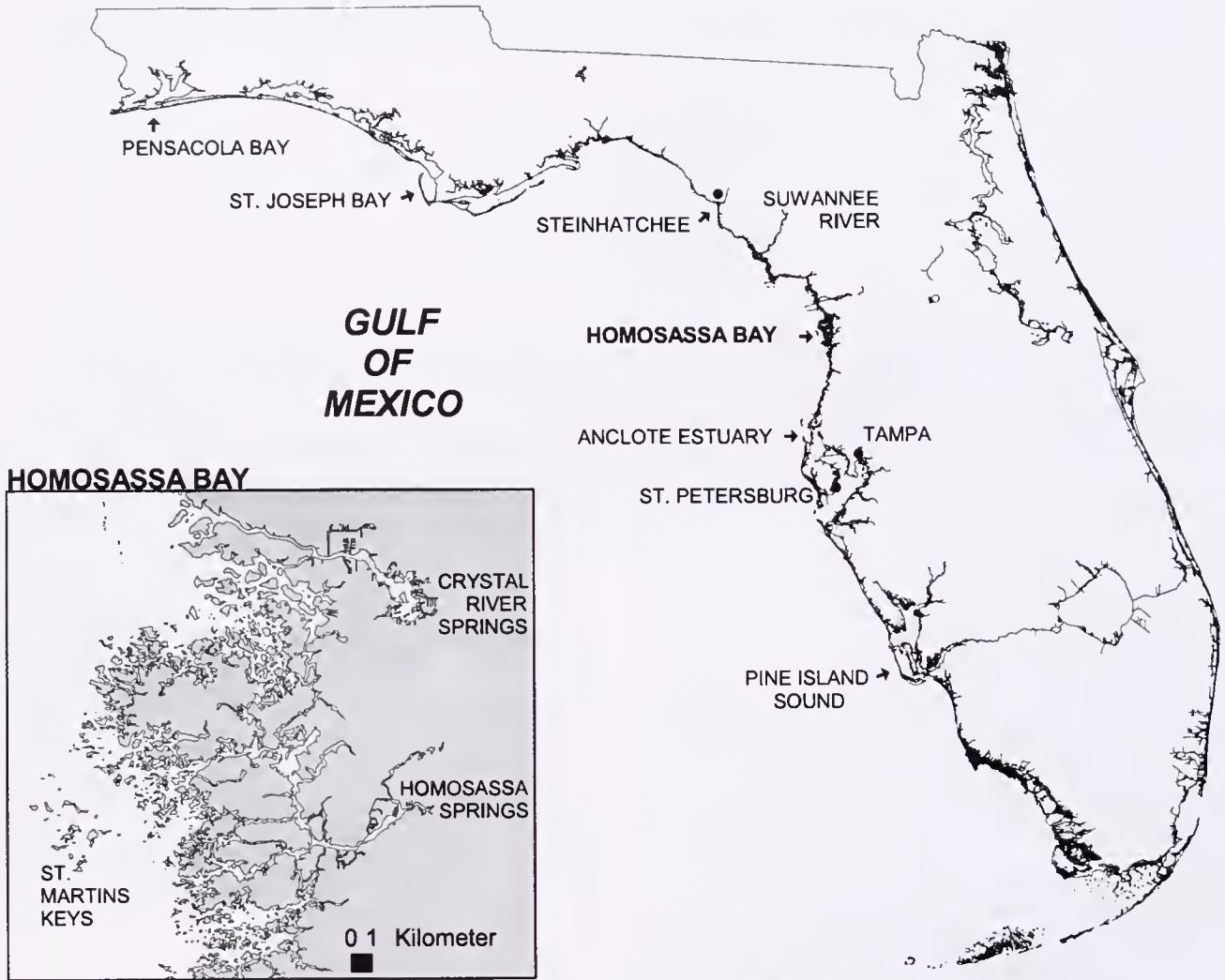


Figure 1. Location of the study area along the Florida Gulf coast. Inset shows detail of Homosassa Bay.

weighted transect line was deployed in a roughly equilateral triangle. A pair of SCUBA divers swam along the transect and counted all scallops within 1 m of each side of the transect. Following the 1992 survey and revision of the study area, 20 new stations were randomly chosen, and these stations were surveyed throughout the remainder of the study. Stations were assigned a posteriori to latitudinal groups: north (n = 6), central (n = 6), and

TABLE 1.

First deployment and final retrieval dates of *Argopecten irradians* recruit collectors in Homosassa Bay, Florida, 1992 to 1997.

Year	First Deployment	Final Retrieval
1992	08/11/92	12/16/92
1993	08/13/93	11/16/93
1994	08/17/94	02/12/95
1995	07/27/95	02/08/96
1996	08/07/96	01/29/97

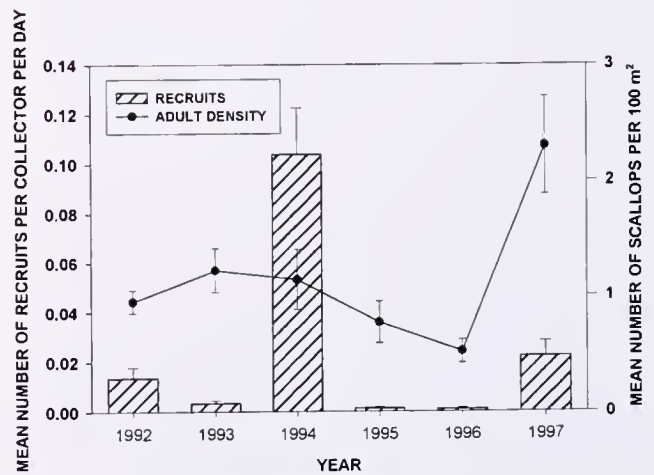


Figure 2. Levels of recruitment and adult abundance of bay scallops (*Argopecten irradians*) in Homosassa Bay, 1992 to 1997. Figures represent mean and standard error for each year.

TABLE 2.

Analysis of variance for effects of year and latitudinal position on density of adult *Argopecten irradians* in Homosassa Bay, Florida, 1992 to 1997.

Source	d.f.	SS	MS	F	P
Year	5	11.99	2.40	8.89	<.0001
Latitude	2	0.52	0.26	0.96	.386
Yr. × lat.	10	3.89	0.39	1.44	.161
Residual	256	69.10	0.27		
Total	275	85.33			

south ( $n = 8$ ). Survey data were natural log-transformed [ $y' = \ln(y + 1)$ ] and were analyzed using a two-way factorial analysis of variance (ANOVA) with factors of year and latitude. Hochberg's GT2 method was used to compare means when F-ratios for main effects were significant ( $P < .05$ ) (Hochberg 1974); this method is useful when variances are equal, but sample sizes are unequal (Day and Quinn 1989).

Contouring software (Surfer version 6, Golden Software, Inc.) was also used to plot the adult survey data so that the relative spatial distribution and density patterns within the Homosassa population could be identified. Bay scallops are generally contagiously distributed, and distribution patterns may be important in casting the distribution of future populations.

TABLE 3.

Analysis of variance for effects of year and latitudinal position on recruitment of *Argopecten irradians* to recruit collectors in Homosassa Bay, Florida, 1992 to 1997.

Source	d.f.	SS	MS	F	P
Year	5	$2.99 \times 10^{-1}$	$5.97 \times 10^{-5}$	25.67	<.0001
Latitude	2	$1.40 \times 10^{-6}$	$1.70 \times 10^{-7}$	0.30	.739
Yr. × Lat.	10	$3.19 \times 10^{-5}$	$3.19 \times 10^{-6}$	1.37	.190
Residual	612	$1.42 \times 10^{-3}$	$2.41 \times 10^{-6}$		
Total	629	$1.75 \times 10^{-3}$			

Recruitment Monitoring

In late August 1992, we deployed three recruit collectors at each of 20 randomly located sites in order to monitor scallop recruitment within the study area. A recruit collector consists of a 1/2-bushel citrus bag containing a 0.135-m<sup>2</sup> rectangle of 4-mm polypropylene mesh (Ambrose et al. 1992, Arnold et al. 1998). Collectors were suspended approximately 0.5 m above the substrate with an external doughnut float and held in position with a concrete anchor. Three weeks after the initial deployment, we placed a second set of three collectors at each site. Collectors were retrieved and replaced with new ones every 6 weeks until mid-December. The sampling period was chosen to maximize sampling

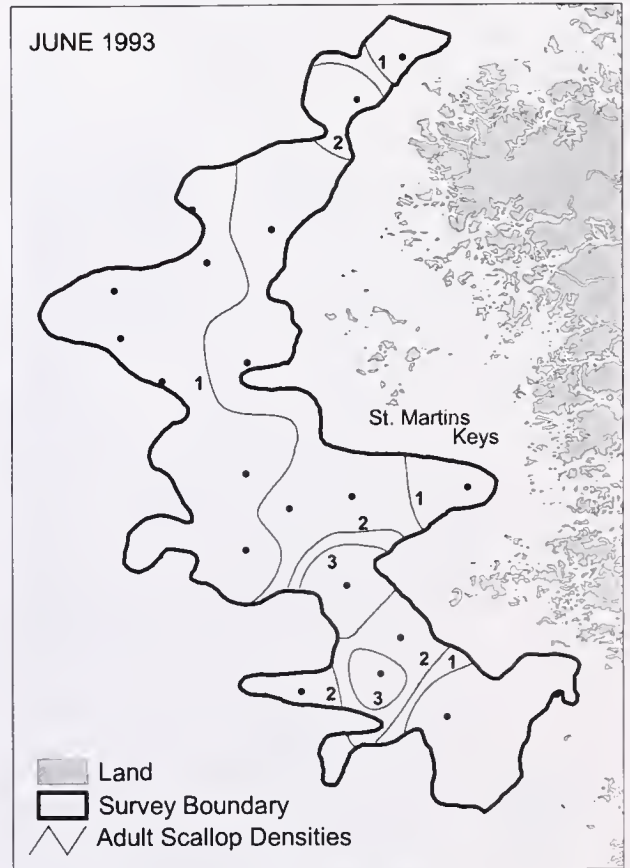
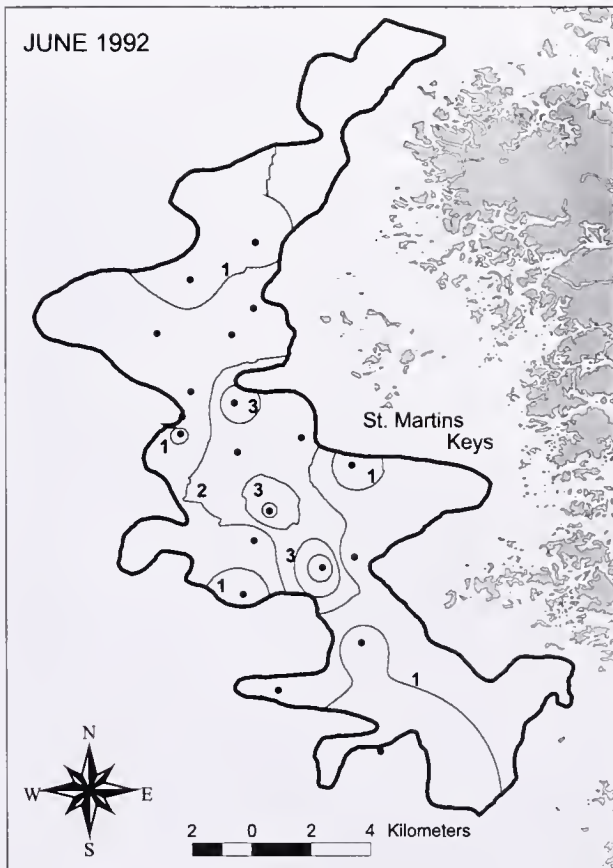


Figure 3. Distribution and abundance of adult bay scallops (*Argopecten irradians*) in Homosassa Bay, June 1992 to 1993. Densities are number of scallops · 100 m<sup>-2</sup>. Survey stations are indicated by (●).

of recruits based on the timing of scallop reproduction near these latitudes (Barber and Blake 1983) and on data from Sastry (1965) concerning the length of larval life. Missing collectors were replaced on the redeployment date. All identifiable bay scallop recruits were counted in the lab, and a recruitment rate (number of recruits per collector per day) was calculated for each trap recovered. In 1993, we reduced the number of recruitment monitoring sites to nine located on three east–west transects in arbitrarily chosen north, central, and southern locations within the survey area. Each transect included three stations located at depths of 0.6 m, 1.2 m, and 1.8 m below MLLW. Collectors were deployed as in 1992: three per station beginning in early August, and three more deployed after 3 weeks. Collectors were retrieved every 6 weeks and replaced until mid-November 1993. The 1993 experimental design was continued from 1994 through 1997 with only slight variations in deployment and final retrieval dates (Table 1).

We organized 1992 recruitment data a posteriori by latitudinal position into three groups—north ( $n = 4$ ), central ( $n = 10$ ), and south ( $n = 6$ )—so that these data could be integrated with the 1993 to 1997 data. The effects of latitude and year on recruitment rate were analyzed using a two-way factorial ANOVA following an arcsin–square-root transformation of the data. Because missing collectors created an unbalanced design, the analysis was conducted using the SAS GLM procedure (SAS Institute 1985). Where F-ratios were significant, Hochberg's GT2 method for comparing means was applied.

Finally, the relationship between recruitment and subsequent

adult densities in Homosassa was examined with a simple linear regression. We also examined the relationship between adult density and subsequent levels of recruitment.

## RESULTS

Adult densities were very low in Homosassa from 1992 through 1997 and were not much greater than  $1 \text{ scallop} \cdot 100 \text{ m}^{-2}$  until 1997 (Fig. 2). Latitude had no significant influence on scallop density ( $P = .39$ ), but the effect of year on density was significant (Table 2). Adult scallop density was greatest in 1997, the only year in which scallop density differed significantly from that in any other year.

Rates of recruitment to the collectors in the Homosassa area were also very low throughout the period we examined (Fig. 2). Recruitment in 3 of these years (1993, 1995, and 1996) was virtually undetectable. Analysis of these data suggested that no significant differences existed between latitudes within the bay ( $P = .74$ ), but that year had a significant effect on recruitment (Table 3). Mean recruitment was significantly greater in 1994 than in any other year examined.

Contour plots of Homosassa scallop density suggest that the population is distributed in patches that have recently had little geographic consistency within the region and that densities have been low throughout the area (Figs. 3–5). Despite interannual variations in density and position, aggregations seem to be a constantly identifiable feature in the population.

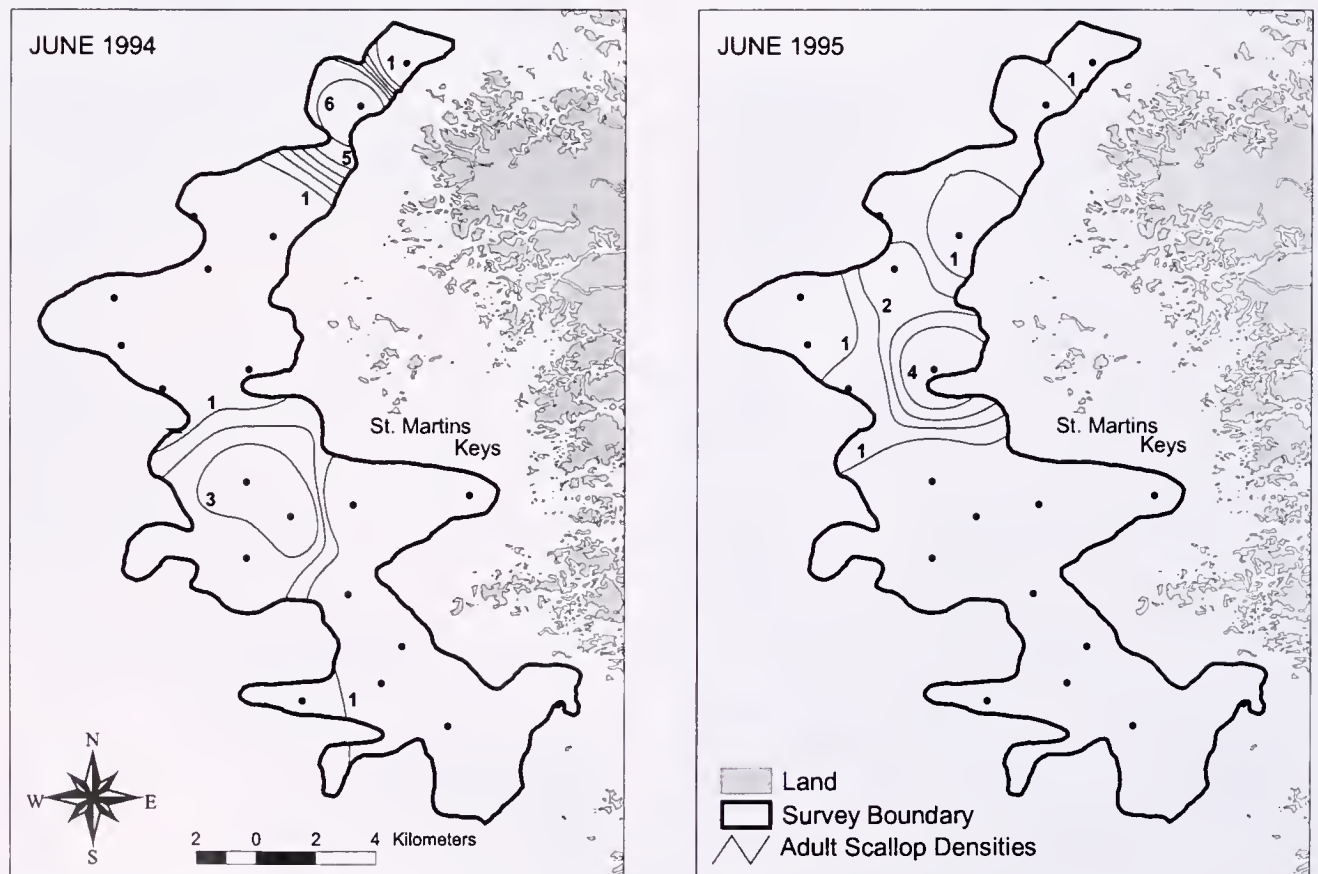


Figure 4. Distribution and abundance of adult bay scallops (*Argopecten irradians*) in Homosassa Bay, June 1994 to 1995. Densities are number of scallops  $\cdot 100 \text{ m}^{-2}$ . Survey stations are indicated by (●).

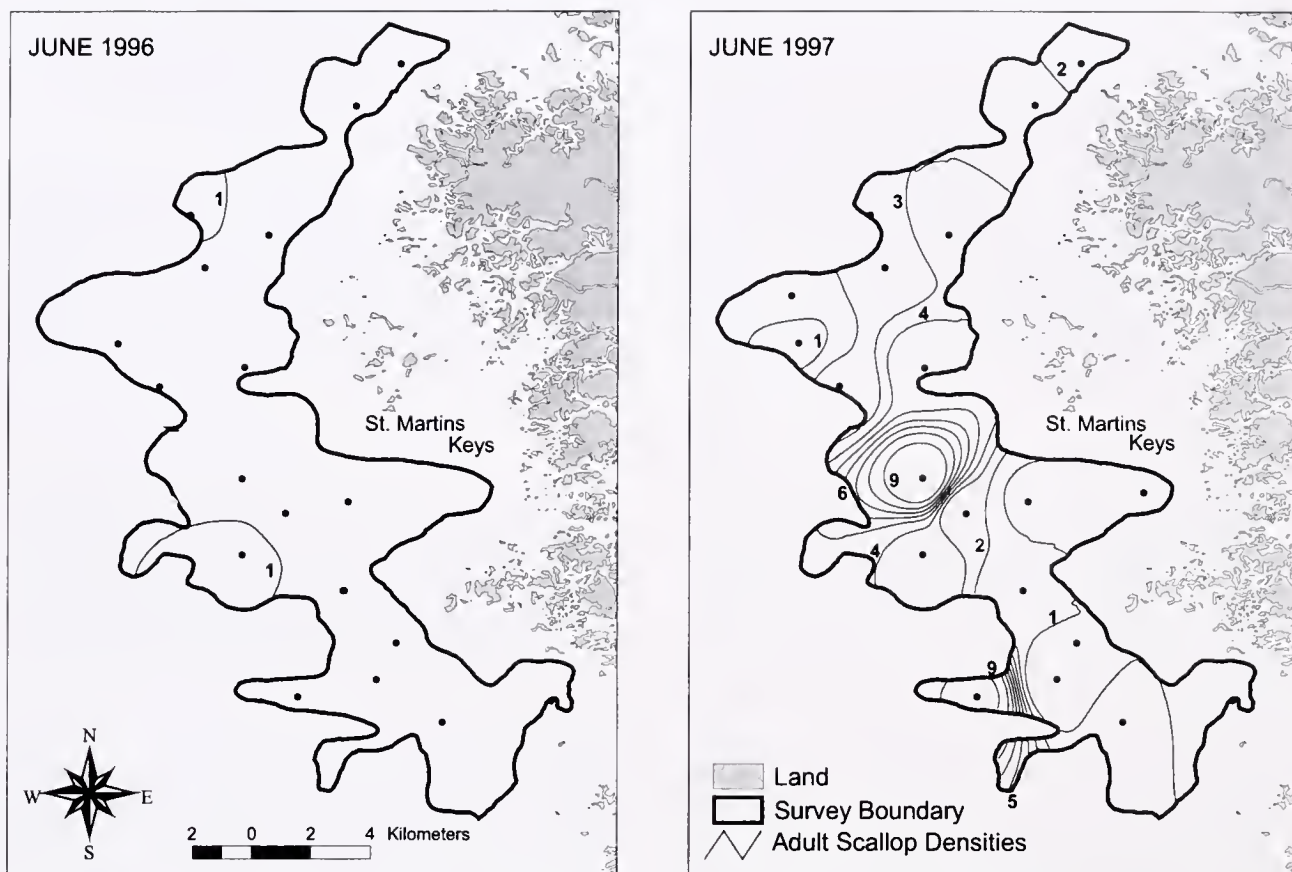


Figure 5. Distribution and abundance of adult bay scallops, (*Argopecten irradians*) in Homosassa Bay, June 1996 to 1997. Densities are number of scallops  $\cdot 100 \text{ m}^{-2}$ . Survey stations are indicated by (●).

Bay scallops in Florida are essentially annual animals (Barber and Blake 1983), and therefore, both recruitment and adult abundance are temporally related. However, our data demonstrated no significant relationship between recruitment and subsequent year-class density ( $r^2 = 0.003$ ) nor any relationship between adult density and subsequent recruitment ( $r^2 = 0.001$ ) (Fig. 2).

#### DISCUSSION

Low recruitment levels and low densities of adults in the Homosassa bay scallop population, with minor, discordant increases and decreases, may suggest a population collapse. Recruitment levels and adult densities in Homosassa have been lower than those of populations in all other areas we have monitored in Florida (Arnold et al. 1998). In addition, levels of bay scallop recruitment and adult abundance in Homosassa were far exceeded by the values of recruitment and abundance seen in the robust Floridian populations of Steinhatchee and St. Joseph Bay (Arnold et al. 1998) as well as some North Carolina populations (Summerston and Peterson 1990, Peterson and Summerston 1992) in which basin-scale coherence of levels of recruitment and adult density have been identified.

Stock-recruitment relationships in annual broadcast-spawning animals such as scallops are generally assumed to be very weak and difficult to demonstrate, and effects of harvest are assumed to be inconsequential (Garcia and LaReste 1981, Sale 1990, McShane 1995). However, there is clearly a relationship between recruitment and adult density when harvest pressure severely re-

duces population abundances, a situation typically described as "recruitment overfishing" (Pauly 1980, Dredge 1988, Breen 1992, Jamieson 1993, Shepherd and Partington 1995).

Low adult densities in the Homosassa population could result in poor recruitment and subsequent year-class strength for such reasons as reproductive failure, fertilization constraints (Clavier 1992, Yund and McCartney 1994), and postsettlement effects, including harvest pressure (Peterson and Summerston 1992). Consequently, it may be extremely difficult to identify stock-recruitment relationships at low scallop densities because of stochastic influences. The most parsimonious explanation for persistent low stock levels is that low densities reduce the probability of fertilization success. Levitan and Petersen (1995) reviewed experiments that have demonstrated this empirically in broadcast-spawning invertebrates, and they suggested that distances in excess of 1 meter may preclude the fertilization rates conducive to successful repopulation.

Low stock abundance in depauperate populations can be perpetuated by unsuccessful fertilization because of the difficulty of locating a mate (Allee effect; Allee 1938, Meyers et al. 1995). Adding the effect of predator saturation (typical of lower latitudes, see Vermeij 1978) can lead to depensation (Allee 1938). Bay scallops may be adapted for dealing with constraints to fertilization, because they are simultaneous hermaphrodites and capable of self-fertilization (Kellogg 1892, Wilbur 1995). Self-fertilization, although probably not adaptive in the long term, may allow a depauperate local population to persist until it is able to overcome

the constraints or until larvae from adjacent local populations resupply the local population and increase fertilization probabilities.

Aggregative spawning by scallops can also mitigate population-wide low spawner densities. Recruits in Homosassa may be the product of very few spawners, because aggregations may have an unduly large influence on fertilization in depressed populations, and because bay scallops are undoubtedly dependent upon larval retention mechanisms in local areas to resupply subsequent year classes (Peterson and Summerson 1992). Recovery of collapsed populations to "normal" levels may take a decade or more (Peterson and Summerson 1992). Management strategies that seek to enhance or restore populations by increasing fertilization success (e.g., Tegner 1992, Quinn et al. 1993, Tettelbach and Wenzel

1993, Blake 1998) or overcoming postsettlement effects (see Ólafsson et al. 1994) may help to restore such depleted populations if ecosystem-wide conditions are otherwise amenable.

#### ACKNOWLEDGMENTS

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## THE TELOMERES OF THE BAY SCALLOP, *ARGOPECTEN IRRADIANS* (LAMARCK)

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**ABSTRACT** The telomeres of the bay scallop, *Argopecten irradians* (L.) were isolated and identified using Southern blotting and chemiluminescent hybridization techniques. Four probes, (TTAGGG)<sub>3</sub>, (TTGGGG)<sub>3</sub>, (TTAGGC)<sub>3</sub>, and (TTAGG)<sub>3</sub> were used to determine the degree of crossreactivity with telomere sequences of other phyla. The bay scallop seems to have the same telomeric repeat sequence found in all vertebrates studied to date; namely, (TTAGGG)<sub>n</sub>. The possible role of telomeres in the short lifespan of this species is discussed.

**KEY WORDS:** telomeres, scallop, *Argopecten*, aging, lifespan

### INTRODUCTION

The bay scallop, *Argopecten irradians* (L.) is a commercially important species found as three subspecies along the east coast of the United States and into the Gulf of Mexico (Belding 1910, Clarke 1965). This species lives less than 2 years and generally reproduces only once in its lifetime (Belding 1910); whereas, other species of scallops may live to 5, 10, and even 20 years or more (Vahl 1981, Shafee and Lucas 1982, MacDonald 1986). Many factors, including fluctuations in eelgrass populations, water temperature, salinity, currents, food sources, predation, siltation, overfishing, coupled with the short lifespan of the scallop, have been blamed for the wide fluctuations seen in annual bay scallop harvests over the years (Belding 1910, Tettelbach et al. 1985, Peterson et al. 1989).

The lifespan of the northern subspecies of the bay scallop, *A. irradians* (L.) has been genetically determined to be between 18–26 months (Belding 1910, Gutzell 1931), with death usually occurring within a window of approximately 4 to 5 months in late winter and early spring in the northern subspecies, with most scallops dead by the end of May. A few, estimated to be between 5–10%, may survive to reproduce again (Belding 1910).

Recent studies have shown that telomeres, tandem repeats of usually five to six bases that are found at the end of chromosomes, may play a key role in determining just how long a species may survive (Harley 1991). With each cell replication, a number of telomeric repeats are lost, until a critically few remain, setting the stage for the beginning of cell senescence and death (Harley 1991, Allsopp et al. 1992, Vaziri et al. 1993).

Previous studies involving telomeres from yeast to humans have found sequences similar or identical to the tandem repeat (TTAGGG)<sub>n</sub> found in all vertebrates studied to date (Moyzis et al. 1988, Meyne et al. 1991). Variations of this sequence include that of the single celled organism *Tetrahymena thermophila*, which has the telomeric sequence (TTGGGG)<sub>n</sub> (Blackburn and Chiou 1981). A few insects studied to date have the sequence (TTAGG)<sub>n</sub> (Okazaki et al. 1993, Meyne and Imai, 1995); whereas, two marine polychaete worms have also been shown to have the vertebrate sequence (An et al. 1995). The sequence (TTAGGC)<sub>n</sub> has been identified in the terrestrial worm, *Ascaris lumbricoides* (Muller et al., 1991).

To date, only one study has been done on the phylum Mollusca, which tentatively identified the telomeric sequence of the oyster, *Crassostrea gigas* (Thunberg) to be (TTAGGG)<sub>n</sub> using fluorescent *in situ* hybridization (FISH) techniques (Guo and Allen 1997), who

also felt that Southern blotting and hybridization with a (TTAGGG) probe would be necessary to confirm the telomere sequence.

It is hypothesized that the bay scallop may begin its life with fewer telomeres, and subsequently, may run out of telomeres sooner than longer-living species. Before performing quantifying studies on the telomeres of long- and short-lived bivalve mollusks, it was first necessary to determine the telomere sequence of the bay scallop.

### EXPERIMENTAL PROCEDURES

Bay scallops were collected from the waters surrounding Nantucket Island off the coast of Massachusetts. Approximately 250 mg of digestive gland (excluding digestive tract), kidney, or gonad tissue were homogenized in 5.0 ml of DNazol obtained from Molecular Research, Inc. (Cincinnati, OH). After remaining at room temperature for 5 minutes, the homogenate was spun at 10,000 × g for 10 minutes, the supernate was poured off into new tubes, and 0.5 ml of 100% ethanol was added. The precipitated DNA was spun at 5,000 × g for 2 minutes and washed 2 times in 95% ethanol. This fraction still contained mucopolysaccharides that were removed by extraction with 200 μl of chloroform. The cleaned-up DNA was reprecipitated with 100% ethanol, washed once in 95% ethanol, and dissolved in TE buffer, pH 8.0. DNA purity was determined to have a 260/280 ratio of 1.8 to 1.9, and the integrity of the genomic DNA checked by electrophoresis in a 0.8% gel at 105 V for 30 minutes.

#### Restriction Enzyme Digestion

Aliquots of scallop DNA were digested with the restriction enzymes *Rsa*I and *Hinf*I obtained from Life Technologies, Inc. (Grand Island, NY), according to the manufacturer's protocol. These enzymes cut genomic DNA into pieces that leave the telomere restriction fragments (TRF) at the end of the chromosomes intact. These fragments contain the tandemly repeated telomeres along with some subtelomeric DNA. The digested DNA was electrophoresed in a 0.8% agarose gel at 105 V for 60 minutes, then stained in 0.5% ethidium bromide for 15 minutes and photographed.

#### Blotting and Hybridization

The gel was alkaline blotted onto a nylon membrane in 0.4M NaOH overnight, rinsed once in 2 × SSC buffer, and dried between sheets of blotting paper. The membrane was rinsed briefly in

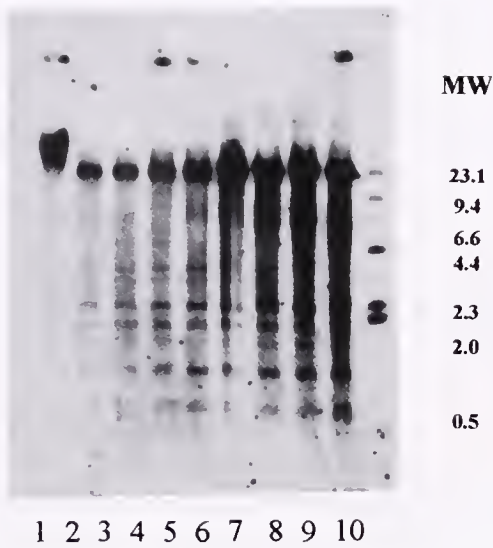


Figure 1. Increasing concentrations of DNA from the bay scallop *A. irradians* digested with *RsaI* and *HinfI* and probed with  $(TTAGGG)_3$ , lanes 2-9; lane 1, undigested DNA; lane 10, lambda molecular weight marker (1kb).

0.25M  $Na_2HPO_4$ , and prehybridized for 1 hour in 25 mL containing 1mM EDTA, 7% SDS, (sodium dodecylsulphate) and 0.25M disodium phosphate, pH 7.2 on a hybridizing rotator at 37 °C. After the addition of 4-5 pmol/mL of biotinylated probe, obtained from Genesys, Inc. (The Woodlands, TX), the membranes were hybridized overnight at 37 °C. Four telomeric probes were used,  $(TTAGGG)_3$ ,  $(TTGGGG)_3$ ,  $(TTAGGC)_3$ , and  $(TTAGG)_3$ . The membranes were then washed in increasingly stringent washes as follows: 2 × minutes in 2 × SSC; 1% SDS at room temperature; 2 × 15 minutes in 1 × SSC; 1% SDS at 37 °C, according to the manufacturer, with the exception that the highest stringency wash was increased from 2 × 5 minutes to 2 × 15 minutes in 1 × SSC at room temperature. The membranes were developed according to the chemiluminescent procedure of Tropix, Inc. (Bedford, MA), which conjugates the enzyme, alkaline phosphatase attached to streptavidin to the biotinylated DNA probe. Alkaline phosphatase

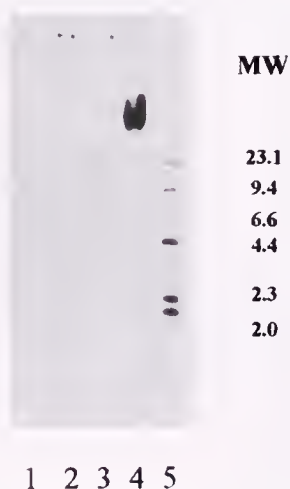


Figure 2. Increasing concentrations of DNA from the bay scallop *A. irradians* digested with *RsaI* and *HinfI* and probed with  $(TTGGGG)_3$ , lanes 1-3; lane 4, undigested DNA; lane 5, lambda molecular weight marker (1kb).

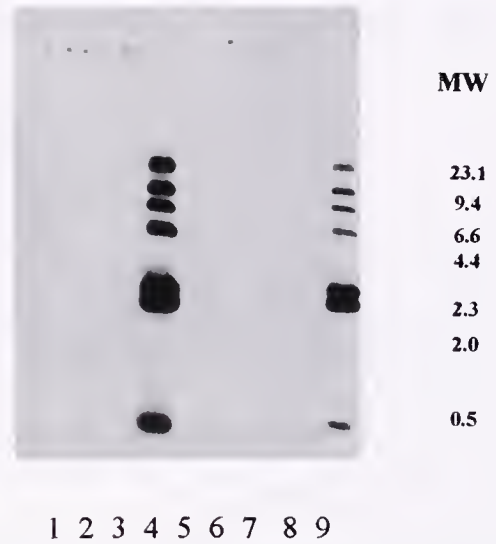


Figure 3. Increasing concentrations of DNA from the bay scallop *A. irradians* digested with *RsaI* and *HinfI* and probed with  $(TTAGG)_3$ , lanes 1-3;  $(TTAGGC)_3$ , lanes 5-8; lanes 4,9 lambda molecular weight marker (1kb).

then produces light as a byproduct of the reaction between alkaline phosphatase and the reagent 1,2 dioxatane. The membrane was inserted into a plastic sleeve and placed in an x-ray cassette and exposed from 2 minutes to 2 hours. The films were automatically developed in a Konica SRX-501A film processor.

#### Bal31 Digestion

Examples of the DNA were digested with the exonuclease, Bal31 to confirm that the DNA identified as telomeres were actually located at the end of the chromosomes. Bal31 removes bases from the ends of DNA one base at a time. A digest consisting of 200 µg of scallop DNA was incubated at 30 °C with fifty units Bal31 in 1.2 mL final volume of Bal31 buffer (Life Technologies, Inc.). At varying intervals, 150 µL of digest were removed, and the digestion was halted with the addition of 10 µL of 0.5M EDTA. DNA was then precipitated with 50 µL of 100% ethanol, reconstituted in TE buffer, pH 8.0, digested with the restriction enzymes *RsaI* and *HinfI*, and hybridized with the probe  $(TTAGGG)_3$ .

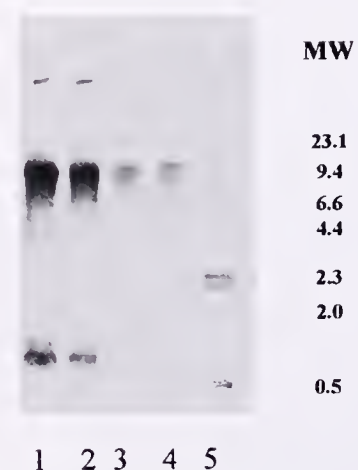


Figure 4. Bal31 digestion of DNA from the bay scallop *A. irradians* probed with  $(TTAGGG)_3$ . Lane 1, T = 0 h; lane 2, T = 1 h; lane 3, T = 3 h; lane 4, T = 4 h; lane 5, lambda molecular weight marker (1kb).

## RESULTS

The telomeric sequence of the bay scallop, *Argopecten irradians* was found to be (TTAGGG)<sub>n</sub> (Fig. 1). Several bands, containing telomere restriction fragments (TRFs) were found to be reproducible from scallop to scallop. These telomere restriction fragments, which contain the terminal telomere arrays plus a subtelomeric fraction to the point where the chromosome is cut by restriction enzymes, seem to be specific in length and are reproducible from scallop to scallop in the same age group (data not shown). Figure 1 also demonstrates the presence of telomeres of varying length found at any one time. Hybridization with the probes (TTAGGC)<sub>3</sub>, (TTGGGG)<sub>3</sub>, and (TTAGG)<sub>3</sub> demonstrated only slight binding, which was essentially lost with high stringency washes (Figs. 2, 3). Membranes were then stripped and rehybridized with (TTAGGG)<sub>3</sub> to confirm that the telomeres were present initially and available for potential binding with the three other probes. Figure 4 demonstrates digestion of the ends of the chromosomes by the exonuclease Bal31, which removes nucleic acids from the terminal end of chromosomes one by one, confirming that the DNA hybridized by the probe actually were telomeres. After 4 hours, all of the telomeres had essentially been removed, leaving only some internal repeat sequences that were not available for digestion by Bal31.

## DISCUSSION

The vertebrate telomeric probe (TTAGGG)<sub>3</sub> was used as a starting point to determine the telomeric sequence of the bay scallop. Guo and Allen (1997) found this to be the telomere sequence in the only mollusk, studied to date, the oyster, *Crassostrea gigas*. Three additional probes, representing three common nonvertebrate telomere sequences; namely, (TTGGGG)<sub>n</sub>, the telomere sequence in *Tetrahymena thermophila* (Blackburn and Chiou 1981),

(TTAGG)<sub>n</sub>, the pentanucleotide form found in several insects; for example, the silkworm *Bombyx mori*, and (TTAGGC)<sub>n</sub>, the telomere sequence in the nematode, *Ascaris lumbricoides* (Muller et al. 1991) were all found not to bind with the bay scallop telomeres. The individual bands, seen in Figure 1, contain the most numerous of the TRF lengths, and many of these telomeres are lost as the scallop ages (see Fig. 4, which is a Bal31 digest of a year 2 scallop, nearing the end of its lifespan). Compare to Fig. 1, which is the DNA of a much younger year 1 scallop.

The bay scallop seems to share the same telomeric sequence as found in vertebrates and many other species studied to date; namely, (TTAGGG)<sub>n</sub> (Meyne et al. 1989). This highly conserved structure may be the mitotic clock that determines the lifespan of a particular species (Harley 1991). People suffering from progeria, a syndrome that involves a rapid aging process, have fewer telomeres than healthy humans (Allsopp et al. 1992) as do those with Down's Syndrome (Vaziri et al. 1993). Both groups fail to live out a normal lifespan.

Recently, Bodnar et al. (1998) were able to extend the lifespan of human cells in tissue culture by increasing the number of telomere repeats through the introduction of the gene that codes for the enzyme telomerase into the cells. It is hypothesized that the bay scallop may have fewer telomeres initially than, for example, the deep-sea scallop *Placopecten magellanicus*, (Gmelin), which can live to 20 years of age, with the bay scallop running out of telomeres sooner. Alternatively, bay scallops may lose telomeres at a greater rate than longer-lived species. Studies are currently underway to quantify the telomeres from both species at different ages.

## ACKNOWLEDGMENTS

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## TEMPORAL VARIATION IN SEA SCALLOP (*PLACOPECTEN MAGELLANICUS*) ADDUCTOR MUSCLE RNA/DNA RATIOS IN RELATION TO GONOSOMATIC CYCLES, OFF DIGBY, NOVA SCOTIA

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**ABSTRACT** This study examines the usefulness of RNA/DNA ratios as an index of nutritional and other stress in wild populations of the sea scallop (*Placopecten magellanicus*). The seasonal variation in the RNA/DNA ratios of the adductor muscle of sea scallops, off Digby, Nova Scotia, were determined, and compared to the seasonal variations in adductor muscle and gonad weights. Results show that the RNA/DNA ratio of the adductor muscle for this scallop population varies with the size or age of the scallop and that there are temporal variations on both interannual and seasonal scales. The seasonal variation in RNA/DNA ratios can be modeled and approximates a sine function. This model explains a maximum of 45% of the variation in RNA/DNA ratios, and the residuals from the model are significantly correlated with subsequent growth. Temporal variations in RNA/DNA ratios are related to growth rate differences and reflect variations in food supply and temperature. RNA/DNA ratios seem to be a useful index of the health of a sea scallop stock, but without prior knowledge of what constitutes a level indicating increased mortality rates, it should not be the only health index used.

**KEY WORDS:** *Placopecten magellanicus*, sea scallop, RNA/DNA, growth, seasonal variation

### INTRODUCTION

RNA/DNA ratios have been used as an index of growth rate and indirectly, health for a variety of organisms (microorganisms Leick 1968; plankton Falkowski and Owens 1982, Dortch et al. 1983; higher invertebrates Sutcliffe 1969; including bivalves Pease 1976, Wright and Hetzel 1985; fish Buckley 1984, Hovenkamp and Witte 1991, Canino 1997; and mammals Monroe and Gray 1969).

The use of the RNA/DNA ratio as an index of growth is based on the fact that the amount of DNA per cell is remarkably constant for all normal somatic cells of a given species; whereas, RNA (ribonucleic acid) varies with the rate of protein synthesis. Because the amount of DNA per cell is essentially constant, and the amount of RNA varies with the level of protein synthesis, the ratio of RNA to DNA (RNA per unit DNA) is a self-calibrating index of the amount of protein synthesis taking place per cell.

Kennington (1994) proposed that the method could be used to monitor the health of scallops in areas closed to fishing activity. The purpose of such a monitoring program would be to provide a warning if the health of the scallops in the closed areas deteriorated to the point that an increased mortality rate was possible. In the first study to examine the potential of RNA/DNA ratios as a general index of health in a wild bivalve stock, Kennington (1994) reported both spatial and interannual variation in the RNA/DNA ratios of the adductor muscle in the sea scallop (*Placopecten magellanicus*) population off Digby, Nova Scotia, Canada. She concluded that the RNA/DNA ratio method would be useful in monitoring the health of the scallops, but that the seasonal variation would have to be determined before the method could be applied. The purpose of this study was to examine the temporal variation in the RNA/DNA ratio in the adductor muscles of the adult *Pla-*

*copecten magellanicus* population off Digby, Nova Scotia in terms of a seasonal and an interannual component. This study compares the seasonal variation in RNA/DNA ratios to the seasonal variations in adductor muscle and gonad weights. The results provide an important piece of information as to the usefulness of this technique as an index of nutritional and other stresses in wild populations.

### MATERIALS AND METHODS

The sampling of scallops for RNA/DNA analysis was attached to a program that had been initiated to examine temporal and spatial variations in adductor muscle weights. The study took advantage of a commercial scallop vessel fishing in the Digby area, whose owner had volunteered to conduct sampling (Vance Hazelton, Hazelton Fisheries Ltd., Digby N.S.). This had the advantage of no cost for chartering a vessel (depths and currents in the area excluded the use of small boats and scuba diving for the collections). It had the disadvantage of an irregular sampling schedule, because the vessel was not always fishing in the area (Table 1). In June of each year, samples were taken with the DFO research vessel, the J. L. Hart, during the annual scallop stock assessment survey on the Digby bed. The RNA/DNA data from the 1990 to 1992 June surveys was that used by Kennington (1994) to look at interannual and spatial variations, with the addition of the 1993 survey data. A preliminary analysis of the seasonal adductor muscle and gonad weight data up to January of 1993 was published by Kennington et al. (1994).

The area sampled was in the commercial scallop bed directly off Digby Gut. This area was divided into two zones, an inside zone (2-6 miles from shore), and an outside zone (6-10 miles from shore). Four random locations were sampled in each zone. At each

TABLE I.  
Sampling dates for RNA/DNA collections.

Year	Month											
	Jan.	Feb.	March	April	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.
1990						Z				Z		
1991				X	X	X					X	
1992			X		X	X		X	X		X	
1993	X				X	X		O				

Z = RNA/DNA samples only; X, both RNA/DNA and meat weight samples; O, meat weight samples only.

location, a tow of approximately 1 nautical mile was conducted using commercial scallop gear. The average depth (corrected for tidal height) was recorded at all stations, and the bottom temperature was recorded with a digital thermometer for the June tows for 1990 to 1993. A sample of up to 72 scallops were randomly collected from each tow for meat weights (except for August 1993, when only 30 were taken). The scallops were shucked at sea, the top shell was retained and the soft parts refrigerated and brought back for weighing onshore. Meat weights for all scallops and gonad weights for the first 30 of each sample were recorded to the nearest 0.01 g.

For the RNA/DNA sampling, six scallops in each of three size groups were taken from each tow location. The three size groups, 80–95 mm, 96–110 mm, and 111–125 mm, were selected to correspond roughly to ages 3, 4, and 5. These sizes are all commercially fished in this area. The scallops were dissected live, and the adductor muscle and top shell were retained. The muscle samples were held and transported in liquid nitrogen and then stored in a  $-85^{\circ}\text{C}$  freezer until analyzed to prevent the degradation of nucleic acids. Shell height was recorded, and the sex of each animal was determined visually when possible. Sex could not be visually determined for some animals in the postspawning period.

#### RNA/DNA Analysis

The tissue sample consisted of a 1-mm transverse section through the center of the phasic portion of the adductor muscle. (The large phasic portion of the adductor will be referred to simply as the adductor muscle and the smaller, tonic portion as the catch muscle). The nucleic acid concentrations were measured fluorometrically following the technique of Karsten and Wollenberger (1972, 1977) as modified by Kenchington (1994). For processing, the sample of adductor muscle was thawed and homogenized with a Brinkman Polytron homogeniser in 7 mL of ice-cold heparin solution ( $3.75\ \mu\text{g mL}^{-1}$ ) and centrifuged at 2000 rpm for 5 minutes at  $5^{\circ}\text{C}$ . This produced three layers, a bottom phase of cellular debris and a top foam layer, neither of which contained appreciable amounts of nucleic acids, and a middle clear phase in which the nucleic acids were concentrated. This middle phase was split into 5–200  $\mu\text{L}$  samples for analysis. Replicate total nucleic acid samples had 400  $\mu\text{L}$  heparin added, replicate DNA samples had 200  $\mu\text{L}$  heparin and 200  $\mu\text{L}$  RNAase. The fifth sample was a tissue blank and received 200  $\mu\text{L}$  heparin and 400  $\mu\text{L}$  distilled water. All five tubes were then incubated in a water bath at  $36\text{--}38^{\circ}\text{C}$  for 30 minutes. This incubation time allowed the RNAase reaction to go to completion without any degradation of DNA. After the incubation time, 200  $\mu\text{L}$  of ethidium bromide (EtBr) was added to the total nucleic acid and DNA samples. EtBr is a fluorophor that

reacts with nucleic acids to produce an increase in fluorescence intensity. It has an excitation wavelength of 365 nm and an emission wavelength of 590 nm. Fluorescence was measured with a Turner 112nm fluorometer using an excitation filter for 320–390 nm and an emission filter of  $>590$  nm. Calf thymus DNA and yeast RNA were used for the calibration of standard curves. Fluorescence readings were converted to nucleic acid concentrations using the calibration curves. The DNA concentration was calculated directly from the RNAase-treated samples, the RNA concentration was calculated as the difference between the concentrations of the total nucleic acid and DNA samples. RNA/DNA was calculated as a mass ratio. A reagent blank, prepared fresh each run, was made with 200  $\mu\text{L}$  EtBr, 400  $\mu\text{L}$  distilled water, and 200  $\mu\text{L}$  heparin. Analytical grade reagents were used for all procedures.

#### DATA ANALYSIS

##### RNA/DNA

A plot of the RNA/DNA data indicated that the mean ratio and the associated variance of scallop adductor muscle RNA/DNA ratios were related (Fig. 1a;  $r = 0.60$ ,  $P < .001$ ). Natural log transformation of the data did not resolve this (Fig. 1b;  $r = -0.37$ ,  $P = .023$ ), and there were still an abundance of outliers (Fig. 1). These factors would compromise the use of a normal probability model for the analysis, and so a generalized linear model (GLM) was used. Generalized linear models differ from such classic linear models as linear regression and analysis of variance (ANOVA) in that the assumptions of normality and constant variance are not required for the error component. Any distribution in the exponential family (i.e., Poisson, binomial, gamma) can be used. These models retain the idea of a predictor based on a linear combination of explanatory variables. In particular, the ideas underlying such factorial models as additivity, interaction, polynomial contrasts, and aliasing are retained in the GLM. Without the assumption of normality for the error component, they estimate parameters by maximum likelihood using an iteratively reweighted least-squares approach. The GLM uses separate functions to model any nonlinearity between the mean and the predictor (the link function), and the relationship between the mean and variance (the variance function). They are closer to a reparameterization of the model than to a re-expression of the response (McCullagh and Nelder 1989, Hastie and Pregibon 1992).

The analysis was carried out using an analysis of deviance with a gamma distribution and an identity link. The gamma distribution is very useful for positive random variables, because it can assume a wide range of shapes from symmetric to highly skewed (McCul-



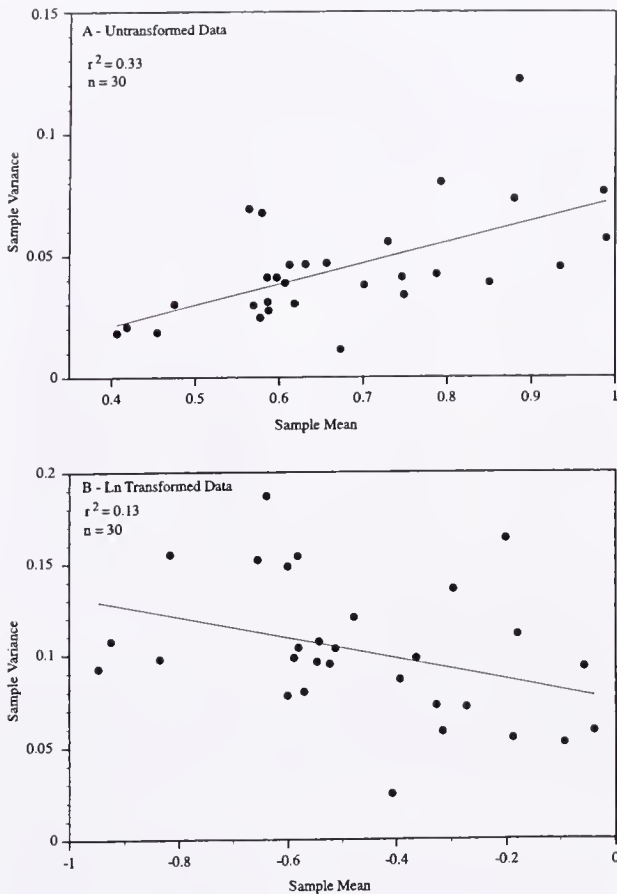


Figure 1. Sample variance versus mean for A raw and B Ln transformed RNA/DNA ratios of scallop adductor muscles.

lagh and Nelder 1989). A constant linear relationship between the mean and variance, as observed here, is a feature of this distribution. The analysis was performed with the S-PLUS statistical package (Statistical Sciences Inc. 1995).

Because the same months were not sampled each year, effects were first tested using *sampling date* (15 samples), *zone* (2–6 miles, and 6–10 miles from shore), *size* (3 classes), and *tow* (four tows, nested within each combination of *sampling date* and *zone*). The full model with all effects and interactions was tested first, and then submodels sequentially deleting nonsignificant terms were tested until all remaining terms were significant (Lawless et al. 1978). At each step, differences between nested models were tested for significance using a Chi-squared test.

The factor *tow* was included in the analysis to examine the significance of differences between tows, but would not be included in a model to examine the general health of a stock. If differences between tows on a single sampling date were larger than seasonal or stress effects, the method would not be as useful for monitoring the health of the stock. As a practical tool, tow effects would be left out of the model, thus incorporating tow-to-tow differences in the error term.

The irregular sampling schedule resulted in 6 of the 9 months sampled in only 1 year, and June the only month sampled in every year. This means that modeling seasonal variation by breaking *sampling date* down into *year* and *month* effects would produce a model in which *month* and *year* are too highly aliased to produce true *month* and *year* coefficients. To examine the seasonal and

interannual components of the variation in RNA/DNA ratios, the model with *sampling date* as a factor (15 sampling dates over the 3.25-year period) was used to predict RNA/DNA ratios for a shell height of 100 mm, and a seasonal model (described below) was then fit to the entire sampling period (see seasonal model below).

Interannual effects were examined using the May and June samples from each year in a model with the *sampling date* factor replaced with *year* and *month* effects. The seasonal model was fit to the gonad, adductor, and catch muscle weights to compare the fit of the seasonal model to the weight data versus the fit to the RNA/DNA data.

#### Adductor Muscle, Catch Muscle, and Gonad Weights

The adductor, gonad, and catch muscle weights were also analyzed with a GLM. Because distinct size classes had not been used for these samples, the log of the shell height was used as a covariate in the analysis with a log link and gamma error functions. The relationship with the covariate was first tested by fitting a common slope and intercept to all data, a common slope with separate intercepts, separate intercepts with a common slope, and separate intercepts and slopes. The best relationship (most significant, minimum deviance) was used for the rest of the model. Factors examined were *sampling date* (14 samples), *zone* (2–6 miles and 6–10 miles from shore), and *tow* (four tows, nested within each combination of *sampling date* and *zone*).

The number of scallops in the adductor and catch muscle datasets varied between samples, because it was not always possible to obtain 72 scallops at each site. To investigate the effects of a highly unbalanced design on the analysis, a random subset of the data taking 30 scallops per tow and three tows were drawn. This subset resulted in a fully balanced design, and this was analyzed and compared with the results from the full dataset.

The subset of the data for which both temperature and depth were available was used to compare the effect of these to the factor *zone*, and the subset of the data for which sex was available was used to examine the effect of this factor.

#### Seasonal Model

Adductor and gonad weights have been shown to follow an annual cycle in *Placopecten magellanicus* (Robinson et al. 1981, Couturier and Newkirk 1991, Kenchington et al. 1994). A sine-wave function was chosen as the model to describe this cycle. It has a well-defined pattern, a small number of parameters to fit to the data, and has been used to incorporate seasonal fluctuations into von Bertalanffy growth functions (Pitcher and MacDonald 1973, Pauly and Gaschultz 1979, Antoine et al. 1979, Hanumara and Hoenig 1987, Allison 1994). A sine function is symmetric and will not model the abrupt decline upon spawning of an individual scallop gonad, but as a model for the population, which has a more protracted spawning event and interannual variation in spawning time, the sine curve should be an adequate model of the annual cycle. The seasonal patterns in the weight and ratio data were, therefore, modeled by fitting the sine function  $\{A + B[\text{Sine}(\text{Julian\_Date}/365 * 2\pi + C)]\}$ , where A, B, and C are constants for the vertical position of the function, the amplitude of the sine wave, and the horizontal offset.

Because significant differences between years were found in the RNA/DNA ratio data, the data were standardized by dividing each year's data by the mean for the June sample in that year. Recall that June was the only month sampled in every year. The

sine wave model was then fit to the standardized data, and the significance of the different components of the model were estimated with partial F tests for parameters added in order (Kleinbaum et al. 1988).

The model was fit to the data with the nonlinear regression (NLR) procedure in the SPSS statistical package (SPSS Inc. Chicago, IL). This procedure fits user-defined models iteratively using the Levenberg-Marquardt algorithm.

If there is a relationship between RNA/DNA ratios and growth rates for scallops, then the residuals from the seasonal RNA/DNA ratio model should be correlated with the residuals from the seasonal adductor muscle growth model in the subsequent time period. That is to say, RNA/DNA ratios higher than the predicted seasonal values should correspond to growth rates that are also higher than predicted levels, and, conversely, low RNA/DNA levels should occur with low growth rates. Residuals for each time period in the seasonal growth model were calculated as the difference in the residuals from the fit of the sine curve to the GLM sample means in successive samples.

The residuals for the RNA/DNA ratios from the fit to the sine curve (Mean GLM Ratio<sub>t1</sub> - Sine Predicted Ratio<sub>t1</sub>) were compared to the residuals from the adductor muscle weight fit to the sine curve for the subsequent time period [(Mean GLM Weight<sub>t2</sub> - Sine Predicted Weight<sub>t2</sub>) - (Mean GLM Weight<sub>t1</sub> - Sine Predicted Weight<sub>t1</sub>)], where mean GLM ratios and weights are the standardized means from the GLM analysis, sine predicted values are those predicted by the sine functions, and the subscripts t1 and t2 indicate successive sampling dates. These data were analyzed to determine if there was any correlation between the residuals from seasonal RNA/DNA ratios and residuals from seasonal growth in the subsequent time period.

## RESULTS

### RNA/DNA Data

The GLM model with all effects and interactions explained a significant portion of the variation in the RNA/DNA ratios. *Zone*, *tow* nested within *sampling date* by *zone*, and the interaction terms were all nonsignificant. The best model includes only the main factors; *sampling date* and *size* (Table 2). The predicted RNA/DNA ratios for the three size classes (Table 3) show a decrease with size. Using the May and June samples to look at interannual variations showed that year was significant ( $P = .0004$ ), *month* and the *month by year* interaction were not. Comparisons of *years* showed that mean RNA/DNA ratios for 1991 were significantly higher ( $P < .01$ ) than those for 1990, 1992 and 1993, which were not significantly different.

TABLE 2.

Analysis of deviance table for the final model for RNA/DNA ratio data using *sampling date* and *size* factors. Response: Ratio; Distribution: Gamma; Link: Identity.

Terms	Df	Deviance	Residual		P (Chi)
			ΔDf	ΔDeviance	
NULL	1437	213.2474			
Date	14	64.5941	1423	148.6533	<.0001
Size	2	9.6559	1421	138.9974	.0080

TABLE 3.

RNA/DNA ratios predicted from final model for sampling dates and size classes.

Sampling Date	80-95 mm	96-110 mm	111-125 mm
June 22, 1990	0.6522	0.5735	0.5159
Oct. 9, 1990	1.0406	0.9618	0.9043
April 9, 1991	0.9084	0.8297	0.7722
May 6, 1991	0.7665	0.6878	0.6302
June 19, 1991	1.0024	0.9237	0.8661
Nov. 7, 1991	0.6991	0.6204	0.5629
March 31, 1992	0.5314	0.4527	0.3952
May 4, 1992	0.6656	0.5869	0.5294
June 6, 1992	0.6394	0.5607	0.5031
Aug. 11, 1992	0.8988	0.8201	0.7625
Sept. 9, 1992	0.7437	0.6649	0.6074
Nov. 10, 1992	0.8302	0.7515	0.6939
Jan. 7, 1993	0.6690	0.5903	0.5328
May 10, 1993	0.5878	0.5091	0.4516
June 6, 1993	0.7324	0.6537	0.5962

### Adductor Muscle, Catch Muscle, and Gonad Weight Data

The analysis of deviance for the three tissue components are shown in Tables 4-6. For all three tissues, the effect of fitting the log of shell height as a covariate was highly significant, and separate fits for each sample were significantly better than a common equation fit to all samples. A common slope with separate intercepts fit best for the meat weights. There was no significant difference between models for the gonad weights, and the minimum deviance model of a common intercept with different slopes was chosen. The model with different intercepts and slopes was significantly better for the catch muscle weights ( $P = .04$ ) than a model with a common intercept and different slopes, although not significantly better than a model with a common slope and different intercepts ( $P = .08$ ). The model with different intercepts and slopes was chosen, on the basis of minimum deviance, for the rest of the analysis. *Sampling date* and *zone* were significant factors in the weight of all three tissues; whereas, *tow*, nested within *sampling date* and *zone*, and the interaction of *sampling date* and *zone* were not. Interannual comparisons using the May and June samples showed significant differences in the catch muscle ( $P = .039$ ), but not for the adductor or gonad samples.

There was no difference in the results using the full dataset for the weight data or the subset used for the fully balanced design. *Shell height*, *sampling date*, and *zone* were significant ( $P < .05$ ), and *tow* nested within *sampling date* and *zone*, and the interaction of *sampling date* and *zone* were not ( $P > .8$ ). The final GLM

TABLE 4.

Analysis of deviance table for the final model for meat weight data. Distribution: Gamma; Link: Log.

Terms	Df	Deviance	Residual		
			ΔDf	ΔDeviance	P (Chi)
NULL	7366	1492.520			
Ln(Height)	1	1241.508	7365	251.012	<.0001
Date	13	81.303	7352	169.709	<.0001
Zone	1	36.336	7351	133.372	<.0001

TABLE 5.

Analysis of deviance table for the final model for gonad weight data. Distribution: Gamma; Link: Log.

Terms	Df	Deviance	Residual		
			ΔDf	ΔDeviance	P (Chi)
NULL	3331	1693.302			
Zone	1	208.998	3330	1481.304	<.0001
Date in Ln(Height)	14	1229.323	3316	254.981	<.0001

models with the full dataset were used to predict means for a 100 mm shell height scallop from each zone (Table 7).

To examine what made the factor *zone* significant in all the tissue weights examined, temperature and depth were compared to *zone*, using the subset of data for which they were both available (June samples, 1990 to 1993). When individually entered into the model after *shell height* and *sample date*, the factor *zone* was the most significant ( $P = .004$ ), followed by *depth* ( $P = .007$ ) and *temperature* ( $P = .0160$ ). The factors are, however, all correlated to the extent that after any one of the three is entered, the others will be nonsignificant, and there is no significant difference between models using any one of the three factors. Taking the scallops for which *sex* could be determined (5,947 of 7,367 scallops), there were no significant differences between males and females for meat weight ( $P = .4548$ ), gonad weight ( $P = .3071$ ), or catch muscle weight ( $P = .5225$ ).

Seasonal Model

The predicted means from the final models of the RNA/DNA ratios (Table 3) and the tissue weights (Table 7) were modeled by fitting a sine function to give a seasonal cycle. Figure 2 shows the fit to the small *size* class (80–95 mm) for the ratio data and the predicted means for the tissue weights for a 100 mm *shell height* scallop from the inside *zone* (Table 7). The  $r^2$  values for the model fit to the data for the inside zone and small size class were: RNA/DNA ratio 0.22; *meat* weight 0.56; *gonad* weight 0.69; and *catch muscle* weight 0.26. The cycles in the meat weight and gonad weight are nearly in opposite phase; when one is dropping the

TABLE 6.

Analysis of deviance table for the final model for catch muscle weight data. Distribution: Gamma; Link: Log.

Terms	Df	Deviance	Residual		
			ΔDf	ΔDeviance	P (Chi)
NULL	7366	1399.552			
Date	13	73.482	7353	1326.070	<.0001
Zone	1	280.931	7352	1045.140	<.0001
Date in Ln(Height)	14	711.058	7338	334.082	<.0001

other is rising. The predicted values for the adductor muscle weight go from a low in June–August to a high in November–January (Fig. 2). The percentage increase from the low to the high for 1991 to 1993, calculated from the standardized means for the inside zone in Table 7, are 31, 37, and 35%, respectively. There is some indication of interannual differences in the timing of the seasonal cycle. In June of 1991, the adductor muscle weight was still high on the 19th of the month, dropping only 5% from the May value of 14.79. The drops from May to June in 1992 and 1993 were 11 and 14%. Because the annual ranges are similar, this indicates an interannual variation in the timing of the cycle. The curve for the RNA/DNA ratio is slightly lagging the gonad cycle, with a peak during the period when the adductor muscle weight is increasing. The fit of the sine function to the RNA/DNA data (Fig. 2) shows that, although there may be a seasonal component, there is a high level of variation, with the RNA/DNA ratios from October 1990 to June 1991 well above the predicted seasonal values. This variation is not a true interannual component, as shown when the data are standardized to the June values within each year (Fig. 2). The fit for the April to June 1991 ratios improves, moving these datapoints closer to the model prediction, but still leaves the Oct. 1990 and November 1991 values as larger outliers.

The partial F test on the ratio data standardized to the June samples each year (Table 8) shows that fitting parameter A, essentially a straight line fit to the mean RNA/DNA ratio value, is highly significant ( $P < .0001$ ). Adding  $B \cdot \text{Sine}(\text{Rdate})$ , which includes the sine wave in the function (Rdate is the Julian date

TABLE 7.

Predicted means for adductor muscle, gonad, and catch muscle weights for a scallop with a shell height of 100 mm.

Date	Means for Inside Zone			Means for Outside Zone		
	Adductor	Gonad	Catch	Adductor	Gonad	Catch
April 9, 19991	14.791	4.041	1.122	12.694	3.379	0.997
May 6, 1991	14.791	6.152	1.187	12.694	5.143	1.055
June 19, 1991	13.980	9.127	1.084	11.998	7.631	0.963
Nov. 7, 1991	18.263	2.799	1.237	15.674	2.340	1.100
March 31, 1992	15.359	3.039	1.167	13.182	2.541	1.037
May 4, 1992	15.359	4.907	1.196	13.181	4.102	1.063
June 6, 1992	13.652	6.956	1.062	11.716	5.816	0.944
Aug. 11, 1992	11.916	9.175	0.907	10.226	7.671	0.806
Sept. 9, 1992	15.556	2.685	1.277	13.350	2.245	1.135
Nov. 10, 1992	16.290	1.965	1.212	13.980	1.643	1.077
Jan. 7, 1993	15.958	1.892	1.153	13.696	1.582	1.025
May 10, 1993	15.388	5.358	1.230	13.206	4.480	1.093
June 6, 1993	13.180	7.115	0.707	11.312	5.949	0.628
Aug. 24, 1993	11.784	4.316	0.935	10.113	3.608	0.831

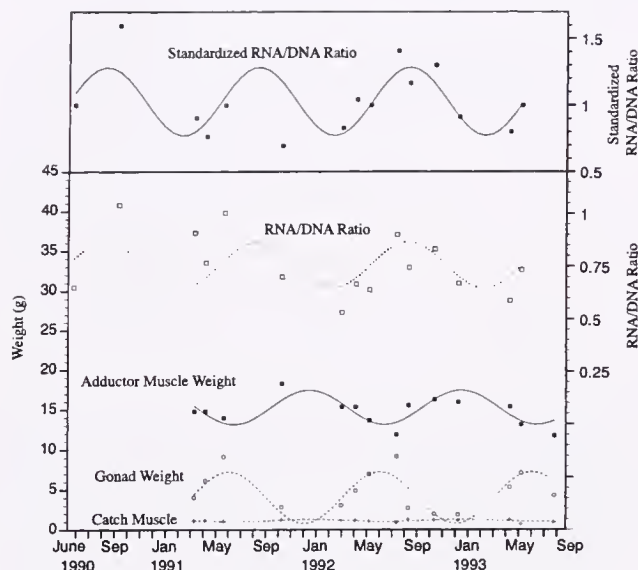


Figure 2. Annual cycle in RNA/DNA ratios, adductor muscle, gonad, and catch muscle weights, as modeled with a sine function for the full time period sampled. RNA/DNA ratios are for adductor muscle tissue from a 80–90 mm shell height scallop. Weights are standardized to a 100 mm shell height scallop from the inside zone. Standardized RNA/DNA ratios are standardized to the June ratio within each year.

converted to  $2\pi$  radians per year), results in a significant ( $P = .01$ ) improvement in the model. This means that the addition of the sine wave has explained significantly more of the variance in the model than the fit of a straight line (model with just parameter A). In the case of the ratio data, parameter C does not significantly improve the model. This is because the sine wave is not shifted horizontally in the case of the ratio data, so parameter C is not significantly different than 0 (actually parameter C will converge to a value, depending on the initial starting estimate, of 0,  $2\pi$ ,  $4\pi$ , etc.). Standardizing the RNA/DNA ratios to eliminate interannual variability increases the  $r^2$  value from 0.22 to 0.45 for the fit of the seasonal model.

The residuals from the predicted seasonal cycle in RNA/DNA ratios (calculated as observed values with GLM size standardization – values predicted by sine function) are correlated with residuals in subsequent growth for the adductor muscle (correlation = 0.484,  $P = .047$ ). This relationship can be seen in Figure 2, where the high ratios in April to June 1991 are followed by a November muscle weight that is well above the seasonal predic-

tion. Low ratios in the spring of 1992 are followed by a low adductor weight in August.

DISCUSSION

This study confirms that the RNA/DNA ratio of the adductor varies with size; the smaller, faster growing scallops having higher ratios than the older ones (Kenchington 1994). This agrees with the link between RNA/DNA ratio and growth rate shown in other studies (Haines 1973, Wright and Hetzel 1985). It is an expected result, because growth rate, and hence, protein synthesis, declines with age in this species (Kenchington et al. 1995).

The significant difference between the RNA/DNA ratios for the inside and outside sampling zones is not in agreement with a high RNA/DNA ratio reflecting a higher growth rate. The inside zone has a higher growth rate than the outside (Robert et al. 1986). The present study shows that the inside zone also has significantly higher meat and gonad weights for comparable shell heights than the outside zone. This greater growth is being achieved at a lower RNA/DNA ratio than in the outside zone. The explanation for this is the influence of temperature on the RNA/DNA ratio. Temperature has been noted to affect the relationship between RNA/DNA ratio and growth in winter flounder by Buckley (1982). He observed that at higher temperatures, an increase in growth rate occurred without an increase in the RNA/DNA ratio, indicating that increased temperatures may result in a higher activity rate for the RNA rather than an increase in the amount present. In the June scallop surveys for Digby, bottom temperature was recorded for each tow. There was a significant ( $P < .001$ ) difference between the bottom temperatures of the inside and outside zone, with the inside zone having a consistently higher temperature (Table 9). The higher growth rate of the inside zone is being achieved with a lower RNA/DNA ratio, but at a higher temperature.

When looking at the interannual variation in the RNA/DNA ratios, 1991 stands out as being higher than the other years. The temperature range for the June samples was broken down into  $0.1^\circ\text{C}$  cells and the mean ratio for each cell plotted against the temperature (Fig. 3). The 1990, 1992, and 1993 samples all show similar ratios over a  $3^\circ\text{C}$  temperature range. The 1991 sample stands out as having a higher RNA/DNA ratio at a similar temperature to 1990. The increased amount of RNA present, and an increased activity of the RNA because of the higher temperatures, should have resulted in an increased growth rate in 1991 as compared to 1992 or 1993. This is supported by the June values for the adductor, gonad, and catch muscle weights, which are all higher in 1991 (Table 7).

Because the temperatures are similar in 1990 and 1991, why are the RNA/DNA ratios so different? On the Digby side of the Bay of Fundy, readings for chlorophyll fluorescence were taken at 1 meter

TABLE 8.

Partial F test for parameters added in order for the sine wave model parameters fit to the RNA/DNA ratio data standardized to the June samples.

Source	DF	SS	MS	F	P
A	1	15.89001	15.89001	404.94419	<.0001
A + B*Sine(Rdate)	1	.34165	.34165	8.70668	.0115
A + B*Sine (Rdate + C)	1	.04249	.04249	1.08282	.3186
Residual	12	.47091	0.3924		
Total	15	16.74506			

TABLE 9.

Means, standard deviations and number of tows for bottom temperatures taken during the June Digby area scallop surveys in each year.

Year	Inside Zone	Outside Zone
1990	7.93 (0.336) 53	7.32 (0.376) 51
1991	7.59 (0.245) 65	7.08 (0.110) 72
1992	5.08 (0.384) 43	4.67 (0.126) 54
1993	6.39 (0.078) 39	6.14 (0.191) 53

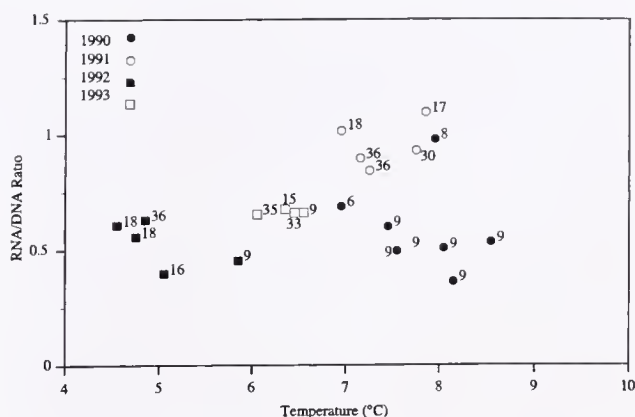


Figure 3. Average RNA/DNA ratio for 0.1 °C cells by temperature. Numbers are sample size for that cell, markers are by year sampled.

from the surface, 1 meter from the bottom, and midwater off the Digby wharf for a sampling program in that area (Fig. 4, Paul Keizer, unpublished data). The readings for April to July, had mean values of chlorophyll *a* ( $\text{mg m}^{-3}$ ) of 2.99 ( $\pm 1.21$ ) for 1990, and 4.15 ( $\pm 2.01$ ) for 1991. The higher RNA/DNA ratios in 1991 may be in response to a higher food supply.

With the irregular sampling schedule, an ANOVA using *year* and *month* factors is too highly aliased to produce realistic annual and seasonal components. Treating each *sampling date* as a separate level and then fitting the sine function to the data would indicate if there is a seasonal component to the variation. It would be possible to improve the fit by adding additional parameters; that is, to improve the fit to the gonad and adductor weight data during the spawning period where Figure 2 shows it underestimating the peak gonad weight and overestimating the minimum meat weight. This would make for a less parsimonious model but may improve its predictive capability. As applied here, to examine the question of a significant seasonal component in the different datasets, the most parsimonious model has proved capable of showing a seasonal component. A linear fit to the RNA/DNA ratio data explains 16% of the variation in the data, but with a nonsignificant slope ( $t = -1.613$ ,  $P = .1306$ ), showing that there is no over-all trend with time running through the data. The partial F test shows that the fit of the sine function is significant, indicating that there is seasonable variation in the RNA/DNA ratio in the scallop population off

Digby, Nova Scotia. The seasonal component, as represented by the sine function, explains a maximum of 45% of the variation in the RNA/DNA ratio, and Figure 2 does not indicate the presence of any consistent pattern in the residuals. The general shape of the sine function models the seasonal component, but there are other factors that have a large influence on the variations in the RNA/DNA ratio, and the data have large outliers, even when standardized within each year. The variation left unexplained by the model, and the large outliers, indicate that the RNA/DNA ratio has variations on a time scale less than seasonal, and that some residuals are as large as the range of seasonal variation predicted by the model.

Seasonal variations in the RNA/DNA ratio of the adductor muscle in oysters was noted by Pease (1976) with the highest values occurring in September to October for the populations studied. Paon and Kenchington (1995) found that the RNA/DNA in the adductor muscles of *Placopecten magellanicus* peaked just before spawning during laboratory conditioning. This agrees with the annual cycle found in this study, with the ratio cycle lagging that of the gonad weight and peaking as the adductor muscle increases after the spawning period in the Digby area.

The adductor muscle is an important site for energy storage in pectinids (Ansell 1974, Taylor and Venn 1979, Idler et al. 1964). Energy, mainly in the form of glycogen and protein, is stored to support gamete maturation and to meet metabolic requirements during winter, when food availability is low. The amount of seasonal variation in adductor muscle weights reported in the literature, and seen in this study, is large. During the annual cycle in adductor weight, Taylor and Venn (1979) report a doubling of the adductor dry weight for *Chlamys opercularis*; whereas, increases of 77% have been reported for *Chlamys islandica* (Sundet and Vahl 1981), and 39% for *Pecten maximus* (Comely 1974). Robinson et al. (1981), working with *Placopecten magellanicus* in Boothbay Harbor, Maine, show an increase of approximately 60%. Comely (1974) showed less than a 5% variation in water content of the adductor muscle for *Pecten maximus*, so the 30–40% variation in adductor wet weight found in this study is not at the high end of the range in pectinids. This seasonal variation in weight has greater implications for management of this species, because most pectinid fisheries outside North America use both the adductor muscle and the gonad; whereas, the majority of the *Placopecten magellanicus* fisheries, including Digby one, land only the adductor muscle. With a quota-based management system, the seasonal pattern of landings should be taken into account when determining the quota.

The timing of the cycle of adductor muscle weight predicted by the sine function (Fig. 2), is unusual, in that it predicts a peak in January. Although there is often a rapid postspawning increase in the adductor weight, it usually occurs during the summer or fall. The adductor weight of *Chlamys opercularis* peaks in September–October following a June–July spawning (Taylor and Venn 1979). *C. islandica* in August–September following June–July spawning (Sundet and Vahl 1981), and *Pecten maximus* in November after protracted spawning starting in the early summer (Comely 1974). In studies of *Placopecten magellanicus*, which spawn August to September in the Digby area, Robinson et al. (1981) reported a spring peak in adductor weight in Maine, with a winter low. Thompson (1977) found a low in total somatic tissue weight from September to March for southeast Newfoundland. The January peak in adductor dry weight predicted from the sine function does not agree with the sample means (Table 7). The peak seems to occur earlier than the sine function predicts, at least in November

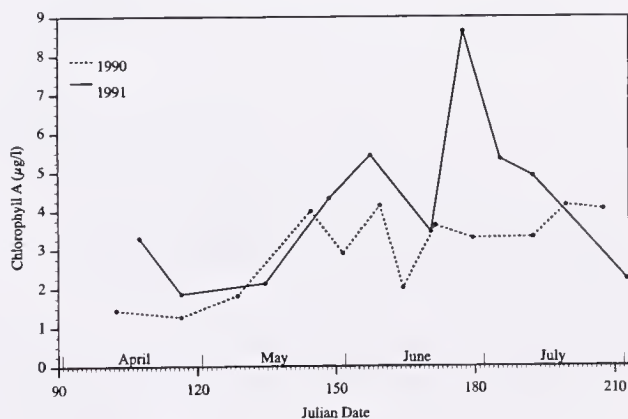


Figure 4. Chlorophyll *a* levels for water samples taken off Digby wharf, April to July in 1990 and 1991 (Paul Keizer, unpublished data).

from the sample means, and perhaps earlier, because there are no data for October. A January peak would be difficult to achieve with the known pattern of plankton in the Bay of Fundy, which has a fall diatom bloom but low plankton concentrations during the late fall and early winter (Martin et al. 1995). This timing differs from the spring peak reported for the Gulf of Maine and Newfoundland areas (Robinson et al. 1981, Thompson 1977).

The catch muscle exhibits little seasonal variation in weight. This is expected, because it is not used as an energy storage site, as the large phasic portion of the adductor muscle is. Robinson et al. (1981) found the concentrations of glycogen in the catch and phasic portions of the adductor to be the same in March and April for *Placopecten magellanicus*, and DeZwaan et al. (1980) found that in July, the glycogen concentration of the quick adductor muscle was over twice that of the catch muscle. The predicted gonad weights produced the best fit to the sine curve, with a peak in July, before the August–September spawning period for the Digby area.

The peak in the adductor RNA/DNA ratio occurs as the adductor muscle weight is increasing. Part of this increase in weight is attributable to increased protein synthesis, which requires a higher RNA/DNA ratio. Pectinids have been shown to use the protein content of the adductor as an energy storage mechanism along with its glycogen content and the lipids in the digestive gland. Thompson (1977) showed a seasonal variation in protein for total somatic tissue of *Placopecten magellanicus*, and Taylor and Venn (1979) showed that the seasonal variation in protein weight is larger than that of glycogen for the adductor muscle of *Chlamys opercularis*. Sundet and Vahl (1981) showed that in *Chlamys islandica*, both mature and immature scallops catabolize adductor muscle protein during the winter, but that it is a much more important energy source for the juveniles. They postulate that the greater use of protein in the juveniles is a growth strategy, allowing for more growth in times of abundant food. On reaching sexual maturity, the production of gametes requires more energy, and the use of glycogen increases, but seasonal variations in protein content continue.

The variations seen in the RNA/DNA ratio of the adductor muscle are not tightly bound to the gametogenic cycle. The seasonal/sine wave model explains 69% of the variation in gonad weight, but a maximum of 45% of the variation in the RNA/DNA

ratio of the adductor muscle. Part of the difference in the fit is that the interannual and subannual variations in the RNA/DNA ratio seem to be larger than those for the gonad or adductor weight. This may be because that the RNA/DNA ratio of the adductor muscle is affected not only by the variable growth rate of the adductor tissue, but also by its use as an energy substrate in support of gametogenesis, and by protein synthesis related to metabolic activities other than tissue protein growth.

The relationship between the RNA/DNA ratio and growth rate is the basis for postulating that RNA/DNA ratios may be a useful index of health for scallop stocks. The fact that residuals from seasonal RNA/DNA ratios are correlated with residual from subsequent adductor growth support the existence of this relationship. This correlation is low under natural conditions as seen in the Digby scallop population, however, and the RNA/DNA ratios show a high level of variation on a short time scale.

## CONCLUSIONS

The RNA/DNA ratio of the adductor muscle for the scallop population off Digby Nova Scotia varies with the size or age of the scallop, and there are temporal variations on interannual and seasonal scales. Temporal variations are related to growth rate differences and reflect variations in food supply and temperature. The seasonal variation in RNA/DNA ratios can be modeled and approximates a sine function. This model explains a maximum of 45% of the variation in RNA/DNA ratio, but the residual from the model are significantly correlated with subsequent growth. The usefulness of RNA/DNA ratios for monitoring the health of a scallop stock will depend on whether or not the large variation seen in this healthy stock during the sampling period is a true reflection of variances in growth rate or would remain high as the health and average RNA/DNA ratio declined. A large variation in RNA/DNA ratios as levels declined would make it more difficult to discriminate healthy stocks that were under normal levels of nutritional stress during periods of low food availability from those that were in danger of increased mortality rates. The method seems to have the potential to indicate when a population is approaching a critical level of stress, but if a monitoring program were established, it could not be recommended as the only method used to indicate the health of the stock.

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## TEMPORAL COINCIDENCE OF THE ANNUAL EELGRASS *ZOSTERA MARINA* AND JUVENILE SCALLOPS *ARGOPECTEN VENTRICOSUS* (SOWERBY II, 1842) IN BAHÍA CONCEPCIÓN, MEXICO

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**ABSTRACT** The possibility that meadows of annual eelgrass *Zostera marina* L. in Bahía Concepción serve as a nursery habitat for Pacific calico scallop *Argopecten ventricosus* has been evaluated. Scallop reproduction occurs mainly in the coldest season of the year, just when annual eelgrass is present. In this study, it was observed that the highest abundance of pectinid larvae was in early March, and scallop spat on artificial collectors was higher in late March. The highest above-ground biomass of eelgrass occurred between February and April and declined in May, just when scallops detach themselves from substrata and settle to the bottom. Our results indicate a high coincidence in timing of the analyzed processes and suggest the possibility that eelgrass beds could be used as nursery grounds by Pacific calico scallop.

**KEY WORDS:** annual eelgrass, scallop spat, nursery habitat, *Zostera marina*, *Argopecten ventricosus*

### INTRODUCTION

The Pacific calico scallop *Argopecten ventricosus* (= *circularis*) (Sowerby II, 1842) supports an important fishery in the state of Baja California Sur, Mexico. Between 1986 and 1991, scallop harvest in Bahía Concepción, the most productive bay for this fishery, had a very high production (up to 5531 t fresh weight in 1989), but there have been large fluctuations in landings since 1991, moving toward critical levels in 1993 (100 t) and 1994 when the fishery was closed. That depletion has prompted increased interest in the factors that affect scallop recruitment and survival (Félix-Pico et al. 1997).

During their early life history, Pacific calico scallops, as well as other pectinids, settle and attach to substrates that elevate them above the bottom (Félix-Pico et al. 1989). These substrates are usually submerged vegetation or even artificial materials, but eelgrass *Zostera marina* L. appears to be the main natural substratum for bay scallop, *Argopecten irradians* (L.) (Thayer and Stuart 1974). Eelgrass beds not only provide juvenile bay scallops with a settlement substrate but also help scallops avoid benthic predators, which results in higher scallop recruitment and survival (Ekman 1987, Peterson et al. 1989, Pohle et al. 1991, García-Esquivel and Bricelj 1993).

Juvenile bay scallops undergo an ontogenic shift in habitat. Scallops often attach themselves in the eelgrass canopy in their earlier life stage until they reach a size of 20–30 mm, after which juvenile scallops settle to the bottom. At this size they have achieved a refuge from some of their major crustaceans predators (García-Esquivel and Bricelj 1993).

Little is known about the association of Pacific calico scallops and eelgrass beds in spite of the fact there are extensive eelgrass meadows in almost all the lagoons of Baja California Sur where scallops occur (Félix-Pico et al. 1989).

*Z. marina* is a widely distributed, temperate seagrass species that occurs in some subtropical regions, such as the Gulf of California (Phillips and Meñez 1988). Although this species commonly

forms perennial beds, in the Gulf of California eelgrass forms only annual beds, which appear in late autumn as seeds, and disappear in mid-spring when all the plants die (Phillips and Backman 1983, Santamaría 1996).

As Pacific calico scallops reproduce mainly in the coolest part of the year, January to March (Villalejo-Fuerte and Ochoa-Báez 1993, Félix-Pico et al. 1997), it is possible to suppose that annual eelgrass beds serve as nursery habitat for juvenile Pacific calico scallops. For this, it is first necessary that spat settlement coincide with the maximum eelgrass standing crop and that scallops reach a sufficient size to be a free living form before eelgrass beds disappear. Direct evaluation of the settlement of juvenile scallops on eelgrass in Bahía Concepción was not possible because scallops were very scarce throughout the bay during the year of study. Thus, the objective of the present study was to determine the timing of *Z. marina* bed presence, pectinid larval abundance, and juvenile scallop settlement on artificial collectors and to determine if eelgrass meadows in Bahía Concepción appear in the time when they could potentially provide scallops with a mechanism to increase survival.

### MATERIALS AND METHODS

Bahía Concepción is located in the Gulf of California on the east coast of the Baja California Peninsula at approximately lat 26°45'N, long 111°45'W (Fig. 1). Usually, the surface water temperature in the Bay varies from 18 °C to 32 °C, but a surface temperature of 16 °C was observed in February 1989 (Villalejo-Fuerte and Ochoa-Báez 1993).

To evaluate the *Z. marina* bed as potential habitat for scallops, data on eelgrass abundance were taken at Punta Arena, Bahía Concepción, during one season of eelgrass development, from December 1994 to July 1995. Samples were taken every 3 weeks via scuba diving. Eelgrass shoot density was measured in 25 × 25 cm quadrats, with 4 to 10 random replicates. Shoot abundance was

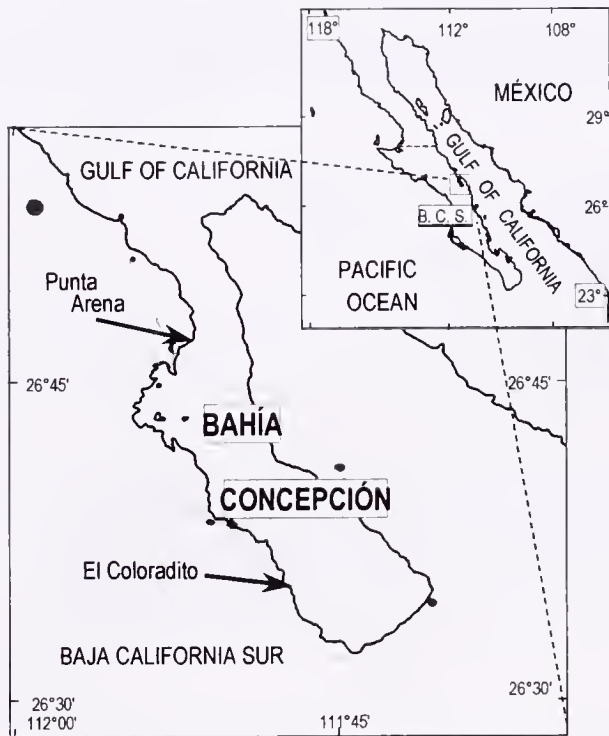


Figure 1. Study area: Bahía Concepción. Sampling sites, Punta Arena and El Coloradito, are indicated by arrows.

extrapolated per  $m^2$ . Percentage cover was examined in 20-m transects, with 4 replicates. Thirty shoots were sampled to determine above-ground biomass and the total height of plants. Plant biomass evaluation was measured by drying individual shoots at  $60^\circ\text{C}$  to constant weight. To evaluate the potential habitat provided by the eelgrass bed, height of the canopy was estimated as the mean height of plants.

Simultaneously, to evaluate abundance of D-veliger (2 to 9 days old), umbonated (10 to 13 days old), and pediveliger (14 to 18 days old) scallop larvae (Avilés-Quevedo 1990, Monsalvo-Spencer 1998), we sampled zooplankton at Punta El Coloradito weekly, using a water-pump and two sieves of 63 and 132  $\mu\text{m}$  mesh. For zooplankton analysis, subsamples were taken by a Folsom Divider. All D-veliger, umbonated, and pediveliger larvae found in both sieves were counted, and the data were standardized per  $m^3$ .

To test the timing of spat settlement in Bahía Concepción, artificial collectors were used due to the low levels of scallop abundance here during this study. Collectors were built with onion sacks filled with 300 g of polypropylene nets. They were hung from a longline, held by buoys and anchors, at 1- and 3-m depth. Ten collectors were installed weekly, 5 at each depth, to monitor spat settlement during the time when scallop spawning was anticipated to occur, from 19 January to 30 March. Each set of collectors was removed from water 2 months after it was installed. Collectors were dried before juvenile scallops were separated through a 5-mm sieve and Pacific calico scallops counted. Mean spat size and number of scallops settled per collector were recorded. Previous data showed that no significant differences were found in collector efficiency between Punta Arena and El Coloradito in 1991 and 1992 (Félix-Pico et al. 1997). Surface seawater temperature was measured at each sampling date.

## RESULTS

The *Z. marina* bed at Punta Arena appeared in early December as seedlings, then all eelgrass plants suddenly died in late May and the meadow disappeared. In July, there were no eelgrass plants, only seeds in the sediment. Shoot density in the bed was about 1,400 shoots per  $m^2$  from December to April when shoot density started to decrease. Highest values of above-ground biomass occurred between February and March 1995 (Fig. 2a), particularly from 15 to 30 March (218 and 235  $\text{g DW}/m^2$ , respectively). There was a decrease in biomass by early March, but this coincided with the highest mean plant height, 71 cm (Fig. 2a), and we suppose that it could be caused by variation in plant morphology. However, shoot density and height of eelgrass bed showed consistently high values from February to May, suggesting that canopy habitat was high during this period.

There were some pectinid larvae in the water column at El Coloradito, on 16 February and 2 March, 95 and 40 larvae/ $m^3$ , respectively, but the maximum concentrations were observed on 9 and 16 March: 400 and 290 larvae/ $m^3$ , respectively (Fig. 2b). Thereafter, larvae were almost absent. Spat caught on collectors were most abundant in collectors installed from 16 to 23 March, averaging 35.5 and 20 individuals per collector, respectively (Fig.

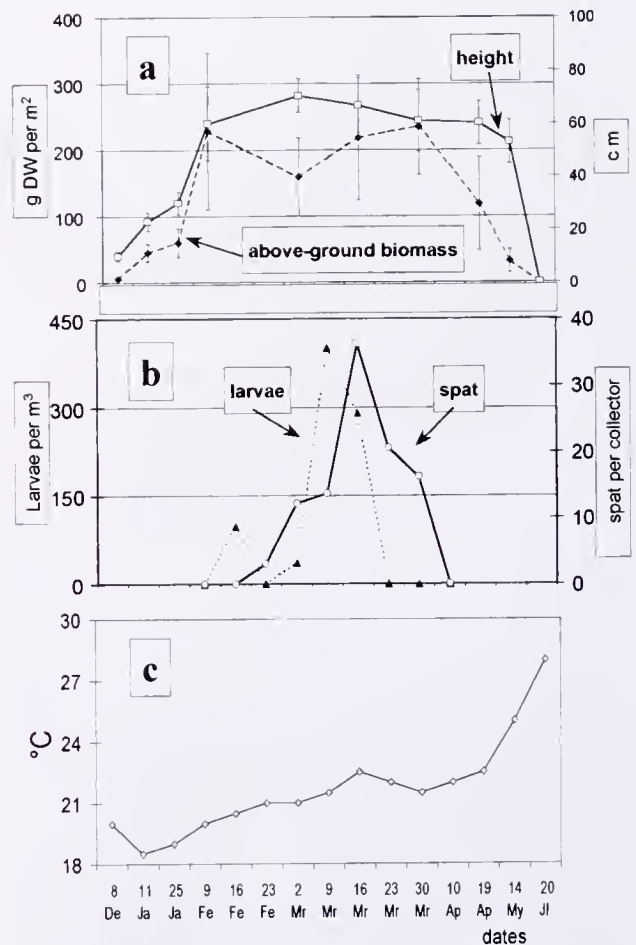


Figure 2. (a) Mean above-ground biomass of eelgrass bed (left scale) and plant height (right scale); error bars = standard deviation ( $n = 30$ ). (b) scallop larvae density (left scale) and new spat abundance on collectors per week (right scale), and (c) seawater temperature during sampling period. All data shown are from December 1994 to July 1995.

2b). Spat in collectors were gradually detached when they reached n 20 to 30 mm, from April to May. Sizes of scallops observed in collectors were between 7 and 34 mm.

Seawater temperature was between 18.2 °C in January and 22.5 °C in April, and reached 28 °C in July (Fig. 2c).

### DISCUSSION

Our data show a high coincidence in timing of scallop spawning, spat abundance, and the permanence of eelgrass beds. Spat abundance peaked in collectors installed one week after we observed peak in larval abundance on 9 March, therefore, it can be inferred that individuals in collectors came from the same cohort as the sampled larvae, because there were no more larvae in the water after that. Efficiency of collectors decreases rapidly after they have been installed, probably due to epibiosis. On the other hand, scallops take about 2 months to reach 20 to 30 mm, when they are able to become a free-living form, but scallop spat are able to detach themselves after reaching about 15 mm (García-Esquivel and Bricelj 1993).

During the time when the scallop spat in Bahía Concepción need to be fixed, the eelgrass bed was present. The height of the eelgrass canopy remained at high levels until plant death, but above-ground biomass began to decline in late March. Previously, it has been observed that most juvenile scallops detach themselves from the substrate to begin a free-living form before all the plants die in May (Félix-Pico et al. 1997). Similarly, populations of *Argopecten irradians* on the east coast of the United States reproduce mainly in the season of major development of eelgrass meadows, although those beds are not annuals but perennials, and it occurs in summer, from July to September (Churchill and Riner 1978, Roman and Able 1988, García-Esquivel and Bricelj 1993).

The absence of juvenile scallops attached to eelgrass shoots in Bahía Concepción during the sampling period was considered to be due to low adult scallop abundance, and spat abundance observed in collectors was also low. Normal levels of spat collections are consistent annual yields of 6,000 spat per collector. In 1994, spat collections were 41 to 852 spat per bag (Félix-Pico et al. 1997), but 2 years later, in spring 1997, a great quantity (about 500 spats/m<sup>2</sup>) of scallop spat were found together with plants sampled in April. Unfortunately it was not possible to quantify the density because distribution inside the bed is very contagious and systematic sampling was not done.

Other types of submerged macrophytes in Bahía Concepción must not be ignored, such as *Sargassum* spp. stands, which are the most abundant macroalgae in the Bay and may also serve as a

substratum to scallop spat. However, the main development of *Sargassum* is in June to July (Casas-Valdez et al. 1993) when scallop spat presence is not significant.

In 1989, an unusually high eelgrass abundance coincided with the lowest temperature recorded in the Bay (Castro-Ortiz pers. comm.), and a boom in catches of scallops was also observed that year (Félix-Pico et al. 1997). As annual eelgrass in the Gulf of California develops in the coolest season of the year because it is a temperate species living in a subtropical locality (Phillips and Backman 1983, Phillips and Meñez 1988, Santamaría 1996), and the main scallop spawning period in Bahía Concepción also coincides with the coolest months of the year (Villalejo-Fuerte and Ochoa-Báez 1993), temperature probably plays a significant role in Pacific calico scallop recruitment and survival. However, other environmental factors that could also affect the abundance of eelgrass beds in their year-to-year variation must be considered in future studies.

Findings obtained in this study are limited and more research is necessary to confirm and evaluate the relation of Pacific calico scallop spat to eelgrass beds, both in the laboratory and field. However, with these results it is possible to propose some suggestions for a better exploitation of Pacific calico scallop. If our hypothesis is confirmed, the protection of eelgrass beds would be necessary to preserve high abundance of scallop populations. Besides, with absolute certainty, eelgrass beds accomplish several other ecological tasks in the Bay (Phillips and Meñez 1988). Avoidance of trawl fishing over the meadows is an urgent matter that should be addressed to protect the meadows. This activity, as we could see during field work in Bahía Concepción, causes severe damage to the eelgrass coverage. Seagrass meadows in other localities have been already protected successfully from trawl fishing (Guillén-Nieto et al. 1994) with benefits for artisanal fisheries (Martínez 1997).

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## OXYGEN CONSUMPTION AND AMMONIA EXCRETION OF LARVAE AND JUVENILES OF THE BAY SCALLOP, *ARGOPECTEN IRRADIANS CONCENTRICUS* (SAY)

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**ABSTRACT** Rates of oxygen consumption and ammonia excretion were determined at 25 °C for larval and juvenile bay scallops *Argopecten irradians concentricus*. Oxygen consumption rate ( $V_{O_2}$ ,  $\mu\text{IO}_2 \text{ h}^{-1}$ ) varied with body size ash-free dry weight (AFDW, mg) according to the relation  $V_{O_2} = 7.199 \text{ AFDW}^{0.921}$  for larvae and  $V_{O_2} = 2.142 \text{ AFDW}^{0.905}$  for juveniles. Mean weight-specific  $V_{O_2}$  ranged from 14.66 to 15.84  $\mu\text{IO}_2 \text{ mg AFDW}^{-1} \text{ h}^{-1}$  for larvae and from 1.80 to 5.28  $\mu\text{IO}_2 \text{ mg AFDW}^{-1} \text{ h}^{-1}$  for juveniles. Weight-specific  $V_{O_2}$  declined with increasing body size at > 2 mm shell height but became independent of body size at > 2 mm shell height. Swimming was estimated to cause 8 to 29% of the total larval oxygen demand. In juveniles of 3.5 to 5.0 mm shell height,  $V_{O_2}$  increased as temperature increased from 15 to 30 °C, being  $1.24 \pm 0.35$ ,  $1.77 \pm 0.70$ ,  $2.33 \pm 0.85$ , and  $2.74 \pm 0.57 \mu\text{IO}_2 \text{ mg AFDW}^{-1} \text{ h}^{-1}$  at 15, 20, 25 and 30 °C, respectively. The  $Q_{10}$  was 2.99 at 15 to 20 °C, 1.74 at 20 to 25 °C and 1.37 at 25 to 30 °C. Ammonia excretion rate ( $U$ ,  $\mu\text{gNH}_4\text{-N h}^{-1}$ ) increased with body size following the equation  $U = 0.161 \text{ AFDW}^{0.928}$ . Energy loss through ammonia excretion was equal to 1.5 to 3.7% and  $13.5 \pm 2.8\%$  of the respiratory energy loss in larvae and juveniles, respectively.

**KEY WORDS:** Bay scallop larvae, oxygen consumption, ammonia excretion

### INTRODUCTION

Metabolism is an important component of physiological energetics, the study of energy gains and losses at the organismal level (Brett and Groves 1979). Metabolic rate is most often estimated as the rate of oxygen consumption, and it represents a major loss of energy in bivalves. Energy loss through ammonia excretion is also very significant (Barber and Blake 1985).

Information on oxygen consumption exists for a variety of adult marine bivalves, but less is known about the respiratory physiology of early developmental stages, with the exception of a few commercially important species (Sprung 1984, MacDonald 1988, Beiras and Camacho 1994). Existing data suggest that, in both larval and adult bivalves, oxygen consumption increases with increasing body size according to the allometric equation  $Y = aX^b$ , where  $Y$  is oxygen consumption,  $X$  is body weight, and  $a$  and  $b$  are fitted parameters (Bayne et al. 1976a). In adult bivalves,  $b$ -values range from 0.65 to 0.84, with a mean of 0.7 (Bayne and Newell 1983); whereas, in larval bivalves,  $b$ -values are often close to 1 (Riisgård et al. 1981, Gerdes 1983, Beiras and Camacho 1994), demonstrating an isometric relationship between metabolic rate and body size in larvae.

In energy balance studies, energy loss attributable to excretion of nitrogenous products is often overlooked (Bayne and Newell 1983), although it may substantially affect the general estimation of the energy budget. In most marine bivalves, ammonia is the dominant end product of protein catabolism, comprising 41 to 94% of the total nitrogen excretion (reviewed by Bayne et al. 1976b). Very limited data are available on ammonia excretion in pectinids; the only available data were collected by Barber and Blake (1985) on adult *Argopecten irradians concentricus*.

The purpose of this study was to determine the energy loss of larvae and juveniles of the bay scallop *A. irradians concentricus* (Say) by measuring their rates of oxygen consumption and ammonia excretion and to determine the relationships between these physiological rates and body size.

### MATERIALS AND METHODS

Bay scallops collected from Homosassa, Florida were spawned at the Department of Marine Science, University of South Florida.

Culture of larvae and juveniles followed the methods described by Lu and Blake (1996). Before each determination of oxygen consumption, larvae and juveniles were filtered onto 35  $\mu\text{m}$  nylon screens, rinsed with, and then released to, 0.45  $\mu\text{m}$  filtered seawater (salinity  $26 \pm 1\%$ ). Larger juveniles were also cleaned with a small, soft pen brush.

Oxygen consumption rates of various size classes of larvae and juveniles were measured at  $25 \pm 0.5$  °C. Larvae and small juveniles were placed in sealed respiration chambers filled with 0.45  $\mu\text{m}$  filtered seawater at a density of 20 to 80 larvae  $\text{ml}^{-1}$  or 1 to 10 juveniles  $\text{ml}^{-1}$ , depending on shell size. Respiration chambers were made from plastic syringes (Torres et al. 1994), whose volume could be adjusted (1–5 ml) according to the number and size of the experimental animals by adjusting the syringe plunger and/or oxygen electrode. Experiments with juveniles larger than 5 mm in shell height were carried out in 25- to 65- ml *Lucite* chambers. Oxygen concentration in the syringes and chambers was measured every 2 minutes using Microcathode oxygen electrodes calibrated with air and nitrogen saturated seawater. Data were recorded using a computer-controlled digital data-logging system. Experiments lasted 4 to 8 hours, during which the oxygen concentration never dropped below 50%.

The effect of temperature on oxygen consumption was determined using juveniles of 3.5 to 5 mm shell height. Experimental procedures were the same as described above, except that experiments were run at four temperatures, 15, 20, 25, and  $30 \pm 0.2$  °C, using circulating water bath to control temperature.

The changing oxygen concentrations recorded for each respiration chamber during the course of a run were regressed against time to obtain a representative slope, a rate. The rate obtained was divided by the number of scallops in the chamber to give the amount of oxygen consumed per individual per hour ( $\mu\text{IO}_2 \text{ h}^{-1}$ ). The oxygen consumption rates were further converted to  $\mu\text{IO}_2 \text{ mg AFDW}^{-1} \text{ h}^{-1}$  using the weight data (total AFDW) of Lu and Blake (1996). All measured oxygen consumption rates were corrected using control runs without animals.

Ammonia excretion was determined by placing larvae or juveniles in 35- and 50-ml capped glass vials filled with 0.45  $\mu\text{m}$  filtered seawater at 20 to 80 larvae  $\text{ml}^{-1}$  or 1 to 60 juveniles per vial. Filtered seawater without animals were used as blanks. Ex-

periments lasted 4 to 7 hours at  $25 \pm 0.2$  °C. At the end of each experiment, a 10-ml sample was drawn from each vial and placed in a 20-ml test tube. In experiments with larvae, the samples were passed through a 35- $\mu$ m mesh screen to remove the scallops. Ammonia concentrations in the samples and blanks were determined using the indophenol blue method (adapted from Solórzano 1969). To each sample and blank, 0.4 ml of phenol-alcohol reagent, 0.4 ml of nitroprusside reagent, and 1.0 ml of oxidizing reagent were added. The blue color that developed in the dark for 1 hour was read on a Cary spectrophotometer at wavelength of 640 nm. Optical densities were converted to ammonia concentrations using a standard curve determined with a solution of ammonium sulfate.

The following factors were used for converting oxygen consumption and ammonia excretion into energy unit:

$$1 \text{ ml O}_2 = 19.9 \text{ J (Elliott and Davidson 1975)}$$

$$1 \text{ mg NH}_4 - \text{N} = 24.8 \text{ J (Elliott and Davidson 1975)}$$

### RESULTS

Table 1 lists the mean oxygen consumption rates of various sizes of larvae and juveniles of the bay scallop. Mean oxygen consumption ranged from  $1.35$  to  $4.10 \times 10^{-3} \mu\text{IO}_2 \text{ h}^{-1}$  for larvae of 120 to 180  $\mu$ m shell length, and  $1.15 \times 10^{-2}$  to  $5.15 \mu\text{IO}_2 \text{ h}^{-1}$  for juveniles of 0.5 to 7.0 mm shell height. The oxygen consumption of larvae and juveniles (up to 10 mm height) is closely related to body size according to the following equations:

$$\text{VO}_2 (\mu\text{IO}_2 \text{ h}^{-1}) = 0.298L^{2.536} \quad r^2 = 0.718 \quad (\text{for larvae})$$

$$\text{VO}_2 (\mu\text{IO}_2 \text{ h}^{-1}) = 0.0444H^{2.410} \quad r^2 = 0.956 \quad (\text{for juveniles})$$

where L is shell length of larvae in mm, and H is shell height of juveniles in mm. The two equations above can be further transformed to the following using the weight data determined by Lu and Blake (1996):

$$\text{VO}_2 (\mu\text{IO}_2 \text{ h}^{-1}) = 7.199 \text{ AFDW}^{0.921} \quad (\text{for larvae})$$

$$\text{VO}_2 (\mu\text{IO}_2 \text{ h}^{-1}) = 2.142 \text{ AFDW}^{0.905} \quad (\text{for juveniles})$$

where AFDW is the total ash free dry weight (including AFDW of the shells) in mg. The measured oxygen consumption rates and the fitted curves are shown in Figure 1 for both larvae and juveniles. Figure 1 was plotted in double logarithmic scales so that oxygen consumption of larvae and small juveniles can be compared on the

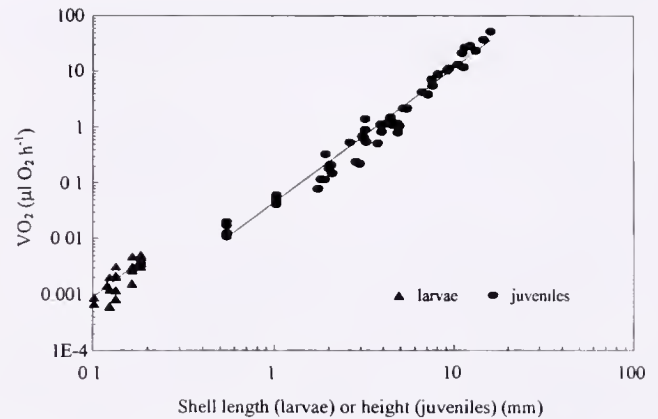


Figure 1. *Argopecten irradians concentricus*. Oxygen consumption rate of larvae and juveniles versus shell size.

same graph. It is clear from Figure 1 that oxygen consumption of larvae is above the extended line fitted to the datapoints of juveniles, suggesting that larvae have higher relative metabolic rates than juveniles.

Weight-specific oxygen consumption decreased with increasing body size at  $< 2$  mm shell height (Fig. 2). Mean values ranged from  $14.66$  to  $15.84 \mu\text{IO}_2 \text{ mg AFDW}^{-1} \text{ h}^{-1}$  for larvae, and from  $5.28$  to  $2.28 \mu\text{IO}_2 \text{ mg AFDW}^{-1} \text{ h}^{-1}$  for juveniles 0.5 to 2 mm shell height. The relationship between weight-specific oxygen consumption rate and body size of juveniles  $< 2$  mm shell height can be best described by the following equation:

$$\text{VO}_2 (\mu\text{IO}_2 \text{ mg AFDW}^{-1} \text{ h}^{-1}) = 3.443H^{-0.648} \quad r^2 = 0.792$$

For juveniles  $> 2$  mm in shell height, weight-specific oxygen consumption rates remained relatively constant, with a mean value of  $2.16 \pm 0.20 \mu\text{IO}_2 \text{ mg AFDW}^{-1} \text{ h}^{-1}$ .

In the temperature experiments, oxygen consumption increased with increasing temperature between 15 to 30 °C (Fig. 3). At 15 °C, the mean weight-specific oxygen consumption was  $1.24 \pm 0.35 \mu\text{IO}_2 \text{ mg AFDW}^{-1} \text{ h}^{-1}$ , representing 37.5% of the rate of oxygen consumption at 30 °C ( $2.74 \pm 0.57 \mu\text{IO}_2 \text{ mg AFDW}^{-1} \text{ h}^{-1}$ ). At 20 and 25 °C, mean rates were  $1.77 \pm 0.70$  and  $2.33 \pm 0.85 \mu\text{IO}_2 \text{ mg AFDW}^{-1} \text{ h}^{-1}$ , being 64.8 and 85.3% of the rate at 30 °C, respectively. The  $Q_{10}$  was 2.99 at 15 to 20 °C, 1.74 at 20 to 25 °C and 1.37 at 25 to 30 °C.

TABLE 1.

*Argopecten irradians concentricus*. Rates (mean  $\pm$  SD) of oxygen consumption and ammonium excretion of larvae and juveniles. n: number of estimates.

Height (mm)	AFDW (mg)	O <sub>2</sub> Consumption Rate			Ammonia Excretion Rate		
		n	$\mu\text{l ind}^{-1} \text{ h}^{-1}$	$\mu\text{l mg AFDW}^{-1} \text{ h}^{-1}$	n	$\mu\text{g ind}^{-1} \text{ h}^{-1}$	$\mu\text{g mg AFDW}^{-1} \text{ h}^{-1}$
0.12	0.0000915	5	$0.00135 \pm 0.00052$	$14.784 \pm 6.334$	6	$(3.51 \pm 0.80) \times 10^{-5}$	$0.384 \pm 0.098$
0.15	0.0001692	2	$0.00268 \pm 0.00074$	$15.839 \pm 4.034$	—	—	—
0.18	0.0002796	6	$0.00410 \pm 0.00077$	$14.664 \pm 2.801$	6	$(4.23 \pm 1.04) \times 10^{-5}$	$0.125 \pm 0.035$
0.50	0.00217	4	$0.0115 \pm 0.0034$	$5.278 \pm 1.099$	5	$(6.35 \pm 1.94) \times 10^{-4}$	$0.292 \pm 0.089$
1.00	0.0138	4	$0.0518 \pm 0.0087$	$3.754 \pm 0.630$	4	$0.00304 \pm 0.00063$	$0.220 \pm 0.045$
1.50	0.0406	2	$0.0972 \pm 0.0270$	$2.394 \pm 0.665$	5	$0.00778 \pm 0.00197$	$0.192 \pm 0.048$
2.00	0.0875	6	$0.1994 \pm 0.0733$	$2.279 \pm 0.838$	4	$0.01540 \pm 0.00763$	$0.176 \pm 0.087$
3.00	0.2576	2	$0.6110 \pm 0.0324$	$2.372 \pm 0.126$	3	$0.07604 \pm 0.00505$	$0.295 \pm 0.019$
4.00	0.5543	3	$1.2528 \pm 0.2060$	$2.260 \pm 0.372$	4	$0.12755 \pm 0.03924$	$0.230 \pm 0.071$
5.00	1.0045	3	$1.8100 \pm 0.6429$	$1.802 \pm 0.640$	5	$0.18289 \pm 0.02421$	$0.182 \pm 0.024$
7.00	2.4616	2	$5.1473 \pm 1.4575$	$2.088 \pm 0.592$	1	$0.42635 \pm 0.00000$	$0.173 \pm 0.000$

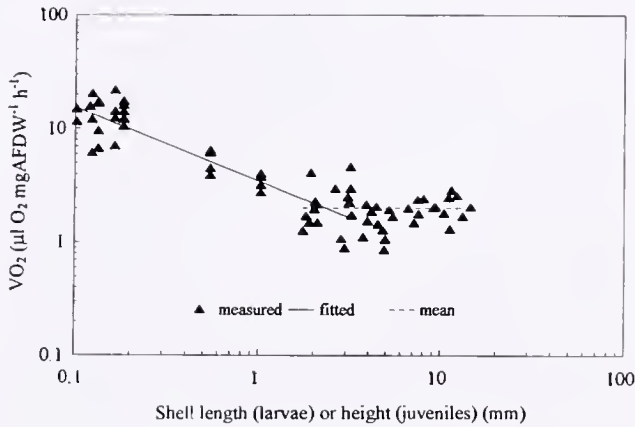


Figure 2. *Argopecten irradians concentricus*. Weight-specific oxygen consumption rate of larvae and juveniles versus shell size.

Mean ammonia excretion rates are also summarized in Table 1. In larvae, ammonia excretion was similar for young and old larvae; whereas, in juveniles, it was an increasing function of body size. The relationship between ammonia excretion rate (U) and body size of juveniles can be described by the following equations:

$$U (\mu\text{gNH}_4 - \text{N h}^{-1}) = 0.00302\text{H}^{2.472} (r^2 = 0.875)$$

$$U (\mu\text{gNH}_4 - \text{N h}^{-1}) = 0.161\text{AFDW}^{0.928}$$

The measured ammonia excretion rates and the fitted U to H curve are shown in Figure 4.

Mean weight-specific ammonia excretion decreased from 0.384  $\mu\text{gN mg AFDW}^{-1} \text{h}^{-1}$  in 120  $\mu\text{m}$  larvae to 0.125  $\mu\text{gN mg AFDW}^{-1} \text{h}^{-1}$  in 180  $\mu\text{m}$  larvae. Energy loss through ammonia excretion was equivalent to 1.5 to 3.7% of the larval respiratory energy loss. In juveniles, mean weight-specific ammonia excretion was relatively constant, ranging from 0.173 to 0.295  $\mu\text{gN mg AFDW}^{-1} \text{h}^{-1}$  with a mean of  $0.220 \pm 0.046 \mu\text{gN mg AFDW}^{-1} \text{h}^{-1}$ . Energy loss through juvenile ammonia excretion equaled  $13.5 \pm 2.8\%$  of the respiratory energy loss.

DISCUSSION

Allometric exponents determined for the relationship between oxygen consumption and body size of larval and juvenile bay

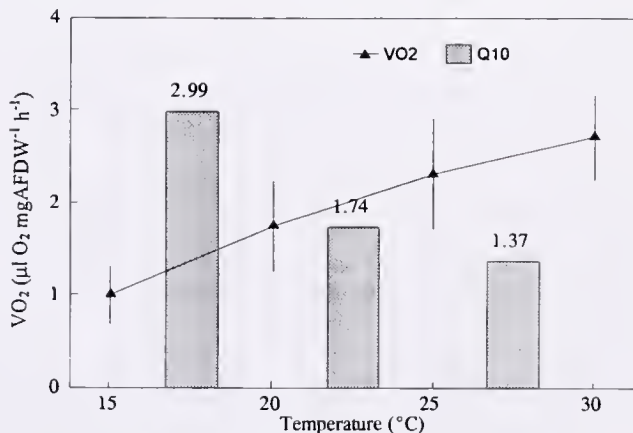


Figure 3. *Argopecten irradians concentricus*. Weight-specific oxygen consumption rate and Q<sub>10</sub> values of juveniles versus temperature.

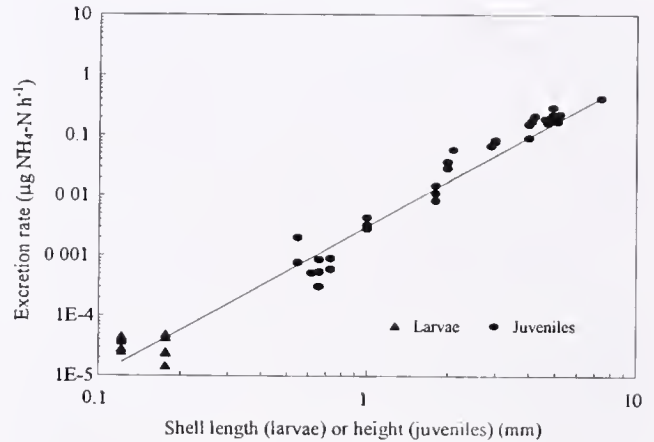


Figure 4. *Argopecten irradians concentricus*. Ammonia excretion rate of larvae and juveniles versus shell size.

scallops are very similar, being 0.921 and 0.905, respectively. These values are higher than those determined for adult bivalves, which range from 0.65 to 0.84, with a mean of 0.72 (Bayne and Newell 1983). A similar trend was found for the Japanese scallop *Patinopecten yessoensis*, in which a b-value of 0.81 was found for adults (Fuji and Hashizume 1974); whereas, a b-value of 1.39 was found for larvae (MacDonald 1988). High values of b were also reported for early stages of other bivalves; for example, 0.90 for *Mytilus edulis* larvae (Riisgård et al. 1981) and 1.09 for *Ostrea edulis* larvae (Beiras and Camacho 1994).

Weight-specific oxygen consumption of bay scallop larvae determined in the present study ranged from 3.0 to 6.6  $\mu\text{lO}_2 \text{ mg DW}^{-1} \text{h}^{-1}$  (6.2 to 20.5  $\mu\text{lO}_2 \text{ mg AFDW}^{-1} \text{h}^{-1}$ ). These values fell at the lower end of the range 4.6 to 15.2  $\mu\text{lO}_2 \text{ mg DW}^{-1} \text{h}^{-1}$  determined for larval *Argopecten irradians* (Siddall 1987) but were similar to the values of 1.6 to 10.0  $\mu\text{lO}_2 \text{ mg DW}^{-1} \text{h}^{-1}$  determined for larvae of other bivalves (reviewed by Holland 1978; Sprung 1984). Similar oxygen consumption was found for larvae of the Japanese scallop *P. yessoensis*, with a range of 5.2 to 11.6  $\mu\text{lO}_2 \text{ mg DW}^{-1} \text{h}^{-1}$  (calculated from MacDonald 1988).

Despite the similar values of b for larvae and juveniles of the bay scallop, the weight-specific oxygen consumption of larvae is 3 to 9 times higher than that determined for juveniles. High metabolic rates observed for larvae are likely a reflection of the energy expended in swimming during this planktonic stage. Swimming activity of bivalve larvae generally represents 8 to 50% of respiration loss (Zeuthen 1947). Sprung (1984) assumed that the energy expenditure of swimming larvae of *Mytilus edulis* was twice the amount needed to overcome the force of sinking (assuming the horizontal component equals the vertical component) and calculated that the energy used in locomotion was less than 2% of the respiration loss. He pointed out that his values were low, because metabolic effort is transferred to the action of motion with certain efficiencies (Klyashtorin and Yarzhombek 1973), and the cost of swimming could be much higher.

Projected oxygen consumption rates of bay scallop larvae were calculated by extrapolation using the allometric equation for small juvenile bay scallops of 0.5 to 2 mm shell height. If the calculated rates are assumed to be equivalent to the metabolic rates of non-swimming larvae, by comparing them with the metabolic rates measured for swimming larvae, we can estimate that the energy expended in swimming is 8.0% and 27.8% of the total metabolism

of larvae 120  $\mu\text{m}$  and 180  $\mu\text{m}$  in shell length, respectively. Larger larvae spend a greater proportion of energy on swimming than smaller ones, a trend that was also found in *Mytilus edulis* larvae (Sprung 1984). The estimation is in accordance with the observation that in *Crassostrea virginica* and *C. gigas*, oxygen consumption of larvae dropped approximately 40% when exposed to epinephrine, a metamorphosis inducer, probably because of the cessation of swimming activity (Haws et al. 1993).

Although weight-specific oxygen consumption decreases with increasing body size in small juveniles (< 2 mm shell height), it is independent of body size in larger juveniles (2–16 mm shell height), with a mean of 2.16  $\mu\text{molO}_2 \text{ mg AFDW}^{-1} \text{ h}^{-1}$ . This value is higher than the mean rate of 1.11  $\mu\text{molO}_2 \text{ mg AFDW}^{-1} \text{ h}^{-1}$  determined for adult *Argopecten irradians concentricus* (calculated from Barber and Blake 1985, assuming 85% of tissue dry weight is AFDW), consistent with the general trend that larger animals have lower weight-specific metabolic rates.

Juvenile bay scallops are well adapted to temperatures between 20–30 °C, as indicated by the low  $Q_{10}$  values over this temperature range (1.73, 20–25 °C; 1.37, 25–30 °C). This is in contrast with the case at low temperatures, where  $Q_{10}$  is much higher (2.99 at 15–20 °C). In adult bay scallops, a  $Q_{10}$  of 1.38 was obtained from  $\text{Vo}_2$  values published by Barber and Blake (1985) over a temperature range of 21–31 °C (calculated by Bricelj et al. 1987). This value is very close to the  $Q_{10}$  found for juveniles over 20–30 °C in this study. Thus, respiration of juvenile bay scallops responds to temperature in a manner similar to adults.

As compared to oxygen consumption, ammonia excretion has been a neglected area in the study of the physiological energetics of marine bivalves. In the present study, energy loss through ammonia excretion represented about 12% of the total energy loss (respiration + ammonia excretion) in juvenile bay scallops, making a significant contribution to the energy budget. Adult bay scallops *A. i. concentricus* were found to lose a similar percentage (7–15.5%) of energy through ammonia excretion (calculated from Barber and Blake 1985). In contrast, the energy loss in bay scallop larvae attributable to ammonia excretion is much less, comprising only 1.5–3.6% of the total energy loss. This may be a result of the high oxygen consumption rate, because weight-specific ammonia excretion of larvae is comparable with that of juveniles; whereas, weight-specific oxygen consumption is much higher.

The highest weight-specific rate of ammonia excretion was

found for 120  $\mu\text{m}$  larvae. Bay scallop life history begins with a brief lecithotrophic stage, during which energy metabolism is supported by the energy reserve of eggs. As determined by Lu and Blake (1997), larvae of 120  $\mu\text{m}$  shell length have started to feed on phytoplankton. The observed high ammonia excretion rate may indicate that these larvae cannot assimilate sufficient energy for their metabolic demand and still must partially depend on the energy reserves from the eggs, which consist mainly of protein (Lu 1996). In eyed larvae, protein metabolism is dramatically reduced, as shown by the low weight-specific ammonia excretion of larvae at this stage. Heavy utilization of protein occurs during metamorphosis, and, thus, a high rate of ammonia excretion would be expected for metamorphosing larvae. Data from the present study show that the smallest juveniles tested (500  $\mu\text{m}$  shell height) did not display significantly higher rates of ammonia excretion than larger ones as expected, probably because they had finished metamorphosis and already lost the characteristics of metamorphosing larvae. Direct measurements on metamorphosing larvae may provide some evidence on this matter.

Weight-specific ammonia excretion found for juvenile bay scallops in the present study (146–250  $\text{ngN mg DW}^{-1} \text{ h}^{-1}$ ) is higher than that found for adults (72 to 140  $\text{ngN mg DW}^{-1} \text{ h}^{-1}$ ). This is consistent with the findings that weight-specific physiological rates (feeding and respiration) are higher in juveniles than in adults.

Oxygen consumption measured for larvae and juveniles of the bay scallop is comparable to that found for other bivalve larvae and juveniles (reviewed by Sprung 1984; Beiras and Camacho 1994), despite the fact that our measurements were made at a higher temperature (25 °C). Because metabolic rates are often determined at the optimum temperature range for each species, this may simply indicate that the bay scallop is adapted to the temperature encountered in its natural environment. Our data show that weight-specific ammonia excretion of larvae and juveniles is slightly higher but comparable to those determined for adult bay scallops, following the general trend that weight-specific physiological rates decrease as animals develop.

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## BIOCHEMICAL UTILIZATION DURING EMBRYOGENESIS AND METAMORPHOSIS IN THE BAY SCALLOP, *ARGOPECTEN IRRADIANS CONCENTRICUS* (SAY)

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**ABSTRACT** Protein, total lipid, and carbohydrate were measured for spawned eggs, D-shaped larvae, premetamorphic, and metamorphic larvae of the bay scallop *Argopecten irradians concentricus* (Say). Spawned eggs were composed of 64.0% protein, 26.5% lipid, and 9.5% carbohydrate. After 48 hours of embryogenesis, 13.6% of the protein, 46.3% of the lipid, and 20.8% of the carbohydrate mass had been lost, providing 25.5%, 69.8%, and 4.8% of the total energy expenditure of 0.176 mJ per embryo. During 48 hours of metamorphosis, lipid was utilized first, followed by a heavy consumption of protein; protein, lipid and carbohydrate lost 55.6%, 59.4%, and 67.3% of their mass respectively. Protein and lipid supplied a comparable amount of energy for metamorphosis, 47.9% and 43.5%, respectively; whereas, carbohydrate contributed only 8.6%, to the 4.35 mJ per larva metamorphic energy expenditure.

### INTRODUCTION

Early survival of marine bivalves is limited by two critical developmental stages: embryogenesis and metamorphosis. Because of the difficulties in obtaining such information from the natural environment, our knowledge on these subjects comes primarily from laboratory studies and hatchery production of commercially important species. Low survival of early developmental stages in laboratory studies has often been found to be associated with embryogenesis and metamorphosis (Castagna and Duggan 1971, Heffernan et al. 1992), with fluctuations in environmental factors often increasing mortalities (Tettelbach and Rhodes 1981, Lu 1989, Lu and Blake 1996).

Embryogenesis of most marine bivalves occurs in surrounding waters rather than in the female and, thus, represents an environmental stage of negative energy balance, because embryos do not have a digestive system and cannot feed on particulates. Endogenous energy reserves of eggs are mobilized to supply the energy necessary for embryogenesis, and this process lasts to at least the formation of a digestive tract. In bivalves, this represents a development from eggs to straight hinge veligers or D-shaped larvae. Early veligers of the bay scallop *Argopecten irradians concentricus* still need to rely partially on endogenous reserves in addition to energy obtained from feeding (Lu 1996).

Metamorphosis represents the second phase of development, wherein stored energy reserves are consumed for metabolism during early development (Whyte et al. 1990). Energy reserves are accumulated by the planktonic larvae through feeding on organic particles and are subsequently used for supporting metamorphosis (Rodriguez et al. 1990, Haws et al. 1993). During metamorphosis, the larval velum disappears, and larvae lose their ability to feed until the development of gill filaments (Sastry 1965, Bayne 1965, Hickman and Gruffydd 1971).

Success in completing embryogenesis and metamorphosis is determined to a large extent by the amount of energy reserves inherited from the female and/or accumulated through larval feeding. High survival has been found in the large eggs of *Mercenaria mercenaria* and *Argopecten irradians* (Kraeuter et al. 1982), probably because of their higher energy content. Survival may depend upon the ability of embryos or larvae to complete development of feeding structures before energy reserves are depleted (Haws et al. 1993).

The southern bay scallop was reported to produce smaller oo-

cytes (Barber and Blake 1981) than its northern counterpart. Low energy reserves of small eggs and higher metabolic demand associated with higher temperature in its natural habitat could be disadvantageous to the early development of the southern bay scallop. However, information on the energy metabolism of early development in the bay scallop is lacking. The objective of this study was to investigate changes in biochemical composition of eggs, larvae, and juveniles, and the energy expenditures associated with embryogenesis and metamorphosis of the southern bay scallop *Argopecten irradians concentricus* (Say).

### MATERIALS AND METHODS

#### Sample Collection

Bay scallops were collected from Homosassa, Florida and were spawned in the lab of the Department of Marine Science, University of South Florida. Culturing of larvae and juveniles followed the methods described by Lu and Blake (1996). Larvae were raised at a density of 4–8 mL<sup>-1</sup> and fed daily with 10,000–30,000 cells mL<sup>-1</sup> of *Isochrysis galbana*, depending upon larval size. Seawater was replaced every day in the amount of 1/3 of the total volume. Daily food ration for juveniles was increased gradually from 30,000 to 100,000 cells mL<sup>-1</sup> of *I. galbana*.

Fertilized eggs were collected onto a 28 µm nylon screen. A portion of the eggs were used for biochemical analysis, and the rest were released in 1.2 µm filtered seawater (25% S) and allowed to develop at 26 °C. After 48 hours, D-shaped larvae were collected on a 35-µm nylon screen. The egg and larval samples were washed with 1.2-µm filtered seawater and pipetted to a graduated cylinder. Seawater was added to bring the volume to 100 mL and total eggs or larvae were determined by counting five 0.5-mL samples. Mean egg diameter and larval shell length were determined by measuring 50 individuals each using a microscope fitted with a micrometer. Subsamples were drawn from the graduated cylinder, washed three times with 3% ammonium formate solution to remove salt, and frozen at -20 °C until analyzed for biochemical composition. Each sample for chemical analysis contains 100,000–150,000 eggs or 150,000–200,000 D-larvae.

Premetamorphic larvae (mean shell height 185 ± 7.8 µm) were kept in 2000-mL plastic beakers containing 1.2-µm filtered seawater without food. A sample of larvae was taken 24 hours later. Development was followed microscopically, and juveniles were observed in the culture at 48 hours. Veligers that settled on the

beakers were brushed into a petri dish. Juveniles were separated from the pediveligers under a microscope. Samples of larvae and juveniles were washed with 3% ammonium formate solution, quantified, and frozen at  $-20^{\circ}\text{C}$  until analyzed. Each sample for chemical analysis contains 3,000–5,000 premetamorphic larvae or 1,000–2,000 postmetamorphic larvae.

Egg and larval samples were homogenized in 1.5-mL DI water using a 4710 series ultrasonic homogenizer (Cole-Parmer Instrument Co.). Subsamples were taken from the slurry for protein, lipid, and carbohydrate analysis.

Juveniles of various shell height (1–10 mm) were collected from a 300 Liter stocking tank at various times. Sample size ranges from 1–50 individuals, depending upon size. For juveniles less than 2 mm, it is difficult to completely separate the soft body from shells; consequently, whole animals were homogenized in DI water with a tissue grinder and then an ultrasonic homogenizer. For larger juveniles up to 10 mm, only the soft body was homogenized. In all cases, samples were analyzed within 1 month of collection.

#### Biochemical Analysis

Under most circumstances, three samples were analyzed for eggs and larvae of each development stage. The exception is with juveniles (>2 mm shell height), which were analyzed individually.

Protein analysis followed the Folin phenol method of Lowry et al. (1951). Three samples of 20- $\mu\text{L}$  homogenate each were transferred to test tubes, to which 60  $\mu\text{L}$  of DI water was added to make final volumes of 80  $\mu\text{L}$  each. Samples were hydrolyzed by adding 0.12 mL of 0.1 N NaOH to each test tube and heating at  $100^{\circ}\text{C}$  for 10 minutes. After cooling, 1.2 mL Reagent B (0.5 mL 1%  $\text{CuSO}_4/5\text{H}_2\text{O}$  + 0.5 mL 2% Na Tartrate + 50 ml 2%  $\text{Na}_2\text{CO}_3$ ) was added and allowed to sit for 10 min. Then 0.12 mL reagent E (phenol reagent diluted to 1 N with water) was added and mixed immediately. After 30 min, the optical density of the blue solution was read at 750 nm on a Cary 2000 spectrophotometer with bovine serum albumin as the standard.

Lipid was extracted according to the method of Bligh and Dyer (1959). Three samples of 0.2-mL homogenate each were placed in test tubes. To each sample, 0.73 mL of 2:1 MeOH: $\text{CHCl}_3$ , 0.24-mL chloroform, and 0.24-mL  $\text{H}_2\text{O}$  were added and mixed after each addition. The samples were allowed to sit for 1 h for separation. The two phase solutions were poured into 0.45- $\mu\text{m}$  PTFE centrifuge filter units, and the lipid fraction passing through was collected in test tubes and dried at  $30^{\circ}\text{C}$  under a flow of nitrogen. Total lipid content was determined using the charring method of Marsh and Weinstein (1966). To each dried sample, 1 mL of concentrated  $\text{H}_2\text{SO}_4$  was added, followed by heating at  $200^{\circ}\text{C}$  for 20 minutes. The charring samples were cooled in tap water and were then diluted with 3-mL  $\text{H}_2\text{O}$ . After cooling, the optical density was read at 375 nm on a spectrophotometer with stearic acid as the standard.

Total carbohydrate content was determined using the phenol-sulfuric acid method of Dubois et al. (1956). Three samples of 0.2-mL homogenate were pipetted to centrifuge tubes, washed consecutively with acetone and ether to remove lipids, and dried at  $60^{\circ}\text{C}$ . The dried samples were hydrolyzed in 2.0 mL 5% trichloroacetic acid at  $100^{\circ}\text{C}$  for 20 minutes. After cooling, the tubes were centrifuged at 5,000 rpm for 10 minutes and 1-mL supernatant was removed from each sample to  $16 \times 150$ -mm test tubes. To each test tube, 0.5 mL of 5% phenol and 2.5 mL of concentrated  $\text{H}_2\text{SO}_4$  were added. After cooling for 30 minutes, the optical

density of the orange-yellow solution was read at 490 nm on a Cary 2000 spectrophotometer with oyster glycogen as the standard.

Energy expenditures for embryogenesis and metamorphosis were estimated by the loss of protein, lipid, and carbohydrate during those two events. Energy conversion factors used were 20.0, 39.5, and 17.5  $\text{mJ } \mu\text{g}^{-1}$  for protein, lipid, and carbohydrate respectively (Brett and Groves 1979).

#### RESULTS

Table 1 gives the content of protein, lipid, and carbohydrate at various developmental stages and shell lengths. The spawned eggs of the southern bay scallop had a mean diameter of  $60.7 \pm 0.8 \mu\text{m}$ , and were composed primarily of protein (64.0%), lipid (26.5%), and carbohydrate (9.5%). Mean energy content of an egg was 0.631 mJ. During the 48 hours of embryogenesis, all three components decreased. Protein dropped 13.6% by mass, lipid 46.3%, and carbohydrate 20.8%, respectively, leading to a loss of 22.9% of the total organic matter (defined here as the total of protein, lipid, and carbohydrate) (Table 2). Lipid was the major substrate utilized for embryogenesis, supplying 69.8% of the total energy expenditure of 0.176 mJ per embryo, while protein and carbohydrate contributed 25.5% and 4.8% respectively.

Lipid was accumulated as the larvae developed, increasing from 18.5% in D-larvae to 26.9% in premetamorphic larvae. Mean energy content of premetamorphic larvae (185- $\mu\text{m}$  shell length) was 7.49 mJ per larva. During the first 24 hours of metamorphosis, protein, lipid, and carbohydrate lost 24.4%, 38.8%, and 53.6% of their original content, respectively. Lipid supplied half of the total energy expenditure, and protein and carbohydrate made up the rest. Carbohydrate was the substrate that lost the highest portion, but it only contributed 12.2% to the total energy expenditure because of its low absolute content.

During the second 24 hours of metamorphosis, more protein was lost (41.3%) than lipid (33.5%) and carbohydrate (29.4%). Protein surpassed lipid as the major substrate in fulfilling the energy demand. The role of carbohydrate as an energy reserve was further reduced.

Overall, the process of metamorphosis consumed 57.9% of the total organic substrate, contributed by 55.6%, 59.4%, and 67.3% of protein, lipid, and carbohydrate reserves respectively. Total energy expenditure during metamorphosis was 4.35 mJ per larva, with

TABLE 1.

*Argopecten irradians concentricus*. Changes in biochemical content (ng ind.<sup>-1</sup>) during embryogenesis and metamorphosis.

Developmental Stages	Length ( $\mu\text{m}$ )	Protein	Lipid	Carbohydrate	Total
Fertilized eggs	61	$16.2 \pm 0.5$ (64.0%)	$6.7 \pm 0.4$ (26.5%)	$2.4 \pm 0.1$ (9.5%)	25.3
D-larvae	98	$14.0 \pm 0.1$ (71.8%)	$3.6 \pm 0.2$ (18.5%)	$1.9 \pm 0.1$ (9.7%)	19.5
Premetamorphic starved 0 hrs	185	$187.4 \pm 6.6$ (62.4%)	$80.5 \pm 7.3$ (26.9%)	$31.8 \pm 0.4$ (10.6%)	299.7
starved 24 hrs	187	$141.7 \pm 2.8$ (68.9%)	$49.3 \pm 5.6$ (24.0%)	$14.7 \pm 0.2$ (7.1%)	205.6
Postmetamorphic	230	$83.2 \pm 1.9$ (65.8%)	$32.8 \pm 1.6$ (25.9%)	$10.4 \pm 0.2$ (8.2%)	126.4

TABLE 2.

*Argopecten irradians concentricus*. Losses of biochemical substrates, their caloric equivalents and contribution to energy expenditures of embryogenesis and metamorphosis.

	Protein	Lipid	Carbohydrate	Total
Embryogenesis				
Wt loss (ng ind. <sup>-1</sup> )	2.2	3.1	0.5	5.8
Wt loss (%)	13.6%	46.3%	20.8%	22.9%
E equiv. (mJ ind. <sup>-1</sup> )	0.0449	0.1229	0.0084	0.1762
E contribution (%)	25.5%	69.8%	4.8%	100%
Metamorphosis				
Wt loss (ng ind. <sup>-1</sup> )	104.2	47.8	21.4	173.4
Wt loss (%)	55.6%	59.4%	67.3	57.9%
E equiv. (mJ ind. <sup>-1</sup> )	2.0841	1.8909	0.374	4.3487
E contribution (%)	47.9%	43.5%	8.6%	100%

protein and lipid providing 47.9% and 43.5%, respectively, and carbohydrate contributing only 8.6%.

Protein, lipid, and carbohydrate content of juveniles are summarized in Table 3. In contrast to the larval biochemical composition, juveniles had significantly higher levels of protein ( $P < .001$ ) and lower levels of lipid ( $P < .001$ ). There was no significant difference in carbohydrate levels between larvae and juveniles. Protein was the major component in the biochemical composition of juveniles, constituting  $72.6 \pm 3.0\%$  of the total organic matter, and mean lipid and carbohydrate components were  $18.6 \pm 1.1\%$  and  $8.8 \pm 2.5\%$ , respectively. As in the larval stage, carbohydrate was the least significant component in juveniles.

## DISCUSSION

Embryogenesis of marine invertebrates is an energy-consuming process during which embryos rely solely on energy reserves within the eggs provided by the female. Existing information has shown that energy stored in other parts of the body is transferred to the gonads during gametogenesis (Gabbott 1976, Bayne 1976, Barber and Blake 1981) and energy stored in eggs as protein, lipid, and carbohydrate substrates are subsequently utilized for growth and development of the embryo (Gallager et al. 1986, Whyte et al. 1990). Protein forms the main constituent of eggs, followed by

TABLE 3.

*Argopecten irradians concentricus*. Protein, total lipid, and total carbohydrate content ( $\mu\text{g ind.}^{-1}$ ) in juveniles.

Mean Height (mm)	Protein	Lipid	Carbohydrate	Total
1.07	8.8 (63.3%)	3.3 (23.7%)	1.8 (12.9%)	13.9
2.1	68.5 (71.6%)	18.0 (18.8%)	9.2 (9.6%)	95.7
2.2	81.3 (68.6%)	25.0 (21.1%)	12.2 (10.3%)	118.5
2.8	112.1 (67.9%)	30.2 (18.3%)	22.7 (13.8%)	165.0
3.0	162.4 (73.7%)	40.7 (18.5%)	17.2 (7.8%)	220.3
3.1	176.8 (74.2%)	43.8 (18.4%)	17.8 (7.5%)	238.4
3.4	248.3 (69.4%)	64.9 (18.1%)	44.7 (12.5%)	357.9
4.8	566.1 (74.7%)	140.5 (18.5%)	51.3 (6.8%)	757.9
5.1	682.8 (74.5%)	164.6 (18.0%)	69.4 (7.6%)	916.8
5.2	685.3 (75.6%)	160.8 (17.7%)	60.7 (6.7%)	906.8
5.5	837.4 (71.6%)	205.4 (17.6%)	74.2 (6.3%)	1170.0
5.8	953.7 (75.1%)	223.7 (17.6%)	92.9 (7.3%)	1270.3

lipid and then carbohydrate in marine invertebrates (reviewed by Holland 1978). The present study on the eggs of the bay scallop *Argopecten irradians concentricus* shows the same trend: protein forms the main biochemical constituent (64.1%), followed by lipid (26.4%). Carbohydrate is the smallest component of the three (9.5%).

Energy reserves in the form of protein, lipid, and carbohydrate were utilized as eggs developed, which was indicated by the reduction of corresponding substrates at the end of embryogenesis. In the bay scallops, lipid was used as the principal energy source for egg development, supplying 69.8% of the total energy expenditure, more than protein (25.5%) and carbohydrate (5.5%) combined. Carbohydrate played a minor role as an energy reserve in the bay scallop because of its low content in eggs. High conversion efficiency ( $W_{\text{final}}/W_{\text{initial}}$ , where W is the weight of a given biochemical constituent (see Holland 1978) of protein (86.4%) and low conversion efficiency of lipid (53.7%) provide evidence that protein is conserved for the formation of planktonic larvae, and lipid is the major energy reserve fueling this process.

Information on the utilization of major biochemical constituents for embryogenesis of marine invertebrates comes mainly from studies on crustaceans. Although some species use lipid as the major energy reserve (Pandian 1967, Pandian and Schumann 1967, Shakuntala 1977), other species rely primarily on protein (Barnes 1965, Lucas and Crisp 1987). The utilization of lipid as the dominant energy reserve in developing eggs was also reported for the Pacific halibut *Hippoglossus stenolepis* (Schmidt) (Whyte et al. 1993) and the red drum *Sciaenops ocellata* (Vetter et al. 1983); whereas utilization of protein dominated in the rainbow trout *Salmo gairdneri* (Oliva-Teles and Kaushik 1987). In bivalves, 69 and 71% of total lipid was lost during embryogenesis of the clam *Mercenaria mercenaria* and the oyster *Crassostrea virginica*, respectively (Gallager et al. 1986), indicating heavy use of lipid; however, lack of information on changes in protein and carbohydrate contents prevents estimation on relative importance between lipid and protein. Whyte et al. (1990) reported that lipid and protein substrates contributed equally to the energy expenditure of embryogenesis of the rock scallop *Crassadoma gigantea*, accounting for 46.7 and 43.5%, respectively, and carbohydrate supplied only 9.8%.

During the planktonic stages of the bay scallop, larvae feed on organic particles, and their organic mass increases as larvae grow (Lu and Blake 1996). As a result, larvae build up energy reserves of protein, lipid, and carbohydrate through feeding (Table 1). Relative lipid content increased from 18.5% in D-shaped larvae to 26.9% in premetamorphic larvae.

Bay scallop larvae use lipid reserves at the beginning of metamorphosis followed by a heavier consumption of protein reserves. During the first 24 hours, metamorphosing larvae derived 50.5% of their energy from lipid and 37.3% from protein. During the next 24 hours, lipid supplied 34.4% of the total energy expenditure of metamorphosing larvae, and protein supplied the bulk of it, 61.6%. On average, protein and lipid provided similar amounts of energy, 47.9 and 43.5%, respectively, during the 48-hour metamorphosis. As in embryogenesis, carbohydrate is the least important constituent in metamorphosing larvae and contributed only 8.6% of the energy expenditure.

Disagreement remains on which substrate serves as the major source of energy for metamorphosis in bivalves. In the oyster *Ostrea edulis*, Holland and Spencer (1973) reported that over half of the neutral lipid reserves were used for metabolism during meta-

morphosis, but the authors had no data for the use of protein, carbohydrate, or phospholipid. However, a later study on the same species by Rodriguez et al. (1990) found protein supplied most of the energy for metamorphosis (62%), more than twice that supplied by lipid (28%). In two other species of oysters, *Crassostrea virginica* and *C. gigas*, 50.2% and 51.1% of their total energy expenditure during metamorphosis came from lipid and 39.9% and 38.5% from protein, respectively (Haws et al. 1993). In the rock scallop *Crassadoma gigantea* (Gray), Whyte et al. (1992) reported that protein formed 59.9% of the total energy expenditure during metamorphosis and lipid 38.5%. However, their data, like those of Whyte et al. (1990), were derived from relative values and were found hard to interpret.

In the bay scallop, metamorphosis consumed 57.9% of the total organic reserves, equivalent to a total energy expenditure of 4.35 mJ per larva. This may represent the minimum level of consumable energy reserve of bay scallop larvae, below which larvae cannot complete metamorphosis without obtaining energy from their surrounding environment. Assuming that metamorphosing larvae have the same respiration rate as eyed larvae ( $14.664 \mu\text{LO}_2 \text{ mgAFDW}^{-1} \text{ h}^{-1}$ ; Lu 1996), energy expenditure during 48 hours of metamorphosis can be calculated to be 4.55 mJ. This value is close to the value of 4.35 mJ determined in this study, to further support our estimation on the energy requirement for metamorphosis of the bay scallop. Those values are comparable to those determined for the oyster *Crassostrea virginica* and *C. gigas* (2.13 and 4.65 mJ per larva, respectively, over a 36-hour period) (Haws et al. 1993). The oyster *Ostrea edulis* seems to lose more energy (5.62–14.65 mJ/larva over a 36–48-hour period) during metamorphosis (Rodriguez et al. 1990).

One of the main difficulties in estimating the biochemical changes and the associated energy metabolism is the lack of

knowledge on when premetamorphic larvae stop feeding and for how long. Our understanding of biochemical energetics in metamorphosing larvae has been based on the assumption that metamorphosing larvae lack the capability to feed and that metamorphic energy demand comes solely from energy reserves accumulated during the planktonic stage. In a recent feeding study on the oyster *Crassostrea virginica*, Baker and Mann (1994) found that all prodissoconchal and dissoconchal metamorphs ingested the experimental microspheres, and, except for only a few hours during the settler phase, feeding was possible throughout the oyster metamorphosis. If this finding holds true for other bivalve larvae, energy obtained through feeding by metamorphosing larvae has to be taken into account in the estimation of energy budget. However, it is not clear what the quantitative contribution of feeding to the total energy expenditure of metamorphosing larvae is. Results of this and other studies (e.g., Rodriguez et al. 1990, Haws et al. 1993) demonstrate that bivalve larvae are able to complete metamorphosis based solely on the accumulated energy reserves of their biochemical substrates.

In the natural environment, larvae may cease feeding at a very late stage of metamorphosis, or larvae may stop feeding for only a few hours during metamorphosis, as observed by Baker and Mann (1994). As a result, the minimum energy level can be substantially lower, and larvae may not need as much energy reserve for metamorphosis as previously thought. Therefore, the estimation made here may represent the upper range of the metamorphic metabolic demand of the bay scallop.

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## CHROMOSOMAL LOCATION BY FLUORESCENCE *IN SITU* HYBRIDIZATION OF THE 28S RIBOSOMAL RNA GENE OF THE EASTERN OYSTER

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**ABSTRACT** The physical location of the 28S ribosomal RNA gene (28S rDNA) was localized to the short arm of chromosome number 2 of the eastern oyster (*Crassostrea virginica*) by fluorescence *in situ* hybridization. The existence of a single locus of the 28S rDNA in the genome of the eastern oyster was concluded based on the findings from metaphase chromosomes prepared from gill, mantle, and embryos and from meiotic chromosome bivalents prepared from ripe gonad tissue. The region of the chromosome occupied by the gene was found to be GC-rich, and the location of probe DNA used to identify the 28S gene corresponded exactly with the location of the nucleolus organizer region. The transcriptional activity of the gene did not vary among different cell types but changed with different phases of mitosis. This study is the first report of physical mapping of a specific gene in mollusks and provides techniques for detection of integration of foreign DNA in the oyster genome.

**KEY WORDS:** fluorescence *in situ* hybridization, 28S rDNA, chromosome, *Crassostrea virginica*

### INTRODUCTION

The ribosomal RNA genes are a group of DNA sequences that produce structural rRNA to support protein synthesis. For example, the 28S ribosomal RNA gene (28S rDNA) encodes a major component of the large subunit of the eukaryotic ribosome. The nucleotide sequences of these genes are polymorphic, which has enabled taxonomic investigation of organisms with ambiguous phylogenetic relationships (Littlewood 1994).

In addition, 28S rDNA is useful for the initiation of physical genome mapping, because the gene exists in multiple copies, which improves the ease of detection. There are various methods for verifying the chromosomal location of 28S rDNA. Fluorescence *in situ* hybridization (FISH) (Pendas et al. 1993) or *in situ* polymerase chain reaction (Zhang et al. 1997) can be used for direct assignment of gene location. The 28S rDNA loci can be revealed indirectly by chromomycin A3 staining to target GC-rich regions (Amemiya and Gold 1987). Moreover, the 28S RNA genes are associated with the nucleolus organizer regions (NOR) (Long and Dawid 1980). By use of silver staining, which targets protein components associated with RNA synthesis (Howell and Black 1980), the active 28S rDNA loci can be detected.

The eastern oyster has been harvested commercially in the United States for hundreds of years. An organized system of oyster leases has been maintained in Louisiana for over 100 years, and harvest in Louisiana was valued at more than \$50 million in 1997 (Louisiana Summary of Agricultural and Natural Resources 1997). Overall, genetic improvement in this species has been hampered by the absence of basic genetic information. Study of the physical location of DNA sequences in oysters is still preliminary, and reports are restricted to nonspecific DNA elements derived from the mammalian genome (Guo and Allen 1997) or oyster genome (Clabby et al. 1996). The procedures for physical mapping of specific genes must be developed and validated in this species.

The chromosomal location of the 28S rDNA in the eastern oyster has been preliminarily investigated using FISH techniques (Zhang et al. 1999a; Xu et al. 1999). Our goal was to use the 28S

rDNA as a first step to verify techniques for physical mapping of genes in oysters. Specific objectives of the present study were to: 1) localize the 28S rDNA on eastern oyster chromosomes by fluorescence *in situ* hybridization; 2) compare these sites of the 28S rDNA to those detected by silver staining and chromomycin A3 staining; and 3) evaluate the transcriptional activity of the 28S rDNA in different cell types.

### MATERIALS AND METHODS

#### Probe Construction

Nuclear DNA was isolated from hemolymph collected from adult oysters ( $n = 5$ ) using a QIAamp blood kit (Qiagen Inc., Chatsworth, CA). Probe DNA was synthesized by polymerase chain reaction (PCR). A pair of primers was designed to target the oyster 28S rDNA gene based on a published sequence (Littlewood 1994) and was synthesized by the Gene Probes and Expression Systems Laboratory, Louisiana State University, Baton Rouge. The primer sequences were (5' to 3'): GCTAAATACTTCCCG-AGTCCGATAGC and GCACCTTCTCCAGCTCTTCTGAC. Conditions for PCR were initial denaturation at 94 °C for 2 min; 35 cycles at 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 1 min, with a final elongation step at 72 °C for 7 min. The PCR products were labeled by inclusion of biotin-16-dUTP in the reaction mixture. Labeling was visualized by agarose electrophoresis, in which the shift of band size was detected because of incorporation of label into the PCR products.

#### Chromosome Preparation

Ten ripe oysters (five males and five females) were used in this study. Chromosomes were prepared from gonad tissues, embryos at 5 h after fertilization (Paniagua-Chavez et al. 1998) and gill tissues with methods reported elsewhere (Zhang et al. 1999b). For obtaining bivalent chromosomes, the gonad tissue was not treated with colchicine, but a prolonged hypotonic treatment in a 1% sodium citrate solution (~5–7 h) was used to promote separation of

chromosomes. Chromosomes were prepared using standard air-drying methods (Zhang et al. 1999b).

#### Fluorescence In Situ Hybridization

Chromosomes were prepared on two-well, Teflon-coated slides (Cel-Line Associates, Inc., Newfield, NJ) and digested with RNase A (100 µg/ml) in 2-x SSC buffer at 37 °C for 1 h. After dehydration with a series of ethanol concentrations (75%, 85%, 95%, and 100%), chromosomal DNA was denatured in 70% formamide at 70 °C for 5 min. The slides were chilled immediately in 70% ethanol at -20 °C, and were dipped in 100% ethanol and dried in a laminar-flow hood.

The hybridization mixture was composed of biotin-labeled probe (1.2 ng/ml), 50% deionized formamide, 2-x SSC buffer, 10-x Denhardt's solution, 0.1% sonicated salmon sperm DNA, and 0.1% sodium dodecyl sulfate (Chen, 1994). The mixture was heated at 95 °C for 10 min and chilled immediately on ice. Twenty µl of the mixture was applied to each well of slides prewarmed to 37 °C, and coverslips were sealed using clear nail polish. The slides were incubated in a wet box at 37 °C for 24 to 36 h. Hybridization was detected with avidin-fluorescein isothiocyanate (FITC) (Zhang et al. 1997). Slides were counterstained with propidium iodide (PI) (0.5 µg/ml) prepared in an antifading medium (100 mg *p*-phenylenediamine in 100 ml glycerol, pH 7.0).

#### Identification of NOR-Bearing Chromosomes by Silver or CMA Staining

Slides were treated with 50% acetic acid for 20 min to remove background materials on embryonic and gonadal chromosomes. To determine the relationship between the 28S rDNA and NOR, chromosomes were stained with chromomycin A3 (CMA) made in a modified McIlvaine's buffer (Amemiya and Gold 1987). After the images of chromosomes were recorded, slides were rinsed gently with 2-x SSC and left in the buffer for 20 min. The slides were dehydrated through a series of ethanol concentrations (70%, 80%, 95%, and 100%) and stained with a one-step silver staining procedure (Howell and Black 1980). Slides were covered with a solution of 33% silver nitrate and 0.7% gelatin and incubated for 8 to 10 min at 50 °C.

#### Image Analysis and Map Construction

Fluorescent images of chromosomes stained with CMA or FITC and PI were examined under a fluorescence microscope (Microphot-SA, Nikon Inc., Garden City, NY) equipped with filters for FITC and CMA (excitation wavelengths of 480 nm) and PI (excitation wavelength of 535 nm). Fluorescent images of nuclei and chromosomes were photographed using Kodak Ektachrome (400 ASA) color slide film. The negatives were scanned into a computer with a slide scanner (SprintScan 35, Polaroid scanner model CS-2770, Needham Heights, MA) for analysis. For silver staining, chromosomal images were captured and analyzed by a computer-based image analysis technique (Zhang and Tiersch 1998). Individual chromosomes were identified based on a previously developed karyotype (Zhang et al. 1999b). Relative length (RL) and centromeric index (CI) of each chromosome were calculated using the following formulae:

$$RL (\%) = (\text{length of the chromosome pair} / \text{total complement length}) \times 100$$

$$CI (\%) = (\text{length of short arm} / \text{total length of the chromosome}) \times 100$$

The location of the 28S rDNA gene was analyzed by microdensitometry, and a map of the chromosome bearing the 28S rDNA was constructed using Microsoft PowerPoint (Office 97 version).

## RESULTS

The location and activity of the 28S rDNA in the genome of the eastern oyster was investigated using four cell sources (Table 1). Two chromosomes from embryo cells were found to hybridize with the 28S rDNA probe (Fig. 1a). The RL of one chromosome was  $6.14 \pm 0.12$ , and the CI was  $42.3 \pm 1.8$ ; whereas, the other chromosome had a RL of  $5.90 \pm 0.10$  and a CI of  $36.5 \pm 2.1$  ( $n = 10$  spreads). Two chromosomes from gill cells (Fig. 1b) were found to hybridize with the 28S rDNA (Fig. 1b & 1c). The same region identified by the 28S rDNA probe was found to stain intensely with chromomycin A3, indicating the presence of GC-rich regions (Fig. 2a), and the location of the 28S rDNA was found to be the same as that of the NOR, as indicated by silver staining (Fig. 2b).

One of 10 bivalent chromosomes prepared from gonad tissue was found to hybridize with the 28S rDNA probe (Fig. 3a). Two hybridization signals sometimes appeared on the same bivalent corresponding to the presence of homologous chromosomes that were in the process of separating (Fig. 3b). The chromosome bivalent was consistently stained with CMA at diakinesis (Fig. 3c) and pachytene stages (Fig. 3d) and was stained positively with silver nitrate for the NOR at pachytene stage (Fig. 3e). However, in most cases (30 out of 50 spreads), this NOR site was not detectable by silver staining of diakinesis and pachytene chromosomes.

Upon more detailed examination of the chromosome in question (number 2, from embryo), the location of the 28S rDNA, as measured by microdensitometry, was identified at the telomeric regions corresponding to the site of maximal hybridization intensity of the 28S rDNA probe (Fig. 4).

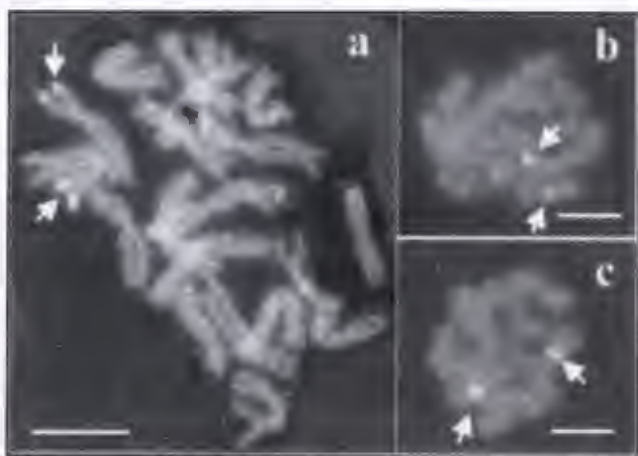
## DISCUSSION

In this study, the 28S rDNA of the eastern oyster was found to be localized to the telomeric region of the short arm of chromosome number 2 by fluorescence *in situ* hybridization. Although the two 28S rDNA-bearing chromosomes from embryo cells were different in size and centromeric index, a single chromosomal location of 28S rDNA was found using meiotic chromosome bivalents. The area of the gene was found to be GC-rich and to

TABLE 1.  
Characterization of the 28S ribosomal RNA gene on chromosomes of the eastern oyster by different techniques.<sup>a</sup>

Tissue Type	Phase of Cell Division	AgNOR Staining	CMA Staining	FISH
Embryo	Prophase	Two, NP	Not studied	Not studied
	Prometaphase	Two, NP	Two, NP	Two, NP
	Early metaphase	Two, NP	Not studied	Not studied
	Metaphase	One	Two, P, AS	Two, P
Gill	Metaphase	One	Not studied	Two, P
Mantle	Metaphase	One	Not studied	Two, P
Gonad	Pachytene	One bivalent	One bivalent	One bivalent
	Diakinesis	One bivalent	One bivalent	One bivalent

<sup>a</sup>Abbreviations: AgNOR, nucleolus organizer regions stained by silver nitrate; CMA, chromomycin A3; FISH, fluorescence *in situ* hybridization; NP, not pairable; P, pairable, and AS, asymmetric staining or different staining intensity between the two chromosomes.



**Figure 1.** Localization of the 28S ribosomal RNA gene (28S rDNA) of the eastern oyster by fluorescence *in situ* hybridization. Metaphase chromosomes were prepared from cells of (a) embryos and (b) and (c) gill. Arrowheads point to the location of 28S rDNA; bars = 10  $\mu$ m.

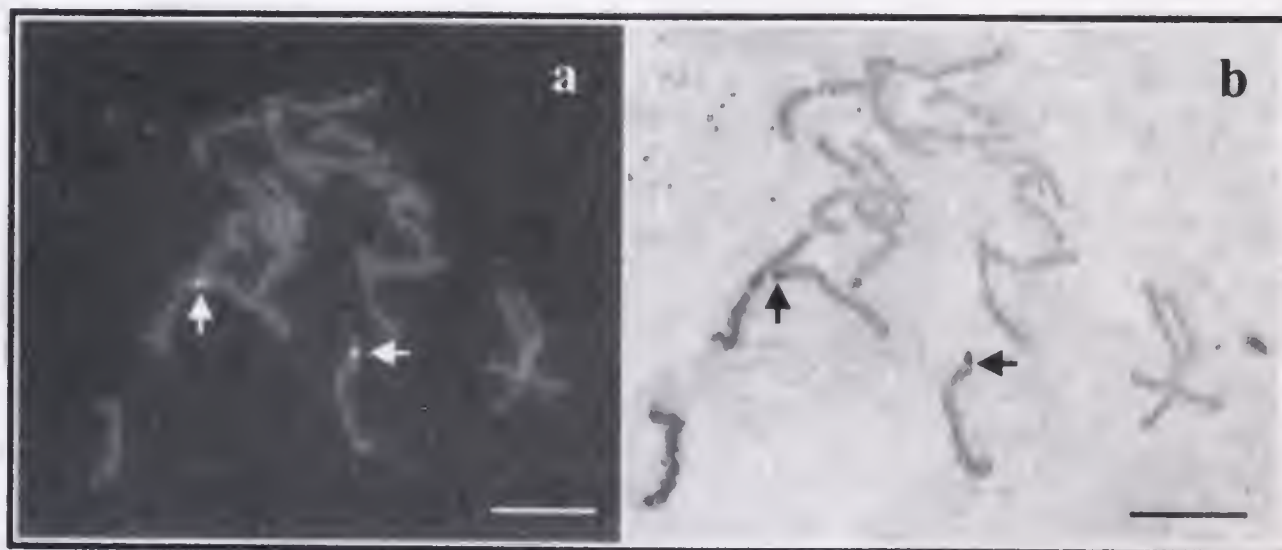
match exactly with the location of nucleolus organizer regions (NOR).

These results indicate that there is a single NOR-bearing chromosome pair in this species. In a previous study, two NOR-bearing chromosomes with measurable differences in size and centromeric index were found in preparations of embryo cells of the eastern oyster by use of silver staining (our unpublished data). Also, the number of the NOR sites was found to be variable among different phases of the cell cycle. Asymmetric features have been observed between homologous chromosomes prepared from embryo cells (Zhang et al. 1999b). Therefore, it was worthwhile to investigate chromosomes prepared from other tissue types for clarification of the NOR sites in embryo cells and to develop methods for physical genome mapping in this valuable species. In the present study, analysis of the location of the 28S rDNA on gonadal chromosomes and the transcriptional activity of the 28S rDNA among different tissues indicates that the two NOR-bearing chromosomes observed in embryo cells are homologous and pair during meiosis.

Variation in chromosome morphology among closely related oyster species has been used to investigate evolutionary relationships (Thiriou-Quievreux and Insua 1992). However, our studies demonstrate that variation in morphology of homologous chromosomes, such as in the pair of NOR-bearing chromosomes, can exist in the eastern oyster, especially in chromosomes obtained from cells of embryos. Centromeric position can change when pericentric inversions involve different lengths of the chromosomal segments on each side of the centromere. This mechanism has been proposed to explain most of the chromosomal variation within oysters of the genus *Crassostrea* (Landron De Guevara et al. 1996) and could be used to interpret the difference of centromeric index between the two homologous 28S rDNA-bearing chromosomes found in this study, although it is likely not appropriate.

The small difference we observed in the homologs of chromosomes would be at the limit of detection when using basic procedures (Longwell and Stiles 1996). In catfish chromosomes, we have reported that a computer-assisted image system can routinely identify a size difference of  $\sim 0.2\%$  in relative length, which is equivalent to  $\sim 0.1 \mu$ m under the microscope (Zhang and Tiersch 1998). The size difference we observed between the two 28S rDNA-bearing chromosomes of the eastern oyster was  $\sim 0.4\%$  in prophase, prometaphase, and early metaphase of embryo chromosomes, but the difference was reduced in late metaphase and was not distinguishable in highly contracted somatic chromosome spreads. Explanations for the size differences between chromosomes include differential activity of the homologs, which may be useful for studying early development and gene expression in oysters.

The results of the present study provide valuable information for physical genome mapping in mollusks. Probe DNA was synthesized by polymerase chain reaction and required only DNA sequence information from two primer regions, eliminating time and labor-intensive cloning and screening procedures. Primers prepared for physical mapping of oyster genes could be derived in some cases from mammalian species because of evolutionary conservatism, which is especially beneficial in such species as the eastern oyster that lack DNA sequence information.



**Figure 2.** Relationship between the 28S rDNA locus and the nucleolus organizer region of the eastern oyster. The same chromosome spread prepared from embryonic cells was subjected to chromomycin A3 staining (a), followed by staining with silver nitrate (b). Arrowheads indicate location of 28S rDNA and NOR; bars = 10  $\mu$ m.

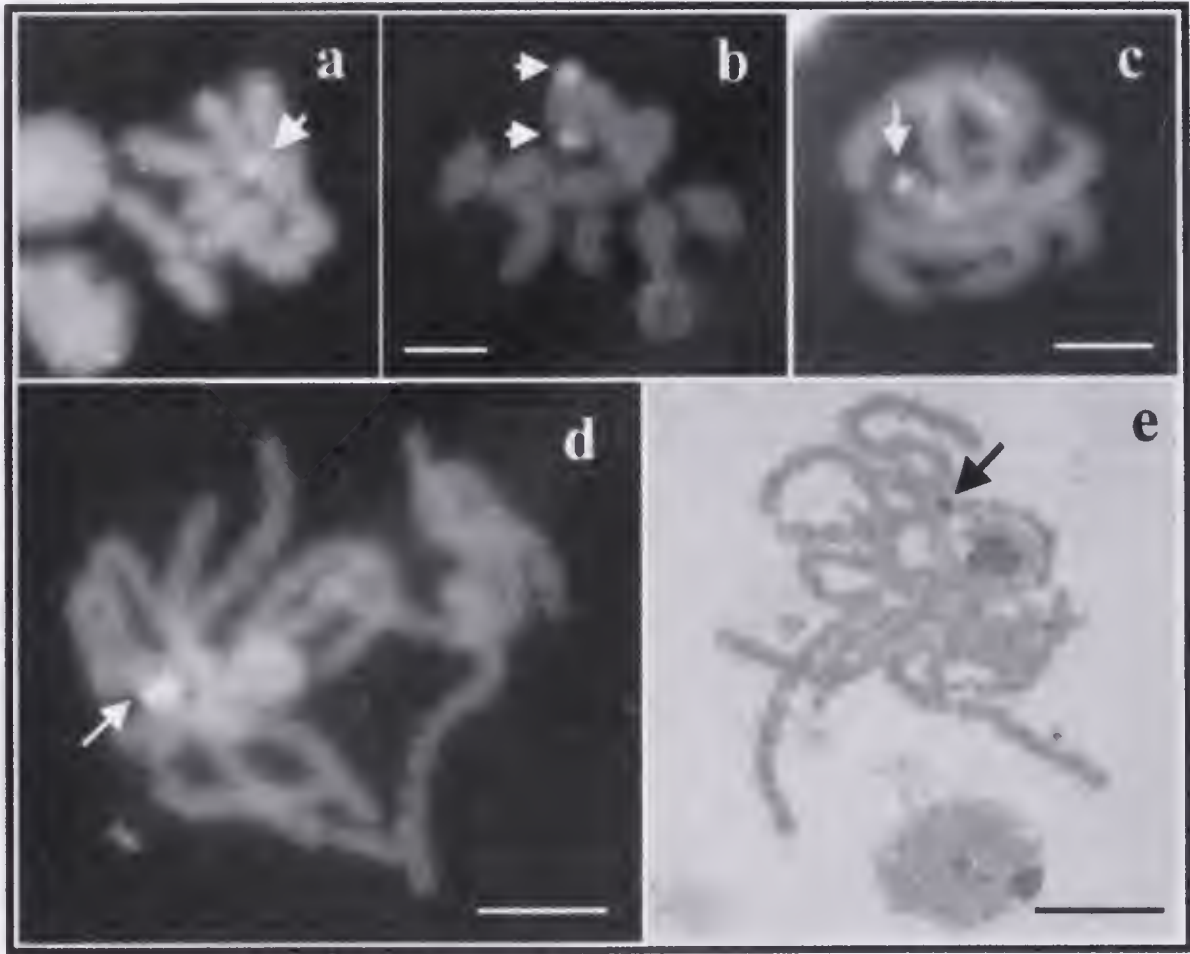


Figure 3. Location and activity of the 28S rDNA on meiotic chromosomes of the eastern oyster. Diakinesis (a, b, and c) and pachytene chromosome bivalents (d and e) were prepared from ripe gonad tissue. Fluorescence *in situ* hybridization (a and b), chromomycin A3 staining (c and d) and silver staining techniques (e) were used to analyze the chromosomal location of this gene (arrowheads); bars = 10  $\mu$ m.

The 28S rDNA, because of its association with the NOR, can be informative for verifying such specific hybridization techniques as FISH and *in situ* PCR and provides an internal positive control for mapping studies. This is useful for such economically important species as the eastern oyster, which are poorly characterized at the chromosome level. Indeed, given the intrinsic difficulties of cytogenetic analysis in oysters, the association between NOR and

28S rDNA may be the only gene-level marker available at present to test physical mapping techniques, although the associations of such other genetic markers as microsatellite loci may prove beneficial in this regard (e.g., McGoldrick 1997). The techniques developed in this study could be applied for identification of chromosomal integration of genetic material foreign to the genome of the eastern oyster, such as that in transgenic studies (Zhang et al. 1998).

Localization of the 28S rDNA represents the first report of physical mapping of a gene in mollusks. More importantly, results of this study provide methodology for examining the validity of hybridization techniques. This is especially important in physical mapping of species without pre-existing genetic information, such as mollusks. Large-scale mapping studies in oysters await improvements of techniques such as probe labeling to increase detection efficiency.

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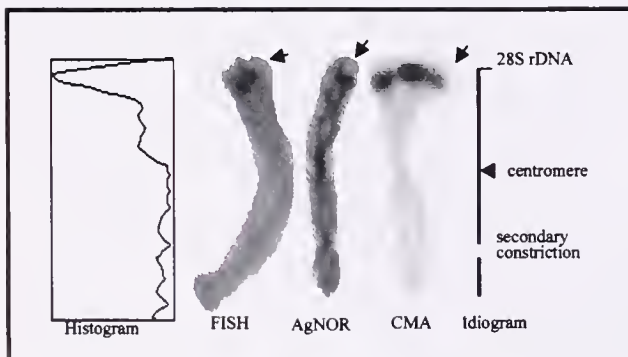


Figure 4. Analysis of the chromosomal location of the 28S rDNA of the eastern oyster by microdensitometry. Arrowheads indicate location of 28S rDNA, revealed by fluorescence *in situ* hybridization (FISH), silver staining (AgNOR) and chromomycin A3 staining (CMA).

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## IMPACTS OF SUSPENDED PEAT PARTICLES ON FEEDING AND ABSORPTION RATES IN CULTURED EASTERN OYSTERS (*CRASSOSTREA VIRGINICA*, GMELIN)

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**ABSTRACT** A large portion of the oyster industry in eastern Canada is located in the northeastern part of New Brunswick. Peat mining industries exist in the same region and concern exists over the release of particulate matter and its potential impact on oyster feeding, growth, and survival. We examined the feeding response and absorption rates of several groups of *Crassostrea virginica* exposed to natural seston, cultured microalgae, and various concentrations of suspended peat (2 to 20 mg L<sup>-1</sup>). Clearance rates declined as the concentration of peat increased above 5 mg L<sup>-1</sup>, but relatively little of the peat cleared was rejected as pseudofeces (<5%); thereby, ingestion rates were maintained as concentrations increased. Absorption efficiency was unaffected by low concentrations of peat (<5 mg L<sup>-1</sup>), although, higher concentrations (>5 mg L<sup>-1</sup>) produced negative absorption and metabolic fecal loss. We conclude that the presence of suspended peat will dilute the natural seston and negatively impact energy gain and growth in the oysters, because they indiscriminately ingest the peat; thereby, filling their guts with material that cannot readily be digested. The concentrations of peat where this dilution effect starts to affect the oysters nutrition adversely will vary with the concentration of the natural seston.

**KEY WORDS:** oyster, *Crassostrea*, feeding, peat impacts, absorption efficiency, metabolic fecal loss

### INTRODUCTION

The northernmost distribution of natural populations and commercial harvesting of the eastern oyster *Crassostrea virginica* (Gmelin 1791) occurs along Canada's eastern coastline. Large populations of the eastern oyster exist in Caraquet Bay (47°50'N, 65°W), New Brunswick and Bedeque Bay (46°22'N, 63°50'W), Prince Edward Island, with smaller populations found in Nova Scotia (Medcof 1961, Sephton and Bryan 1989, Lavoie 1995). Natural eastern populations of oysters have been greatly reduced in size over the past several decades by Malpeque disease, industrial and agricultural encroachment, mining, construction, forestry, and fishing activities (Medcof 1961, Chiasson 1991). Today, the oyster industry is based on both private natural leases and aquaculture operations with an estimated value of \$7.5 million in eastern Canada in 1994 (Boghen 1995, Sephton 1997). Concerns have been expressed by the oyster leaseholders in New Brunswick that increasing harvesting of peatlands may jeopardize the growth and survival of oysters because of increased turbidity in local bays and estuaries (Sephton and Bryan 1989). In addition to the natural erosion of peatlands, peat particles enter the adjacent shallow marine ecosystems from activities associated with peat harvesting, including primarily airborne transport of fine particles and runoff from the mounds of peat drying in the fields (Lavoie 1995). Many of the oyster-producing leases held in New Brunswick are in close proximity to large peat deposits (Fig. 1).

Peat harvests continue to escalate although environmental impacts of harvesting peat are not well known (Glooschenko 1990). It has been suggested that drainage waters from developed peatlands decrease light penetration, change water color through leaching, and increase suspended particulate matter that could be detrimental to downstream aquatic organisms (Dunson and Martin 1973, Mitchell and McDonald 1992, Pattinson et al. 1994). Sub-

stantial increases in turbidity and sedimentation may have obvious impacts on benthic organisms by smothering and killing them. However, it is difficult to assess more subtle sublethal effects of smaller increases in particle concentration, including any reduction in light for phytoplankton production or interference with feeding activity and possible reduction in growth of suspension-feeders (Peterson 1985, Cloern 1987, Grant et al. 1990, Grant and Thorpe 1991). Environmental awareness has led to the installation of sedimentation ponds and stringent guidelines restricting the discharge of peat particles into the environment to <20 mg L<sup>-1</sup> (Gemtech Ltd. 1991, Gemtech Ltd. 1993). Although concern regarding commercial harvesting of peatlands and the impact on receiving waters is increasing, there is little quantitative evidence of the possible effects on local benthic populations.

Increases in particulate matter in the water column could potentially be a problem to suspension-feeding invertebrates especially if the additional particles are poor in nutritional quality, such as inorganic sediments associated with natural resuspension events and anthropogenic activities including, agricultural runoff, dredging, and dumping. In many cases, these additional poor quality particles are thought to dilute the higher quality background seston, consisting of higher proportions of phytoplankton and other nutritious organic particles, possibly interfering with feeding activity and reducing potential energy gain (Widdows et al. 1979, Berg and Newell 1986, Smaal et al. 1986). Many species of bivalves, including *C. virginica*, reduce the potential impact of seston dilution by rejecting poor quality particles before ingestion through the production of pseudofeces (Kiorbøe and Mohlenberg 1981, Newell and Jordan 1983, MacDonald and Ward 1994, Hawkins et al. 1996). *C. virginica* is known to thrive in estuaries where natural concentrations of seston range between 6.0 and 30.0 mg L<sup>-1</sup>, is very tolerant of high concentrations of suspended sediments, and has been shown to maintain high clearance rates at elevated seston concentrations up to 75 mg L<sup>-1</sup> (Berg and Newell 1986, Newell and Langdon 1996, Shumway 1996).

The purpose of this study was to assess the potential impact of suspended peat particles on the ability of *C. virginica* to capture

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**Figure 1.** Distribution of peatlands in the province of New Brunswick. Principle areas for producing oysters (*Crassostrea virginica*) in the province are indicated as black circles.

food particles and gain energy to support growth. Several groups of oysters were exposed to various concentrations of suspended peat, natural seston, and cultured microalgae while we measured clearance and ingestion rates, pseudofeces production, and absorption efficiency. We tested the hypothesis that exposure to concentrations of peat up to  $20 \text{ mg L}^{-1}$  would have no impact on feeding and energy gain in the oyster.

## MATERIALS AND METHODS

### General Procedures

*Crassostrea virginica* (~70 mm in shell length) were collected approximately every 2 to 4 days from an intertidal site in Oyster Point, Caraquet Bay, New Brunswick over a 3-wk period in June 1996 (Fig. 1). This area was selected, because eastern oysters are harvested commercially from this site, and it receives some runoff from channels draining local peatlands. Oysters were maintained in flowing filtered seawater ( $14 \pm 1 \text{ }^\circ\text{C SE}$ ; 25%) at the Marine Science Centre in Shippagan, NB for 2 to 4 days until experiments began. All epibiota were removed from the oyster shell immediately, and the length of the oyster was recorded to the nearest 0.1 mm using vernier calipers.

### Experimental Diets and Apparatus

Ten groups of randomly selected oysters ( $n = 9$ ) were exposed to different experimental diets consisting of natural seston, filtered seawater, filtered seawater supplemented with microalgae (*Isochrysis galbana*, T-iso), and filtered seawater supplemented with various concentrations of suspended peat particles (2, 5, 10, and  $20 \text{ mg L}^{-1}$ ). The natural seston diet was supplied by a direct unfiltered seawater line submerged below the water surface in Shippagan Harbour. Filtered seawater was passed through a sand filter to remove particles greater than  $>10 \text{ }\mu\text{m}$  and stored in large underground tanks (454,000 L) to minimize variation in the experimental diets caused by fluctuations in the background water over the 3-wk experimental period. In this way, any differences observed in the feeding activity may be attributed to the suspended particles rather than to any possible changes in the background water. *I. galbana* was grown in autoclaved *f/2* medium, at  $20 \text{ }^\circ\text{C}$ , under constant illumination and harvested for the diet when they were approximately at their exponential growth phase. Samples of local

peat were dried at  $60 \text{ }^\circ\text{C}$  for 24 h, ground into a fine powder with an ultrafine mill (Retschvibratory Mill, type MM-2), and sieved with stainless steel sieves to a particle size of less than  $<63 \text{ }\mu\text{m}$ . The peat powder was then suspended in seawater, and a peristaltic pump added the desired concentration during the process of acclimating the oysters (10 h) and throughout the experimental period. To determine whether oysters were influenced either by the peat fiber or the peat fiber and any associated microbial assemblages, two groups were also exposed to experimental diets (2 and  $5 \text{ mg L}^{-1}$ ) containing autoclaved peat. Peat was autoclaved at  $20 \text{ psi}$  and  $125.7 \pm 0.6 \text{ }^\circ\text{C SE}$  for 15 min and passed through a  $63\text{-}\mu\text{m}$  sieve.

The experimental apparatus used to measure feeding rates consisted of a 20-L header tank/mixing chamber supplied with natural seston or filtered seawater from the large storage tanks. Seawater from the header tank was gravity fed via Tygon tubing to a series of 11 2-litre plastic holding chambers. Flow rates were held constant ( $220\text{--}280 \text{ mL min}^{-1}$ ) by maintaining head pressure and by inserting plastic flow-restricting plugs in each of the 11 delivery lines. Water exited the holding chambers through upright plastic standpipes fitted through the bottom of the chamber. A plastic baffle was inserted at the front of each chamber ensuring adequate mixing of the particles, and the chamber's oblong shape reduced recirculation of the experimental diet. A slurry of suspended peat particles was pumped to the header tank/mixing chamber using a peristaltic pump at a rate calculated to supply the desired experimental concentration. Submersible stirrers and air stones were used to mix the experimental diet in the header tank and prevent settling. Oysters were placed in the holding chambers and exposed to the experimental diet for 10 h before measurements began to acclimate them to the diet and purge the digestive system. All chambers were cleaned of biodeposits and any settled particles before the beginning of the experimental period.

Nine of the eleven chambers contained individual oysters. The concentration and size distribution of the particles in the experimental diet was determined by sampling the outflowing water from the two remaining "empty reference" chambers. One of the two reference chambers contained only a pair of oyster shells to correct for any possible settlement of particles at the highest experimental concentrations. Water samples were simultaneously collected from the standpipe drain of each chamber every 2 to 3 h over the 10- to 12-h experimental period. Water collection was done over a recorded time interval to allow calculation of flow rates for each chamber. The concentration of particles in the outflowing water was determined using an electronic particle counter (Coulter Multisizer) equipped with a  $100\text{-}\mu\text{m}$  aperture. Differences in particle concentration between the reference chamber containing a shell and the other holding chambers was used to calculate clearance rates (see below).

### Analysis of Seston and Biodeposits

Four replicate water samples were collected at the beginning, middle, and end of each experiment to verify the particle concentration ( $\text{mg L}^{-1}$ ) and percentage organic content of the experimental diets. Samples of each diet mixture were collected by filtering a known volume of water through a pre-ashed, pre-weighed 2.4-cm GF/C filter under low vacuum. To remove any salts, all filters were washed with 10 mL isotonic ammonium formate before drying at a constant weight at  $80 \text{ }^\circ\text{C}$  and then frozen at  $-25 \text{ }^\circ\text{C}$  and stored for later analysis. The percentage organics in each diet was determined by combusting them at  $475 \text{ }^\circ\text{C}$  for 24 h in a muffle



furnace and reweighing them ( $\pm 0.01$  mg) after cooling in a desiccator.

Samples of feces were collected with a micropipette from each holding chamber at the end of the experimental period. The percentage organic content of feces was determined using the same collection, treatment, and weighing methods as those used for the water samples. All biodeposits were removed from each holding chamber and discarded once during the experimental period to facilitate the quantitative collection of all pseudofeces produced during a subsequent recorded time interval. Subsamples of the pseudofeces were collected with a micropipette and placed in vials with 20 mL of filtered seawater, treated with several drops of Coulter Electronics type IC dispersant and shaken vigorously for 5 to 10 min to disperse the particles so they could be counted using the Multisizer (Ward et al. 1992). If coincidence was high ( $>10\%$ ), samples were diluted with filtered seawater and recounted. The total volume of the sample was determined before counting so we could calculate the total number of particles above the background concentration expelled as pseudofeces. The weight of the pseudofeces were determined using the same collection, treatment, and weighing methods as those used for the water and feces samples.

#### Measurements of Standardized Clearance Rates

Clearance rate (CR), one indicator of feeding activity, is defined as the volume of water cleared of suspended particles  $>2$   $\mu\text{m}$  in diameter per unit time (Bayne et al. 1977). Differences in the concentration of particles between the reference chamber and the holding chamber containing an oyster were used to calculate CR as follows:

$$\text{CR} = \text{FR} \times (C_1 - C_2)/C_1,$$

where FR is the flow rate of water through the experimental chambers ( $\text{L h}^{-1}$ ),  $C_1$  is the particle concentration (number  $\text{mL}^{-1}$ ) in the inflowing seawater (determined from the reference chamber), and  $C_2$  is the particle concentration (number  $\text{mL}^{-1}$ ) in the outflowing water from a holding chamber after it has been processed by the oyster. The CR ( $\text{L h}^{-1}$ ) for each oyster was the minimum of three consistent rates measured over the experimental period. Although all the oysters were similar length ( $\sim 70$  mm), it was necessary to correct for any weight differences among the oysters to compare CR and other indicators of feeding activity between the groups of oysters exposed to different experimental diets. This was done by standardizing all rates ( $\text{CR}_S$ ) to an oyster with 1.0-g body dry weight using the following equation:

$$\text{CR}_S = (W_S/W_O)^b \times \text{CR}_O$$

where  $\text{CR}_S$  = the clearance rate for an oyster of standard weight,  $W_S$  = the standard weight of the oyster,  $W_O$  = the observed weight of the oyster,  $\text{CR}_O$  = the observed or measured clearance rate,  $b = -0.70$ , the weight exponent for oyster clearance rate (Newell and Langdon 1996). At the end of each experiment, the soft body tissues were removed from the shell and dried to a constant weight ( $\pm 0.01$  g) at  $90^\circ\text{C}$ .

#### Ingestion and Absorption Rates

Ingestion rates ( $\text{mg h}^{-1}$ ) standardized for a 1.0-g oyster ( $\text{IR}_S$ ) were calculated as the product of  $\text{CR}_S$  ( $\text{L h}^{-1}$ ) and particle concentration or total particulate material (TPM;  $\text{mg L}^{-1}$ ) after subtracting particles rejected as pseudofeces (PS;  $\text{mg h}^{-1}$ ) using the following equation:

$$\text{IR}_S = (\text{CR}_S \times \text{TPM}) - \text{PS}.$$

Absorption efficiency (AE) was calculated using the Conover (1966) ratio, which compares the organic content of the food and feces as follows:

$$\text{AE} = (F - E) \times 100 / (1 - E) \times F$$

where F = ash-free dry weight:dry weight ratio in the food or experimental diet, and E = ash-free dry weight:dry weight ratio in the egesta or feces. Absorption rates ( $\text{mg organics h}^{-1}$ ) standardized for a 1.0-g oyster ( $\text{AR}_S$ ) were calculated as the product of  $\text{IR}_S$  ( $\text{mg h}^{-1}$ ) and absorption efficiency (%).

#### Statistical Analysis

All statistical procedures were carried out using the General Linear Model (GLM) procedure of the Statistical Analysis System (SAS Institute Inc.). One-way analyses of variance (ANOVA) were used to determine if oyster clearance rates, ingestion rates, absorption efficiencies, and absorption rates were significantly different for the various diet treatments. The null hypothesis for these experiments was that the addition of peat would have no effect on oyster feeding activities. For all comparisons, a significance level of  $\alpha = 0.05$  was used. If significant F values were observed in any ANOVA tests, a Student–Newman–Keuls test was conducted to determine which means were significantly different from one another. Before statistical analyses, data were tested for normality and homogeneity of variance using a Shapiro–Wilk (W) test (Zar 1984) and an F-max test (Sokal and Rohlf 1981), respectively. If one of the above ANOVA requirements was not met, the data were  $\log_{10}$  transformed, and the ANOVA was repeated. If the assumptions of the ANOVA were still not met after transformation, a nonparametric Kruskal–Wallis test was used.

A paired t-test using a repeated measures design (Zar 1984) was performed to determine whether feeding activities would differ when a group of oysters was exposed to the same concentrations of natural and autoclaved peat. The null hypothesis was that the presence of the living microbial community would have no impact on feeding activity in oysters.

## RESULTS

Particle concentrations measured for each diet during the experimental period with the targeted concentrations are presented in Table 1. There were significant differences in clearance rates (CR) between the 10 experimental groups of oysters ( $F = 15.62$ ,  $df = 9$ ,  $P < .001$ ; Table 2). Clearance rates on the nonpeat diets, filtered water, natural seston, and algae (T-Iso), ranged from 2.5 to 3.0  $\text{L h}^{-1}$  and were not significantly different (Fig. 2). Relatively low concentrations of peat (2  $\text{mg L}^{-1}$ ) did not significantly reduce clearance in *C. virginica*, making it comparable to values observed for the nonpeat diets. Concentrations of suspended peat particles  $>2$   $\text{mg L}^{-1}$  reduce CR significantly. Generally, CR decreased as concentrations of peat increased to between 2 and 20  $\text{mg L}^{-1}$ , with values dropping from about 2.25 to about 0.50  $\text{L h}^{-1} \text{g}^{-1}$  (Fig. 2).

Ingestion rate is a better indicator of feeding activity than CR, because it factors out the particles filtered from suspension but rejected as pseudofeces [e.g.,  $\text{IR} = (\text{CR} \times \text{TPM}) - \text{PS}$ ]. There were no significant differences in percentage of particles rejected as pseudofeces among the groups of oysters ( $F = 1.88$ ,  $df = 9$ ,  $P < .066$ ; Table 2). The percentage of particles rejected as pseudofeces was relatively low, typically ranging from 1 to 2% by number for the nonpeat algal and natural seston diets (Fig. 3). Although a

TABLE 1.

Target diet concentrations ( $\text{mg L}^{-1}$ ) and concentrations actually obtained during the experiments.

Treatment	Target Concentration ( $\text{mg L}^{-1}$ )	Actual Concentration ( $\text{mg L}^{-1}$ ) $\pm$ SD
Algae (T-iso)	1.5	1.51 $\pm$ 0.09
Natural seston	1.7	1.65 $\pm$ 0.22
Filtered seawater	0.66	0.66 $\pm$ 0.46
Peat	2	2.15 $\pm$ 0.19
Peat	2*	2.10 $\pm$ 0.05
Peat	5	5.18 $\pm$ 0.20
Peat	5*	4.17 $\pm$ 0.16
Peat	5**	4.92 $\pm$ 0.14
Peat	10	10.23 $\pm$ 0.15
Peat	20	21.28 $\pm$ 1.21

\* Autoclaved peat.

\*\* Same oysters used in a repeated measures design.

higher percentage of the particles may be rejected for the peat diets, percentage rejection values of less than 5% indicate that the majority (95%) of the peat cleared from suspension is ingested. There were significant differences in ingestion rates for oysters exposed to the 10 experimental diets ( $F = 10.39$ ,  $df = 9$ ,  $P < .001$ ; Table 2). With the exception of the 20  $\text{mg L}^{-1}$  peat diet and the filtered seawater diets ( $\sim 1.5 \text{ mg h}^{-1}$ ), ingestion rates ranged between 2 and 5  $\text{mg h}^{-1}$  (Fig. 4). Overall, ingestion rates did not increase with increasing concentration, because CR continued to decrease. The exception to this trend was observed for oysters exposed to 20  $\text{mg L}^{-1}$  of peat that maintained higher CR values that were comparable to oysters exposed to lower concentrations (Fig. 2). We do not have sufficient evidence to reject the hypothesis that exposure to various concentrations of peat will have no impact on feeding activity in the oyster.

There were significant differences in the oysters' ability to extract organic material (absorption efficiency) from the various experimental diets ( $F = 5.81$ ,  $df = 9$ ,  $P < .001$ ; Table 2). Absorption efficiencies (AE) were highest on the nonpeat diets but decreased as peat concentrations increased up to about 5  $\text{mg L}^{-1}$  until negative values were observed at 10 and 20  $\text{mg L}^{-1}$  (Fig. 5). The only significant differences in AE were found between the algae and the 5, 10, and 20  $\text{mg L}^{-1}$  groups. The organic content of carbon and nitrogen of peat in this study was 53.65%  $\pm$  1.86 SD and 1.76  $\pm$  0.68 SD (respectively) and is comparable to previously published values (Table 3). The values recorded for the nonpeat diets are also comparable to published reports for bivalves in gen-

TABLE 2.

Summary of nonparametric one-way ANOVA procedure for testing clearance and ingestion rate, absorption efficiency, absorption rate, and rejection rates (pseudofeces) of *Crassostrea virginica* exposed to various concentrations of suspended peat particles ( $\alpha = 0.05$ ).

Response Variable	F-Value	df	Error	Pr > F
Clearance rate	15.62	9	80	0.001
Ingestion rate	10.39	9	80	0.001
Absorption efficiency	5.81	9	39	0.001
Absorption rate	59.87	9	80	0.001
Pseudofeces (% rejection)	1.88	9	80	0.066

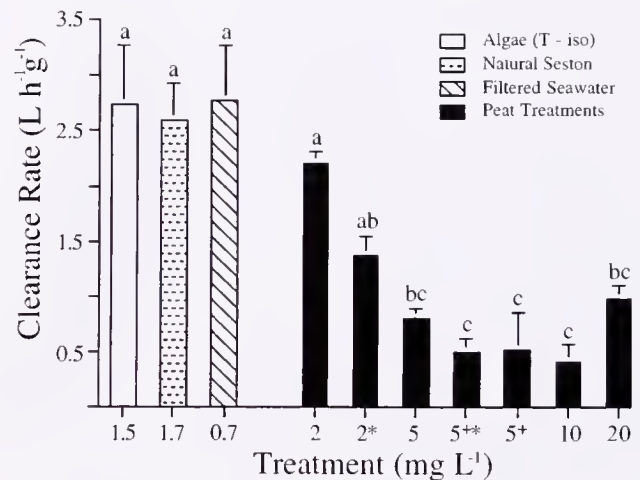


Figure 2. Mean clearance rates ( $\pm$ SD) standardized to a 1.0-g body size for *Crassostrea virginica* exposed to various treatments of microalgae (T-iso), natural seston, filtered seawater, and peat ( $\text{mg L}^{-1}$ ). Groups with the same letter are not significantly different from one another. See text for details. \* Indicates autoclaved peat, and + indicates the same oysters were used in a repeated measures design.

eral and *Crassostrea* in particular (Langdon and Newell 1996). Negative values of AE indicate a higher percentage of organic matter recorded in the feces than present in the respective experimental diet. Absorption rate (AR) considers not only ingestion rate but also absorption efficiency. AR significantly decreases with increasing concentrations of peat ( $F = 59.87$ ,  $df = 9$ ,  $P < .001$ ;

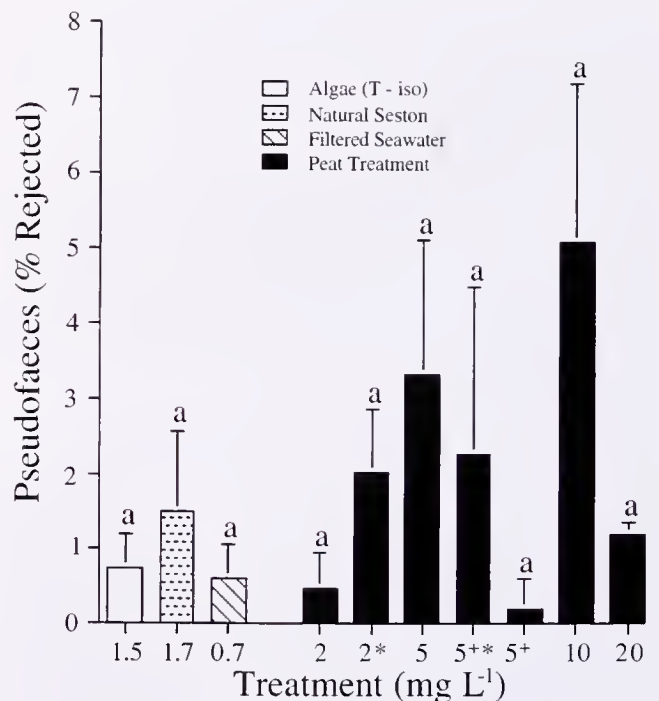


Figure 3. Mean pseudofeces rejection rates ( $\pm$ SD), expressed as a percentage of particles cleared by *Crassostrea virginica*, exposed to various treatments of microalgae (T-iso), natural seston, filtered seawater, and peat ( $\text{mg L}^{-1}$ ). There were no significant differences among the groups (Table 2). \* Indicates autoclaved peat, and + indicates that the same oysters were used in a repeated measures design.

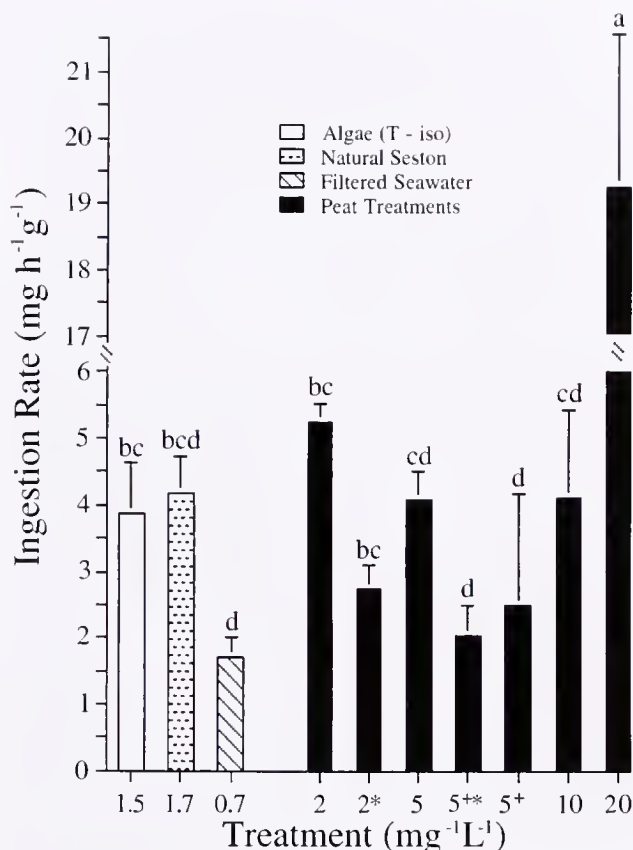


Figure 4. Mean ingestion rates ( $\pm$ SD) standardized to a 1.0-g body size for *Crassostrea virginica* exposed to various treatments of microalgae (T-iso), natural seston, filtered seawater, and peat ( $\text{mg L}^{-1}$ ). Groups with the same letter are not significantly different from one another. See text for details. \* Indicates autoclaved peat, and + indicates that the same oysters were used in a repeated measures design.

Table 2). Negative values of AR were observed at 10 and 20  $\text{mg L}^{-1}$  (Fig. 6). The AR values for the filtered seawater diet did not differ from the other nonpeat diets (algae and natural seston). We reject the hypothesis that exposure to various concentrations of peat will have no impact on absorption and energy gain in the oyster.

*Microbial Assemblages Associated with Peat*

The ANOVA and posteriori tests revealed no significant differences in CR, PS, IR, or AE, measured separately, when oysters were exposed to autoclaved peat containing dead microbial assemblages and non-autoclaved peat complete with living microbial assemblages with dry peat (Figs. 2–6). These results suggest that oysters clear, ingest, and absorb peat fiber at similar rates regardless of whether or not a living microbial community was associated with the peat. However, lower values of AR were observed for oysters exposed to 2\* and 5\*\*  $\text{mg L}^{-1}$  of autoclaved peat (Fig. 6). Although not statistically different individually, the combination of lower estimates of CR and AE produced this result in the oysters at 2\*  $\text{mg L}^{-1}$ ; whereas, lower AE values were probably the reason for the lower AR in the 5\*\*  $\text{mg L}^{-1}$  group. The paired t-test using repeated measures had the same result between the group of oysters exposed to both autoclaved and non-autoclaved peat.

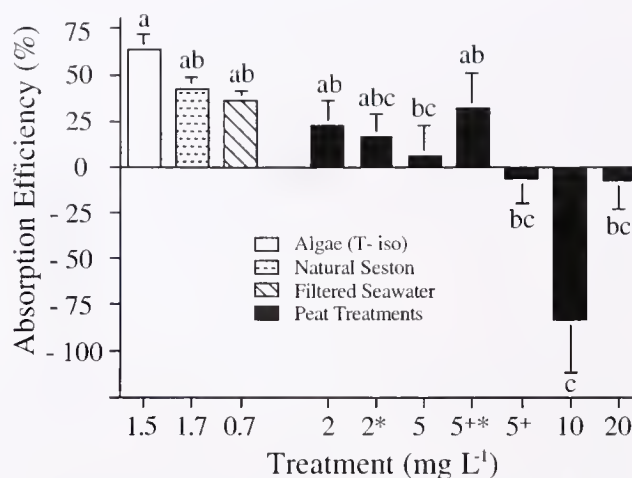


Figure 5. Mean absorption efficiencies ( $\pm$ SD) for *Crassostrea virginica* exposed to various treatments of microalgae (T-iso), natural seston, filtered seawater, and peat ( $\text{mg L}^{-1}$ ). Groups with the same letter are not significantly different from one another. See text for details. \* Indicates autoclaved peat, and + indicates that the same oysters were used in a repeated measures design.

DISCUSSION

Unlike many other species of bivalves, *Crassostrea virginica* have consistently been shown to possess the ability to maintain high clearance rates at relatively high seston concentrations (Nelson 1938, Galtsoff 1964, Newell and Langdon 1996). Clearance rates in *C. virginica* increase to a maximum at seston concentrations between 5 and 10  $\text{mg L}^{-1}$  before particle capture exceeds the rate of particle ingestion and pseudofeces are produced (Newell and Langdon 1996). Further increases in seston concentration result in increasing amounts of pseudofeces being voided until clearance rates begin to decline at concentrations above 25  $\text{mg L}^{-1}$  (Haven and Morales-Alamo 1966, Newell and Langdon 1996). However, in our study *C. virginica* regulated ingestion as the concentration of particles increased both by producing pseudofeces and reducing clearance rates even at relatively low particle concentrations, 5  $\text{mg L}^{-1}$  (Fig. 2).

A reduction in clearance rates in response to modest increases in concentration (20  $\text{mg L}^{-1}$ ) has been reported for a greater variety of other species including *Mercenaria mercenaria* (Bricelj and Malouf 1984), *Mytilus edulis* (Bayne et al. 1987), *Cerastoderma edule* (Navarro et al. 1992), and *Mya arenaria* and *Pla-*

TABLE 3.

Summary of average peat carbon (%) and nitrogen (%) content of surface peat collected from the Shippagan-Caraquet area located in northeastern New Brunswick and compared to various published peat organic values.

Author	Carbon (%)	Nitrogen (%)
Strychar and MacDonald	53.65 $\pm$ 1.86 SD	1.76 $\pm$ 0.68 SD
Allison 1973	52.5	0.84
Riley 1989	50.25	2.2
Thiessen 1925	58.88	2.58
Schnitzer and Khan 1978	53.8–58.7	0.8–2.4
Visser 1964	54.98	4.33

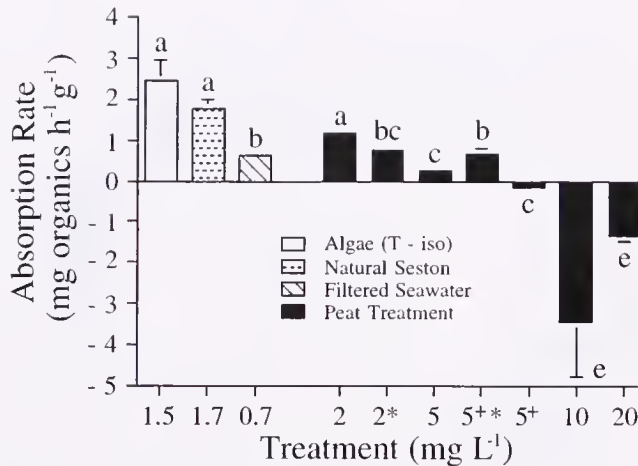


Figure 6. Mean absorption rates ( $\pm$ SD) standardized to a 1.0-g body size for *Crassostrea virginica* exposed to various treatments of microalgae (T-iso), natural seston, filtered seawater, and peat ( $\text{mg L}^{-1}$ ). Groups with the same letter are not significantly different from one another. See text for details. \* Indicates autoclaved peat, and + indicates that the same oysters were used in a repeated measures design.

*copecten magellanicus* (Bacon et al. 1998). Bivalves have very complex feeding responses to variations in ambient temperatures and the concentration and quality of the particles suspended in their natural environment. Oysters from this area in New Brunswick may be well adapted to much lower water temperatures and seston concentrations than oysters studied in the warmer more turbid environments, such as the Chesapeake Bay. Jordan's (1987) model predicted reduced clearance rates (e.g., biodeposition rates) at lower temperatures, but more significantly, that any increase in seston concentration may influence clearance in *C. virginica* differently at temperatures below 20 °C than at temperatures above 20 °C (Newell and Langdon 1996). The feeding activity of the oysters used in this study was measured at relatively low temperatures for this species (14 °C) and may account for some of the differences observed.

In this study, *C. virginica* maintained relatively constant ingestion rates as particle concentration increased by significantly reducing clearance rates and producing some pseudofeces. Pseudofeces production is an important mechanism to regulate ingestion and has typically been shown to increase with elevated seston concentrations in most species of bivalves studied. There was some evidence of increasing pseudofeces production with increasing particle concentration for *C. virginica* in this study; however, high variability in these types of measurements may have prevented the trend from being significant (Fig. 3, Table 2). *Mytilus edulis* and *Cerastoderma edule* have been shown to reject as much as 20–40% and 58%, respectively, of the particles cleared as concentration increased and quality decreased (Bayne et al. 1993, Navarro et al. 1994). Other species have been shown to reject much smaller fractions of the particles cleared, including *Merccenaria mercenaria* (<18%), *Placopecten magellanicus* (7–14%), and *Mya arenaria* (2–4%) (Bricelj and Malouf 1984, Bacon et al. 1998). *C. virginica* in this study probably rejected few peat particles as concentration increased (<5%) because of their high organic content (Table 3) and potential food value. This result is interesting, because it suggests that the gill/palp of *C. virginica* is not selecting against the peat particle. Ward et al. (1998), however, have shown that *Spartina alterniflora* compared to algae are being

selected against on the gill, which suggests that oysters may perceive *S. alterniflora* and peat as different.

Maintaining relatively constant ingestion as particle concentration increases may be advantageous in helping to regulate the efficiency of absorption (Navarro et al. 1994). Absorption efficiencies (AE) for *C. virginica* exposed to natural seston and cultured microalgae are comparable to those observed for other species fed similar types of particles (Bayne and Newell 1983). The addition of small amounts of peat ( $2 \text{ mg L}^{-1}$ ) did not significantly reduce AE in oysters (Fig. 5). However, the addition of increasing amounts of peat ( $>5 \text{ mg L}^{-1}$ ) did reduce absorption efficiency until negative values were observed. Negative values for AE have been observed before and have been described as metabolic fecal loss (Hawkins et al. 1983). Possible explanations for negative absorption efficiencies include, ingestion of diets with very low organic content (Hawkins and Bayne 1985) or feeding on diets with high organic content but short retention times in the gut, reducing enzymatic degradation of ingested food (Bricelj and Malouf 1984, Bayne et al. 1987). It is unlikely that gut retention times were reduced in these oysters, because clearance was reduced, and ingestion remained relatively constant. Peat particles, because of their composition, may simply be more resistant to degradation in the digestive system than typical particles in the seston. Willows (1992) suggests that bivalves exposed to poor diets after short acclimation times could experience negative AEs; however, after longer acclimation periods, AE could become positive as gut residence times increase. In this experiment, we exposed the oysters to the experimental diets for 10 h before initiating the experiment, but this may not be long enough to adjust gut residence times and absorb the peat, or perhaps the peat was simply too refractory.

Negative AE seen in this study at high peat concentrations could be related to the absorptive quality of the peat. Peat consists of large porous hyaline cells that help this fibrous material absorb large quantities of water and nutrients (Warner 1992). It is possible that high concentrations of ingested peat will absorb organic material within the gut and, thereby, increase the organic content of the feces expelled. Bayne et al. (1987) suggested that the reasons for negative AEs could be the presence of exocytosed mucus material from the digestive cells or the adsorption of nitrogenous compounds to the fecal strip.

It has long been thought that nonalgal particles, such as bacteria or plant detritus, suspended in the water column could represent an important supplementary food source for suspension-feeding bivalves when concentrations of phytoplankton are low. This will depend upon the capabilities of the different species to accumulate bacteria and digest detritus. *C. virginica* has been shown to have a limited ability to absorb carbon from refractory cellulosic material ( $\sim 3\%$ ); however, in the presence of cellulolytic bacteria, the efficiency of absorption was increased to 10% (Langdon and Newell 1990). These authors concluded that oysters may supplement their nitrogen requirements during the summer months through the ingestion of bacteria, but the use of detritus and bacteria does not meet the carbon and nitrogen requirements of this species. Our results are somewhat similar when seston concentrations are very low (e.g., filtered water  $<0.7 \text{ mg L}^{-1}$ ). The addition of  $2 \text{ mg L}^{-1}$  of peat actually increased the ingestion and absorption rate significantly above the rate for oysters fed on filtered water alone (Figs. 4 and 6). This observation suggests that peat may introduce a compliment of microbes favored by the oysters, the sand filter may not have efficiently removed all natural background seston (filtered seawater versus natural seston is approximately 60%, Table 1), and/or the peat fiber may have contributed additional organic

nutrients (e.g., carbon and nitrogen, Table 3). We also found that *C. virginica* does not have the ability to reduce the organic content of peat ingested at higher concentrations (>5 mg L<sup>-1</sup>), but the presence of a living microbial community increased the absorption rate in oysters above the rates observed for oysters fed autoclaved peat (Fig. 6). Microbes isolated within dry peat included 28 different genera of bacteria and fungi, with *Bacillus sp.*, *Nitrosomonas sp.*, *Nitrobacter sp.*, and *Clostridium sp.* the most common bacteria and *Penicillium sp.*, *Fusidium sp.*, *Aspergillus sp.*, and *Paecilomyces sp.* the most abundant fungi (Strychar and Johnson, submitted).

In summary, *C. virginica* clears and ingests peat particles at similar rates to those observed for microalgae and other suspended particles but is incapable of efficiently absorbing peat, resulting in metabolic fecal loss. *C. virginica* has previously been shown to reduce the impact of seston dilution by poorer particles, before ingestion, through the rejection of proportionately more of these particles in pseudofeces (Newell and Jordan 1983). However, unless the pseudofeces are produced in quantities sufficient enough to significantly alter the quantity and quality of the ingested ration, it is not likely to be ecologically meaningful (Newell and Jordan 1983, Iglesias et al. 1992, MacDonald and Ward 1994). In this study, oysters produced very small amounts of pseudofeces, resulting in virtually indiscriminate ingestion and, consequently, a dilution of the ingested ration by inert peat particles. The ratio between suspended peat particles and the concentration of back-

ground seston may be a more useful indicator of the potential impact of peat on oysters than simply using an absolute concentration of peat. For example, the addition of 2 mg L<sup>-1</sup> of peat to very low background seston concentrations (<1.0 mg L<sup>-1</sup>) may have a neutral or positive impact; whereas, the addition of 5.0 mg L<sup>-1</sup> at the same time of year may have a very negative effect. However, if background seston concentrations were higher (5–10 mg L<sup>-1</sup>), the addition of 5.0 mg L<sup>-1</sup> or even higher concentrations of peat may not have adverse effects.

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## ASSESSMENT OF REPRODUCTIVE HEALTH IN THE WILD SEED OYSTERS, *CRASSOSTREA GIGAS*, FROM TWO LOCATIONS IN KOREA

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**ABSTRACT** Spawning Pacific oysters, *Crassostrea gigas*, were collected monthly over 1 year from two distinct local seed grounds in southern coastal waters of the Korean Peninsula. One site, Tongyoung, is considered favorable for the collection of oyster seeds, the other, Koje, unfavorable. The reproductive health of the oysters was evaluated by means of reproductive stage and three condition indices. The health of the spawners was further evaluated by comparing the abundance and lipid content of D-shaped larvae they produced. Tongyoung oysters were distinct in spawning pattern, as compared to Koje oysters, which were characterized by a prolonged spawning stage, lower spawning prevalence, and earlier appearance of reproductive arrest. Spawns were dispersed rather than two cycles at both grounds, but D-shaped larval occurrence was considerably higher in Tongyoung oysters than in Koje oysters. The lipid content of D-shaped larvae was also higher in Tongyoung than in Koje. The condition indices of the two groups of oysters were similar except for the higher overall levels and earlier postspawning recovery time in Tongyoung oysters as compared to Koje oysters. The condition indices decreased after the start of spawning, but fluctuations were also noticed during the spawning period. These fluctuations were particularly significant ( $P < .05$ ) in Tongyoung oysters, suggesting stronger spawning activities in the oysters. These results imply that reproductive health of spawning oysters might be determined by reproductive cycles and condition indices for a new seed ground of *C. gigas* seed. The abundance and lipid content of D-shaped larvae also seem to be indicators of physiological health of the spawners from which they were produced.

**KEY WORDS:** Pacific oyster, *Crassostrea gigas*, nursery ground, reproductive health, condition indices, biochemical composition, reproductive cycle

### INTRODUCTION

Koje and Tongyoung, located on the south coast of the Korean peninsula, have long been used as farming grounds for Pacific oyster, *Crassostrea gigas*, one of the most commercially important bivalves cultured in the country. Oyster culture in these areas has shown a marked increase, including increasing application of advanced technology, after FDA designation of these areas as blue belts (clean areas) for oyster culture in 1972. However, since production reached 288,000 M/T in 1987, it has shown a decrease, largely attributable to local shortages of healthy seed (NFRDI Report 1997). The local failure to collect healthy seed has been attributed to the appearance of apparently reproductively unhealthy broodstock. This is believed to be related to increased water pollution, intensified farming, and frequent outbreaks of pathogenic infection that endanger the ecological integrity of the local spawning areas (Choi et al. 1997, NFRDI Report 1997, Park et al. 1998). Thus, successful farming of the oyster has become strongly dependent on selection of a good spawning ground where there are apparently healthy wild broodstock.

Oyster health condition indices have been routinely measured to grade the farming grounds. Typically, the biometry and biochemical composition of oysters undergo marked seasonal changes associated with both environmental factors and the annual reproductive cycles (Giese 1969, Sastry 1979, Gallagher and Mann 1986, Ruiz et al. 1992). Lucas and Beninger (1985) reviewed several

condition indices for assessing health conditions related to adult bivalve reproduction. The condition indices reviewed by them have been widely used (and, in some cases, modified) for bivalve condition (Brown and Hartwick 1988, Fisher et al. 1996). Biochemical composition alone has also been a good indicator for evaluating bivalve health (Gallagher et al. 1986, Ruiz et al. 1992, Robinson 1992). Other indicators have been studied for assessment of bivalve health, as related to the growth and reproduction. These are lipid and glycogen content (Holland 1978, Pieters et al. 1979, Holm and Shapiro 1984), digestive tubule condition (Winstead 1995), vesicular connective tissue condition, and RNA/DNA ratio (Kenchington 1994, Paon and Kenchington 1995). In general, the biochemical composition of bivalves changes conspicuously with season in association with the reproductive cycle (Giese 1969, Sastry 1979). The total content of lipid and/or glycogen has, therefore, been continuously monitored to get valuable information concerning general condition, metabolic state, and gonadal development of bivalves (De Zwaan and Zandee 1972, Helm et al. 1973, Barber and Blake 1981, Gabbott 1983). These seasonal changes have been well documented for *C. gigas* (Walne 1970, Whyte et al. 1990, Ruiz et al. 1992, NFRDI Report 1997).

The present study is aimed at providing measurements of the physiological condition of *C. gigas* broodstocks from two different grades of spawning ground to see if any act as indicators of reproductive health. If so, such measurements may help select better sites for wild seed collection and enhancing culture of this valued oyster.

## MATERIALS AND METHODS

### Oysters and Experimental Sites

Thirty ropes with 500 Pacific oysters, *Crassostrea gigas* per rope were suspended from a long line situated at different localities in Koje and Tongyoung in the southern part of the Korean Peninsula (Fig. 1). The two locations were chosen to evaluate how condition indices and other indicators related to reproduction of the broodstocks from an apparent poor oyster ground for seed collection (Koje) compared with apparently normal seed collection (Tongyoung). Sixty oysters were randomly sampled from the 30 ropes on a monthly basis during 1997. Care was taken to minimize sample variation in size class differences by selecting oysters of similar sizes.

### Reproductive Cycle

Shucked oysters were fixed in Bouin's fixative for 24 h. The visceral mass (gonad included) were washed in overflowing tap water for 24 h, dehydrated in an ethyl alcohol series dilution, and embedded in paraffin. Sections 3- to 5- $\mu$ m thick were made and stained with hematoxylin-eosin on slides. The reproductive process of the adult oysters was divided into stages, based on micro-histological observation of the slides (Barber 1996) in which the spawning stage of the oysters was represented by the appearance of mature ova in the follicles, and the arrest stage was identified by the appearance of shrunken follicles and refractory ova.

### Occurrence of D-Shaped Larvae

The occurrence of D-shaped larvae produced from the adult oysters was measured on a daily basis for 4 months from June 1997. Plankton samples to a depth of 3 m were collected three times from each ground using a plankton net (75- $\mu$ m mesh size), and fixed in 2% of neutral buffered formaldehyde solution. The total number of D-shaped larvae collected were counted under the microscope and divided by 3 as an average for each sample.

### Lipid Content of the D-Shaped Larvae

The lipid content of the D-shaped larvae from the two oyster grounds was measured every 10 days for 4 months, starting in the middle of June 1997. The larval lipid content was measured three times using the technique of Mann and Gallager (1985) and then

averaged for each sample. In brief, larval lipid was extracted twice in 1:2 v/v chloroform : methanol and 2:1 v/v chloroform:methanol, respectively, after being freeze-dried, and then purified in 0.7% w/v NaCl solution before being quantified gravimetrically at 500 g for 10 min.

### Ratio of Dry Flesh Weight to Wet Flesh Weight

The flesh and adductor muscles from the shells were weighed after the mantle fluid was removed with a paper towel. The flesh and adductor muscles were freeze dried for 36 h to achieve dry weight. The index was calculated as the ratio of dry flesh weight to wet flesh weight multiplied by 100.

### Ratio of Dry Flesh Weight to Dry Shell Weight

The shells of oysters transported to the laboratory were scrubbed to remove any attached epifauna and adhering sediment. The flesh and adductor muscles were removed from the shells and freeze dried in the drier (EYELA FD- 1) for 36 h after mantle fluid was removed with paper towels. The shells were rinsed with tap water and dried at 110°C until the weights were no longer reduced. The index was calculated as the ratio of dry flesh weight to dry shell weight multiplied by 100.

### Glycogen Content

Wet flesh for glycogen analysis was processed immediately after the total weight was determined. The wet flesh was added to a mixture of 50 ml of chloroform:methanol:water (2:4:1, v/v) and homogenized for 1 min with a Sorvall omni-mixer. After centrifugation at 4000 rpm for 10 min, the supernatant was decanted, and the residue was extracted with a further 50 ml of solvent mixture. This step was repeated twice. The residue was then dried at 60°C for 48 h and weighed as the polymeric fraction containing glycogen (Whyte and Englar, 1982).

## RESULTS

### Spawning Cycle

Spawning periods of the oysters from the two sites were determined (Table 1). Koje oysters were characterized by a longer spawning period and an earlier appearance of reproductive arrest, as compared to Tongyoung oysters. In Koje, a partial spawning first occurred in May (8%), and the spawning continued to October (12%). However, the prevalence of the spawning stage never exceeded 90%. The first spawning in Tongyoung oysters occurred a month later than in Koje oysters, but it happened synchronously. The spawning in Tongyoung was first observed in June with a prevalence of 92%. In September, 95% of oysters were still available as spawners in Tongyoung; whereas, only 46% remained

TABLE 1.

The occurrence of spawning and resting stages of *C. gigas* in two spawning sites from APR to NOV.

Oysters	% spawning (degenerative/resting)							
	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV
KO	0(0)	8(0)	86(0)	90(8)	78(16)	46(54)	12(88)	0(100)
TO	0(0)	0(0)	92(0)	95(0)	94(0)	95(5)	3(97)	0(100)

KO and TO stand for Koje and Tongyoung oysters, respectively.

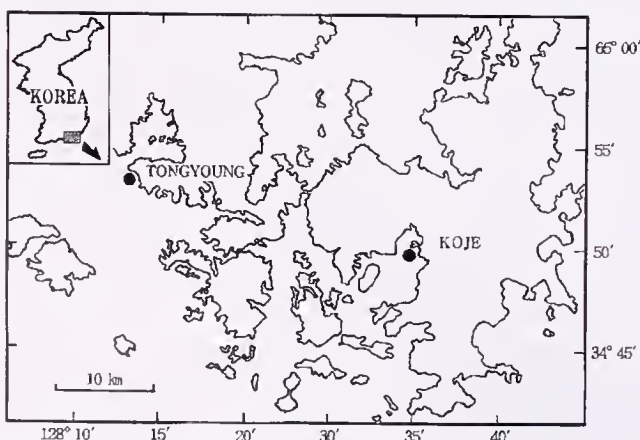


Figure 1. Map showing the two locations of Tongyoung and Koje on southern coast of Korean Peninsula.



TABLE 2.

Lipid content (ng/larva) of D-shaped larvae of *C. gigas* from two seed grounds for 3 months of spawning period.

Site	JUN			JUL			AUG		
	M	L	E	M	L	E	M	L	
KO	3.67 (0.31)	2.97 (0.15)	2.83 (0.26)	3.10 (0.20)	3.27 (0.19)	2.73 (0.25)	2.87 (0.24)	2.67 (0.32)	
TO	4.63 (0.41)	3.77 (0.35)	4.23 (0.28)	5.03 (0.41)	3.47 (0.25)	4.23 (0.21)	3.74 (0.40)	3.06 (0.15)	

Abbreviations: KO, Koje oysters; TO, Tongyoung oysters; E, early in the month; M, middle of the month; L, late in the month. The number in parentheses is standard error mean (SEM).

reproductively viable in Koje. Some Koje oysters entered into a period of reproductive arrest (degenerative and resting stages) 2 months earlier than Tongyoung oysters. Fifty-four percent of Koje oysters showed reproductive arrest by September; whereas, only 5% of Tongyoung oysters were at the same stage.

#### Occurrence of D-Shaped Larvae

Occurrence of D-shaped larvae from the two spawning grounds was measured on a daily basis for the 4-month spawning period. Four peaks in larval production were observed during this time at both locations; however, the abundance of larvae differed significantly (Fig. 2). At peak abundance, there were about 30,000 larvae/net for Tongyoung and about 6,000 for Koje. At Tongyoung, the first three spawning cycles provided sufficient larvae for seed

collection; none of the spawning cycles in Koje provided sufficient larvae.

#### Lipid Content in the D-Shaped Larvae

Lipid content of the two D-shaped larvae was measured for 3 months starting in the middle of June (Table 2). The lipid content of Tongyoung larvae ranged from 3.1 to 5.0 ng/larva while those from Koje ground ranged from 2.7 to 3.3 ng/larva.

#### Dry Flesh Weight to Dry Shell Weight Ratio (Condition Index I)

Condition index I, calculated as a ratio of dry flesh weight to dry shell weight multiplied by 100, exhibited similar patterns at the two sites (Fig. 3), with an initial increase followed by a decrease over the year cycle. Despite their similar patterns, the over-all levels of the condition index I in Tongyoung oysters were higher

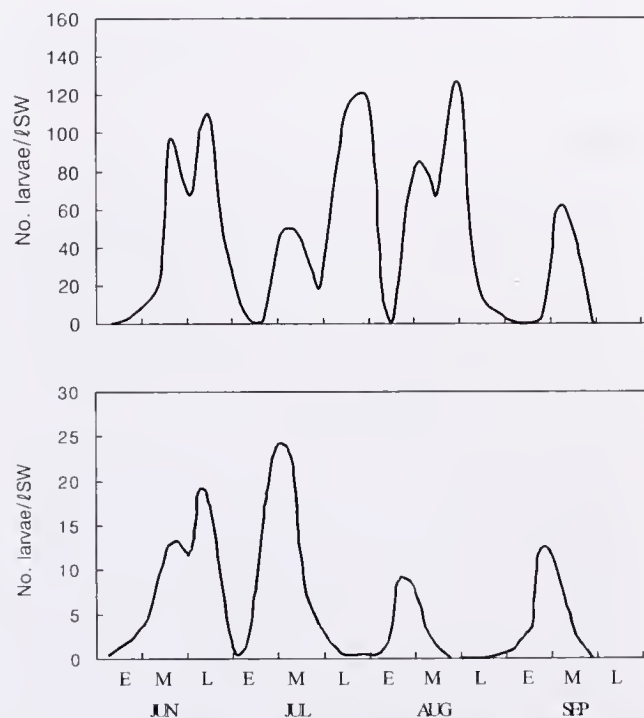


Figure 2. D-shaped larval occurrence during the spawning season of *C. gigas* from two seed grounds, Tongyoung (upper), a favorable, and Koje (lower), an unfavorable seed ground. D-shaped larvae to a depth of 3m were collected three times from each ground using plankton net (75 $\mu$ m mesh size) and averaged by dividing by three. Abbreviations: E, early; M, middle; L, late of the month.

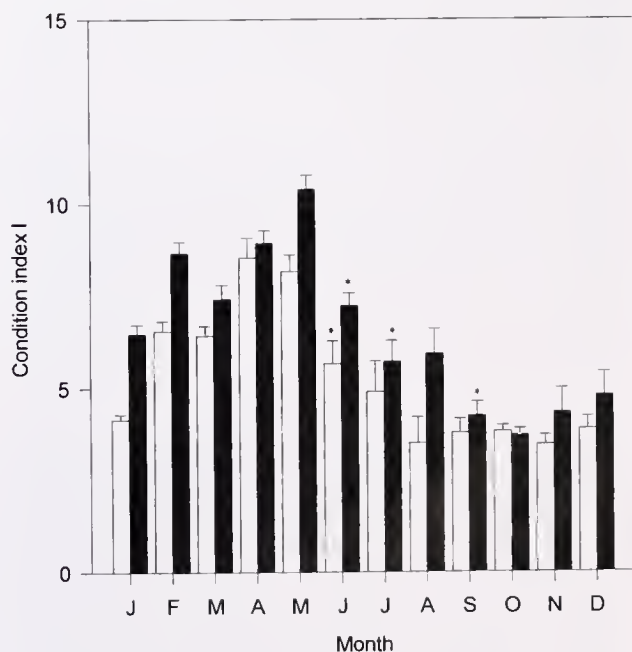


Figure 3. Condition index I of *C. gigas*, expressed as dry flesh weight to dry shell weight multiplied by 100 (mean  $\pm$  SEM) (Solid bars = Tongyoung oysters, open bars = Koje oysters). A total of 60 oysters, 30 from each seed ground (Koje and Tongyoung), were measured monthly over a year. The shells were removed for measurement of dry shell weight. The flesh weight includes all tissues removed from the shells. Asterisk indicates significant difference ( $p < 0.05$ ) from previous value during spawning season.

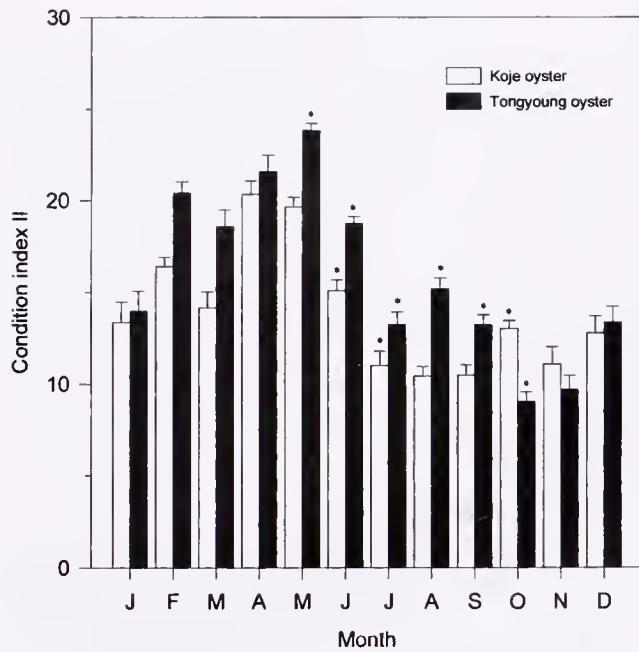


Figure 4. Condition index 2 of *C. gigas*, expressed as dry flesh weight to wet flesh weight multiplied by 100 (mean  $\pm$  SEM) (Solid bars = Tongyoung, open bars = Kojie). A total of 120 oysters, 60 from each ground (Kojie and Tongyoung), were measured monthly over a year. The flesh weight includes all tissues removed from the shells. Asterisk indicates significant difference ( $p < 0.05$ ) from previous value during spawning season.

than in Kojie oysters. This was particularly true when the off-spawning season was considered. In the off-spawning season (January to May), Tongyoung oysters were significantly higher than Kojie oysters in the value of condition index 1 over 4 months ( $P < .05$ ). With the commencement of spawning season (June to October), condition index 1 at both oyster grounds were marked by a decreasing trend. In the decreasing trends, only one significant decrease of condition index 1 between successive months ( $P < .05$ ) was noticed in Kojie oysters whereas, three significant decreases were observed in Tongyoung oyster, suggesting mass spawnings in Tongyoung oysters during the spawning season. The maximum values of condition index 1 were 10.4 for Tongyoung and 8.55 for Kojie oysters, and minimum values were 3.7 for Tongyoung and 3.5 for Kojie oysters.

#### Dry Flesh Weight to Wet Flesh Weight Ratio (Condition Index 2)

Condition index 2, expressed as a ratio of dry flesh weight to wet weight multiplied by 100, was similar to condition 1 at both sites, although greater fluctuations were noted in condition index 2 (Fig. 4). Condition index 2 showed an increase from January to May, and then a decrease from June, the time when most of the oysters started to spawn. Significant decreases in condition index 2 between successive months ( $P < .05$ ) were noticed once (July) in Kojie oysters and twice (July and October) in Tongyoung oysters. Interestingly, significant elevations of condition index 2 ( $P < .05$ ) from the previous month were also noted at both locations during the over-all decreasing trend. This significant fluctuation of condition index 2 in Tongyoung oysters was reminiscent of spawning events, although spawning abundance was different at the two localities. The maximum values of condition index 2 were 23.8 for Tongyoung and 20.3 for Kojie oysters, and minimum values were 9.0 for Tongyoung and 10.4 for Kojie oysters.

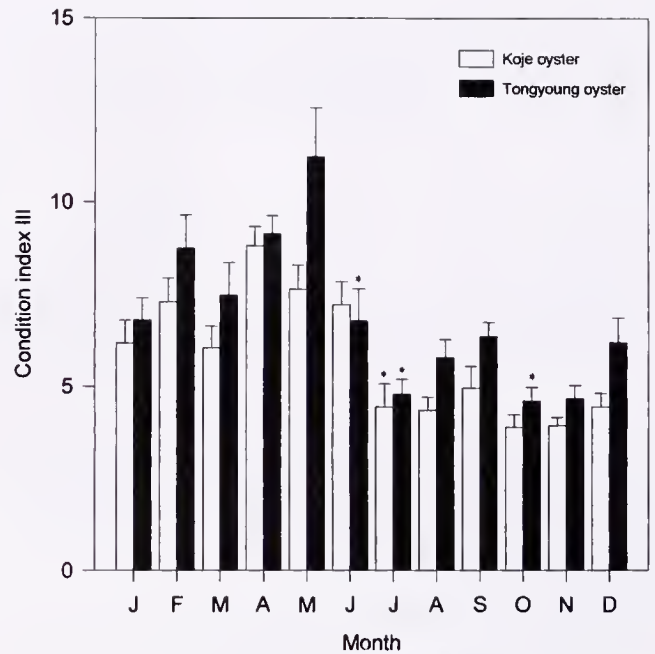


Figure 5. Condition index 3 of *C. gigas*, expressed as total glycogen content to dry flesh weight multiplied by 100 (mean  $\pm$  SEM) (Solid bars = Tongyoung, open bars = Kojie). A total of 120 oysters, 60 from each ground (Kojie and Tongyoung), were measured monthly over a year. The flesh weight includes all tissues removed from the shells. Asterisk indicates significant difference ( $p < 0.05$ ) from previous value during spawning season.

#### Glycogen Content (Condition Index 3)

Condition index 3, represented by total glycogen content to dry flesh weight multiplied by 100, was similar to condition indices 1 and 2 at both sites (Fig. 5). Condition index 3 also decreased significantly ( $P < .05$ ) with the start of spawning season of the oysters. Unlike condition index 2, however, no significant increases of condition index 3 were observed during the spawning seasons of the two oyster localities. However, significant decreases of condition index 3 ( $P < .05$ ) were marked once for Kojie oysters (July) and twice for Tongyoung oysters (July and October) during their spawning seasons. The maximum values of condition index 3 were 11.2 for Tongyoung and 8.8 for Kojie oysters, and minimum values were 4.6 for Tongyoung and 3.8 for Kojie oysters.

#### DISCUSSION

Pacific oysters, *Crassostrea gigas* from two distinct spawning sites were studied over 1 year to assess their reproductive health. The timing and duration of gametogenesis of *C. gigas* are different in the different localities of the world (Ventilla 1984, Sphigel 1989). Generally, the reproductive strategy of *C. gigas* can be considered as an adaptation to ambient environmental factors, with temperature and nutrition as principal factors (Lubet 1976, Ruiz et al. 1992). Kojie oysters were characterized by a prolonged pre-spawning stage, a lower prevalence of spawning oysters, and an earlier appearance of reproductive arrest, as compared to Tongyoung oysters (Table 1). In addition, Kojie oysters exhibited extremely low production of D-shaped larvae. However, although the occurrence of D-shaped larvae at Tongyoung was high enough for seed collection, they were dispersed across four spawning cycles (Fig. 2). In general, spawning peaks of *C. gigas* are marked by two and, in some cases, three cycles in a season (Koganezawa 1972,

Ruiz et al. 1992). Yoo and Ryu (1985) also observed two distinct cycles of D-shaped larvae.

Lipid has been considered one of the principal energy sources for gametogenesis of adult bivalves (Gabbott 1983) and for normal development of eggs (Gallager and Mann 1986). Good survival of bivalve larvae also depends upon the optimum lipid level (Holland 1978, Whyte et al. 1987, Helm et al., 1991, Lim et al. 1999). Bayne (1972) and Bayne et al. (1975) reported reduced growth in larvae of *Mytilus edulis* that developed from gametes of nutritionally stressed adults. Gallager and Mann (1986) also reported that a minimum threshold lipid level in eggs was necessary for optimal survival through nonfeeding embryonic and early larval stages. Lipid content of the D-shaped larvae from the two locations was studied for 3 months (Table 2). Tongyoung larvae ranged from 3.1 to 5.0 ng/larva on average; whereas, Koje larvae ranged from 2.7 to 3.3 ng/larva. The lower larval lipid content, together with lower larval occurrence suggested that the eggs produced from Koje oysters were not as healthy as Tongyoung oysters.

Lucas and Beninger (1985) reviewed the physiological condition indices most commonly used in bivalve aquaculture. Three of these were applied in the present study: condition index 1 for dry flesh weight to dry shell weight; condition index 2 for dry flesh weight to wet flesh weight; and condition index 3 for total glycogen content to dry flesh weight. Condition index 1, as indicated by Rheault and Rice (1996), was performed using the method described by Lucas and Beninger (1985) rather than that of Lawrence and Scott (1982) to avoid problems related to varied shell thickness and morphology. Condition indices 1 and 2 express the proportions of dry matter to whole tissues. High proportion of water in the tissues reflects an energy-depleted state of an organism attributed to losses of insoluble ash, lipid, glycogen, sugars, protein, and nitrogenous compounds from body tissue (Whyte et al. 1990). Condition 3 also signifies an energy status of the oysters.

Generally, glycogen has been regarded as the main source of energy in bivalves (De Zwaan and Zandee 1972, Barber and Blake 1981). The natural gametogenic cycle in bivalve mollusks is closely linked to cycles of glycogen storage and subsequent de novo synthesis of lipid during vitellogenesis at the expense of stored glycogen (Gabbott 1975). Interruption of this cycle by artificial conditioning may force the development of eggs before sufficient glycogen has accumulated for the synthesis of lipid. Thus, the consequence would be the production of either fewer eggs or eggs of suboptimal quality (Gallager and Mann 1986). All condition indices declined from May or June ( $P < .05$ ) with the commencement of spawning. The condition indices remained low until the beginning of winter. The patterns for condition indices at the two oyster localities were similar except for the higher magnitude and earlier rebound in Tongyoung oysters. The condition indices declined throughout the spawning period but showed some month-to-month fluctuation. These fluctuations were particularly significant ( $P < .05$ ) in condition indices 2 and 3 of Tongyoung oysters, perhaps indicating stronger spawning activities in Tongyoung oysters.

These results imply that the reproductive health of the spawners might be determined from measures of reproductive cycles and condition indices. This could be very useful for the new ground selection of *C. gigas* seed. The abundance and lipid content of D-shaped larvae were greater from the site that exhibited a shorter spawning season with higher prevalence of spawning organisms and a quicker physiological recovery.

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## GROWOUT OF BLACKLIP PEARL OYSTERS, *PINCTADA MARGARITIFERA*, ON CHAPLETS IN SUSPENDED CULTURE IN SOLOMON ISLANDS

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**ABSTRACT** This study, conducted in the “open” reef systems of Solomon Islands, assessed growth and survival of blacklip pearl oysters (*Pinctada margaritifera*, L.) on chaplets in suspended culture. Oysters were robust and mortalities after handling and drilling were low (<0.6%). Survival of an initial hatch of *P. margaritifera* was 87% after 1 year. Groups of oysters with mean dorsoventral measurements (DVM) of 63 and 71 mm showed mean ( $\pm$  SE) annual growth rates of  $64 \pm 2$  and  $60 \pm 1$  mm, respectively. Growth rates compared favorably with those reported for *P. margaritifera* in Polynesia and indicate that oysters caught as spat (approx. 11 mm DVM) would reach acceptable size for “seeding” (110 mm DVM) in around 16 months. Oysters on chaplets were subject to significant fouling by algae, however, survival of oysters cleaned every 2, 3, 4, and 6 weeks was 96–97% over a 7-month period. Growth of oysters cleaned every 3 weeks was significantly greater than those cleaned every 2 or 6 weeks. Oysters became detached from chaplets (through drill-hole breakage) in significant numbers; this problem was greater for smaller oysters. When oysters were attached singly on chaplets, approximately 54% made byssal attachments to the rope; however, 90% of oysters held in pairs on chaplets made byssal attachments to each other. Although paired oysters could be cleaned more rapidly than oysters hung singly, shell growth (DVM) of paired oysters was significantly reduced.

**KEY WORDS:** pearl oyster, *Pinctada*, growth, survival, chaplets, suspended culture, open reefs

### INTRODUCTION

Production of cultured black pearls from *Pinctada margaritifera* is seen as an appropriate, sustainable industry for remote regions of the Pacific (Lucas et al. 1995), and has expanded rapidly in eastern Polynesia over the last decade (Fassler 1995). French Polynesia has been at the forefront of this development and currently earns approximately US \$145 million annually from the sale of black pearls (Remoissenet 1996, Doubilet 1997). This success has not gone unnoticed by other small island nations in the region (Lucas et al. 1995). Historically, these nations have relied on more modest incomes from the sale of *P. margaritifera* shell for its nacre or “mother-of-pearl” (MOP) (Gervis and Sims 1992, Richards et al. 1994). In Cook Islands, pearl culture started with one family in 1982 (Sims 1993a), and, by 1994, pearl sales generated an annual income of US\$ 4.5 million (Fassler 1995). At present, black pearl culture is also underway, being attempted or assessed in Japan (Lintilhac 1987), Marshall Islands (Sims pers comm. 1998), China (Meng and Xing 1991), Vanuatu (Anon 1996), Fiji (Ward 1995, Mercier and Hamel 1998), and Solomon Islands (Friedman et al. 1996, Mercier and Hamel 1998, Friedman et al. 1998).

In the atolls of Polynesia, *P. margaritifera* are generally harvested from collectors as spat and cultured on dropper ropes or “chaplets” (see Fig. 1) when their dorsoventral measurement (DVM, Nicholls 1931) reaches 65–90 mm (AQUACOP 1982, Preston 1990). This method is also widely used for scallops in Japan (Ventilla 1982). Oysters are drilled through the base of the shell (dorsal posterior region) and attached to chaplets with wire or monofilament fishing line. Oysters are grown on chaplets for the rest of their time in culture, only being removed temporarily when

they reach 110 mm DVM, to be “seeded” for pearl production (Lintilhac 1987).

Oysters large enough to be held on chaplets have “size refuge” from all but the largest fish and invertebrate predators (Coeroli et al. 1984), and, because longlines are set in relatively deep water, they are isolated from predators associated with reefs (Swift 1985, Sims and Sarver 1995). However, survival and growth of pearl oysters in suspended culture is also influenced by fouling (Alagarswami and Chellam 1976, Mohammad 1976, Doroudi 1996, Taylor et al. 1997). In Cook Islands, chaplets are removed from the water once a year for washing with pressure hoses. At this time, algae (“soft” fouling) and such organisms as cementing bivalves and tubular polychaetes (“hard” fouling) are removed. Before oysters are returned to the water, the fastening of the oyster to the chaplet is checked for wear, and the oyster is re-drilled if necessary (J. Lyons, pers comm. 1997).

Oysters are sometimes lost from chaplets when drill holes break, and oysters fall to the bottom. In Cook Islands, farmers lose ~5% of stock in this way (R. Newnham, pers comm. 1995); however, because atoll lagoons generally have a hard substrate (Sims 1992, Coeroli et al. 1984), a large percentage of these oysters can be recovered (J. Lyons, pers comm. 1997).

Recently, experiments were conducted in Solomon Islands to determine whether collections of wild spat and culture of juvenile oysters would be successful in the “open” reef systems that are characteristic of that region (Friedman and Bell 1996, Friedman et al. 1996, Friedman et al. 1998, Friedman 1998). However, there is a paucity of published information on growth of *P. margaritifera* held on chaplets for open reef systems of the Pacific. This paper describes growth and survival of *P. margaritifera* held on chaplets in Solomon Islands. In addition, experiments were conducted to: (1) determine a cleaning regime that optimizes growth of oysters held on chaplets; and (2) assess the rate of oyster losses from chaplets because of failure of drill holes.

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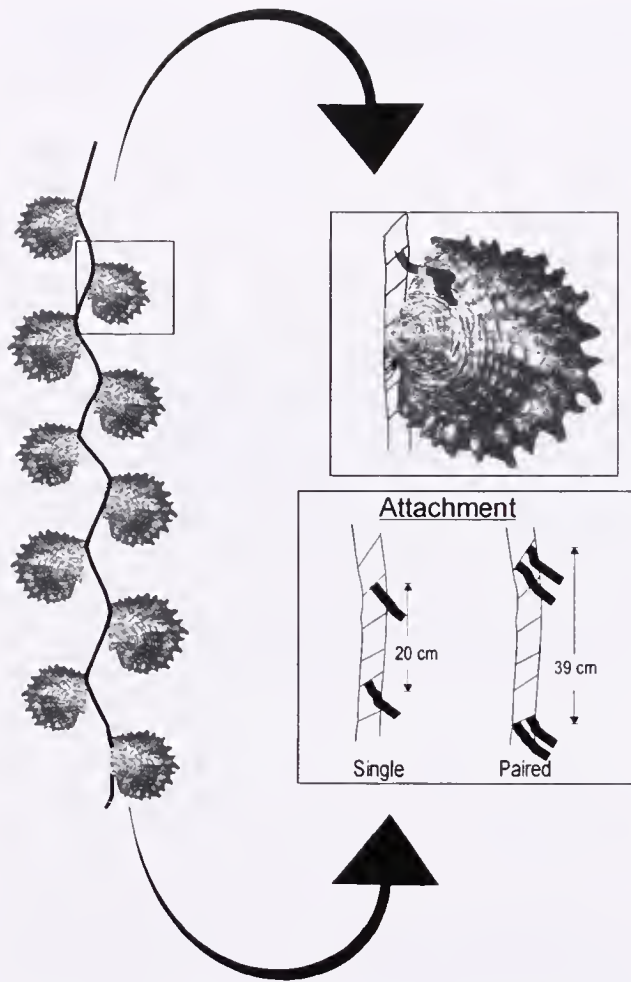


Figure 1. Chaplet system for culturing *P. margaritifera*.

## MATERIALS AND METHODS

Growout of *P. margaritifera* on chaplets was carried out within Gizo lagoon in the Western Province of Solomon Islands (Fig. 2). The section of the lagoon chosen for oyster culture was approximately 1 km across with a mean depth of ~40 m, and numerous passages and sections of submerged reef linking the lagoon to the open ocean. Currents in this area measured over a representative tidal cycle with a Schiltknecht Mini Air 20 current meter ranged between 0.0–0.15 m s<sup>-1</sup>.

*P. margaritifera* with a DVM of ~65 mm were drilled (2-mm drill bit) and tied to 4-mm polypropylene rope chaplets using monofilament fishing line (37 kg breaking strain) of a similar width to the drill hole (Fig. 1). All oysters were threaded onto single pieces of monofilament and tied so that the flatter of the two valves lay against the chaplet rope (Fig. 1). Oysters tied singly, were attached at ~20 cm intervals, when attached singly along the chaplet and 40 cm apart when in pairs (Fig. 1). Chaplets held 10 single oysters (or five pairs) and were attached to submerged longlines at 1.5-m intervals. The 100-m longlines (Fig. 3) were held at a depth of approximately 9 m and sited at least 50 m clear of fringing reef, over a sandy substrate. During the growth trials, oysters were brushed *in situ* by divers using SCUBA, to remove fouling on a monthly basis. Oysters that became detached from

chaplets during the trials were re-drilled and replaced in their original position on the chaplet.

### Growth and Survival of Oysters on Chaplets

A trial group of 90 oysters were drilled and hung singly on chaplets at the end of August 1996. These oysters were divided into two size classes: 58–65 mm and 66–78 mm DVM, with a mean ( $\pm$  SE) of 63  $\pm$  0.3 mm ( $n = 51$ ) and 71  $\pm$  0.7 mm ( $n = 39$ ), respectively. These oysters were removed from the water and measured every 3 months. A second group of 1,440 oysters with a mean (SE) DVM of 66  $\pm$  0.3 mm, were drilled and hung singly on chaplets between 11 November 1996 and 5 December 1996. These oysters were removed from the water and measured in February and again in September 1997.

At the end of these trials (September 1997), oysters from experiments were measured for wet weight and shell thickness (Gervis and Sims 1992), as well as DVM. Additional data from pearl oysters grown on chaplets under similar conditions, but not included in experiments, were collated with data from experimental animals and used for analysis of morphometric relationships. Morphometric analyses of pearl oysters were conducted on both experimental animals and other oysters grown under similar conditions.

### Modifying Cleaning Regimes of Oysters on Chaplets

To identify a cleaning regime that provided satisfactory growth and survival, with acceptable labor input, 100 oysters that were hung singly on 10 replicate chaplets were cleaned every 2, 3, 4, or 6 weeks, for 7 months (20 February to 22 September 1997). At the start and end of this experiment, all oysters were measured (DVM). The mean DVM at the start of the experiment was 85  $\pm$  0.4 mm ( $n = 400$ ). Fouling algae were saved from 25% of the oysters from each cleaning treatment in June 1997, at the time when oysters were scheduled for cleaning. Algae from individual oysters were placed into individual fine-meshed bags and rinsed to removed fine particulate matter (silt) and contaminants (e.g., shell, crabs) before being sun dried for up to a week. The samples were then oven dried for 24 h at 65 °C and weighed.

### Retention of Oysters on Chaplets

Chaplets made up in November to December 1996 ( $n = 1440$ ) and immersed for 7 months were assessed to determine any relationship between the size of oysters at drilling and their retention on chaplets. All data used in this assessment were from chaplets where oysters had been hung singly.

Strong byssal attachment reduces the chance of oysters being lost from chaplets. Observations that oysters readily made attachments to other oysters prompted an experiment to monitor byssal production and attachment of oysters hung in pairs and oysters hung singly. In April 1997, eight replicate chaplets (10 individuals per chaplet) for each treatment were deployed on longlines. The mean ( $\pm$ SE) DVM at the start of the experiment was 67  $\pm$  0.5 mm ( $n = 160$ ). At the end of 4 months immersion, retention of oysters, byssal attachment, and growth (DVM and wet weight) of oysters were recorded. Chaplets holding "paired" and "single" oysters were cleaned monthly, and the time required for cleaning was determined. Following this experiment, byssal threads of all oysters were severed, and re-attachment was examined by SCUBA divers after 1, 2, 7, 14, and 21 days.

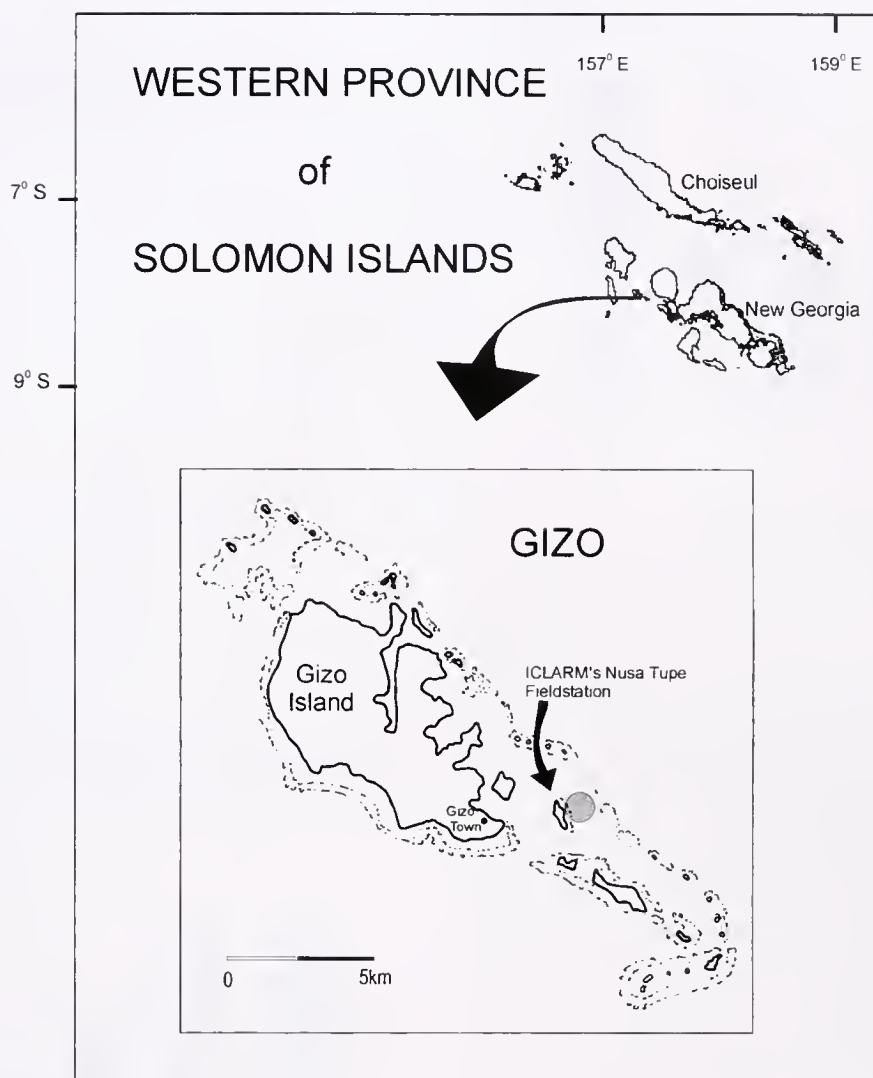


Figure 2. Gizo Lagoon in the Western Province of Solomon Islands. *P. margaritifera* were held in suspended culture east of Nusa Tupe Island (circled area).

#### Data Analysis

To examine differences in oyster growth (mm DVM) among cleaning regimes, a nested analysis of variance (ANOVA), (cleaning schedule  $\times$  chaplet [cs]) was used with data from six oysters per chaplet. To examine differences in dry weights of algae among cleaning regimes, a similar analysis was conducted using algal weights from five oysters from four chaplets within each treatment.

To compare byssal attachment between oysters hung singly and in pairs, the number of oysters on each chaplet forming attachments with each other, or the rope, were compared for each treatment using a t-test. Growth (mm DVM and g wet weight) of oysters in each treatment were analyzed using t-tests. To analyze variation in the time taken to brush all oysters on a chaplet for each treatment, a two-way ANOVA, (method of attachment  $\times$  diver) was used for two divers each cleaning four chaplets within each treatment.

Before t-tests or ANOVA, data were checked for homogeneity of variance using Levene's or Cochran's test, respectively, and

transformed to  $\log_{10}(x + 1)$  to meet this assumption where necessary. Significant differences among means were identified using Tukey's HSD test.

## RESULTS

#### Growth and Survival of Oysters on Chaplets

From the initial batch of 90 oysters, 78 (87%) were alive 1 year later. For the 12 oysters that were lost, only two dead shells were found attached to chaplets. The two subgroups drilled at sizes between 58–65 mm and 66–78 mm, had a mean ( $\pm$  SE) annual growth rate of  $64 \pm 1.9$  mm and  $60 \pm 1.4$  mm DVM, respectively (Fig. 4). Two of the oysters from each of these subgroups showed very low growth rates ( $<39$  mm  $y^{-1}$  DVM).

Of the 1,440 oysters drilled and hung in November to December 1996, 1,342 (93.2%) were live in September 1997. Of the 98 that were lost, only eight dead shells were found on chaplets. Growth of these oysters is represented in Figure 4, by four subgroups of oysters, delineated by their mean ( $\pm$  SE) sizes at drilling

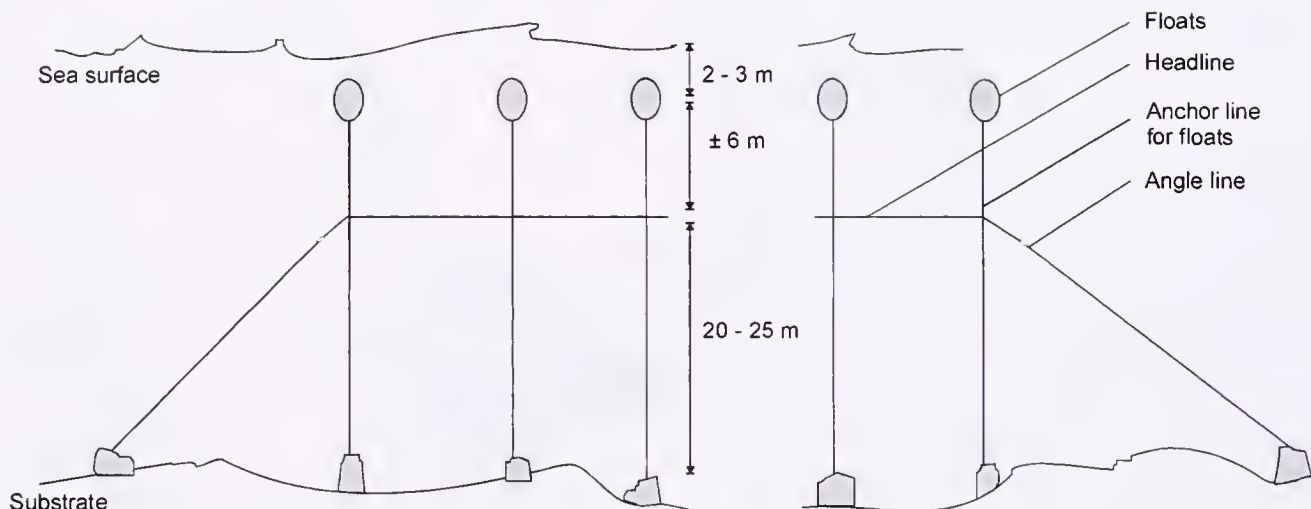


Figure 3. Diagram of longline system for suspended culture of *P. margaritifera* on chaplets.

(DVM):  $52 \pm 0.2$  mm ( $n = 64$ );  $60 \pm 0.2$  mm ( $n = 225$ );  $69 \pm 0.2$  mm ( $n = 206$ ); and  $78 \pm 0.3$  mm ( $n = 63$ ). Growth of these oysters was not as fast as the 90 oysters drilled in August 1996 (Fig. 4). Those hung on chaplets at a size of approximately 65 mm DVM attained 110 mm DVM, the size required for seeding, in 8 months. When oysters were attached to chaplets at a size of 77.8 mm DVM, they were large enough to seed in 6.5 months (Fig. 4).

Morphometric relationships between wet weight and DVM and shell thickness and DVM are shown in Fig. 5 a & b. Graph a) is useful to farmers of *P. margaritifera*, because it allows for comparisons of size/weight ratios between stocks of oysters grown in Solomon Islands and those grown elsewhere. The graph can also help farmers of *P. margaritifera* in Solomon Islands who want to calculate flotation needs for longline culture. Graph b) is added for general reference and will be of value should a relationship be established between shell thickness and the capacity for oysters of greater thickness to accept and retain larger nuclei.

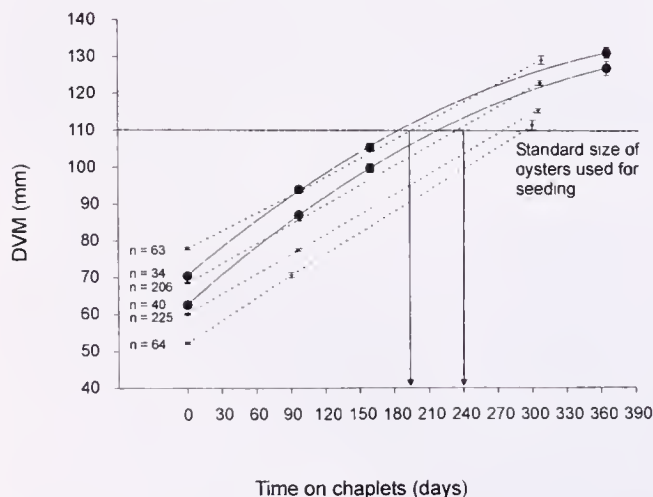


Figure 4. Growth (DVM SE) trajectories for subgroups of *P. margaritifera* of different sizes placed on chaplets for growout. The two long-dash lines depict growth of the 90 oysters drilled and hung on chaplets in August 1996, and the four dotted lines are from the bulk of oysters drilled and hung on chaplets in November to December 1996.

Modifying Cleaning Regimes of Oysters on Chaplets

Survival of oysters cleaned every 2, 3, 4, and 6 weeks was 96, 96, 96, and 97%, respectively, despite the fact that algae grew heavily on oysters, sometimes covering them completely. Analysis of dry weight of algae on oysters cleaned every 2, 3, 4, or 6 weeks showed a progressive increase in weight of algae from 2 to 4 weeks, but no significant increase in weight between 4 and 6

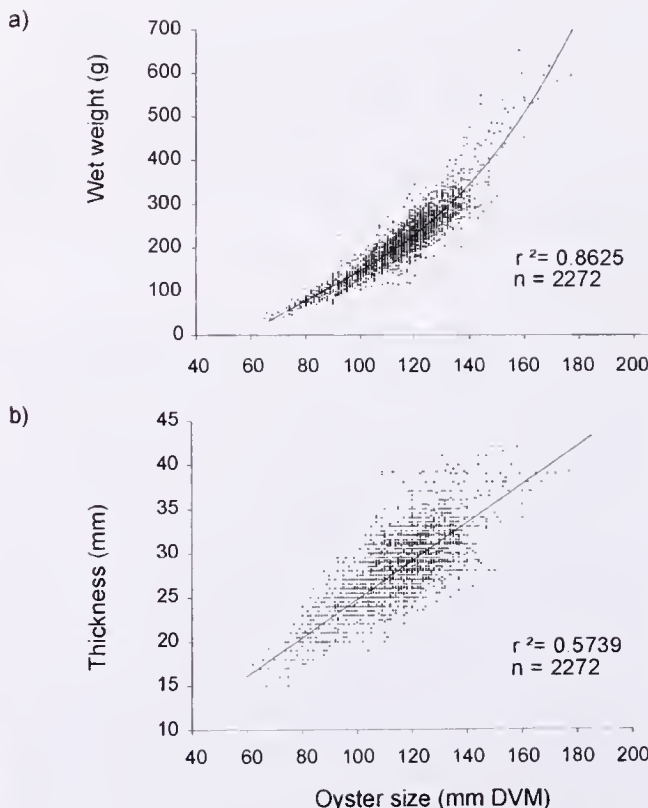


Figure 5. Size of *P. margaritifera* (DVM) relative to: a) wet weight; and b) thickness.



TABLE 1.

Results of the nested ANOVA for effects of cleaning schedule (fixed factor) and chaplet (random factor) on a) dry weight of algae on collectors, and b) growth of *P. margaritifera* (DVM).

Source of Variation	df	MS	F	P
a) Dry weight of algae				
Cleaning schedule	3	1.270	82.452	0.0000
Chaplet [clean schedule]	12	0.015	7.630	0.0000
Residual	64	0.015		
b) Growth of oysters				
Cleaning schedule	3	100.117	3.182	0.0354
Chaplet [clean schedule]	36	31.468	1.048	0.4037
Residual	200	31.468		

weeks (Table 1, Fig. 6a). The average mass of algae on oysters not cleaned for 6 weeks was  $7.8 \pm 0.8$  SE g per oyster.

The growth increment of oysters among the four cleaning treatments differed significantly (Table 1), with oysters cleaned every 3 weeks growing significantly faster ( $p < .05$ ) than those cleaned on a 2- or 6-week schedule (Fig. 6b).

#### Retention of Oysters on Chaplets

Mortalities of oysters after handling and drilling were low (< 0.6%). However, greater proportions of smaller oysters were lost

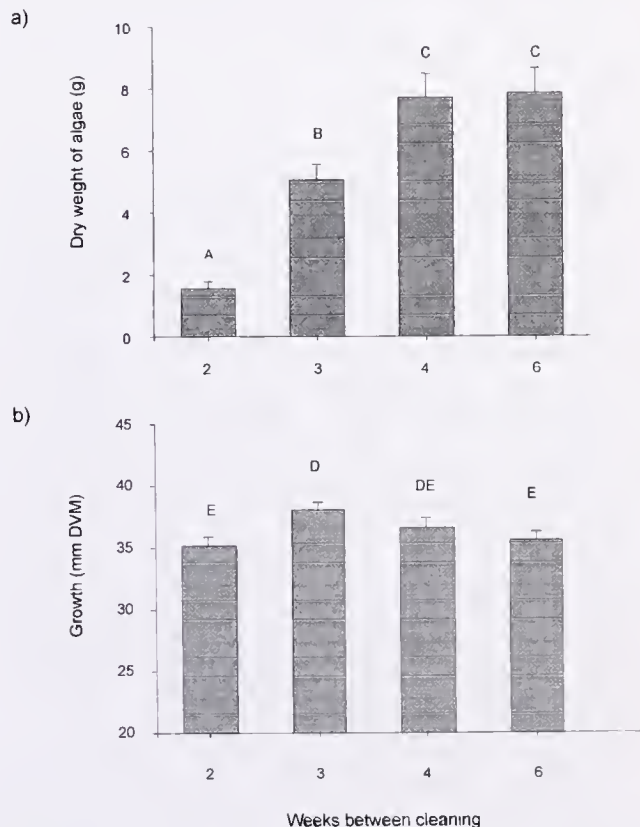


Figure 6. Variation in: a) mean ( $\pm$ SE) dry weight of algae removed from *P. margaritifera*; and b) growth of *P. margaritifera* (mean DVM  $\pm$ SE) cleaned at 2, 3, 4, and 6 week frequencies. Means with different superscripts were significantly different ( $p < .05$ ).

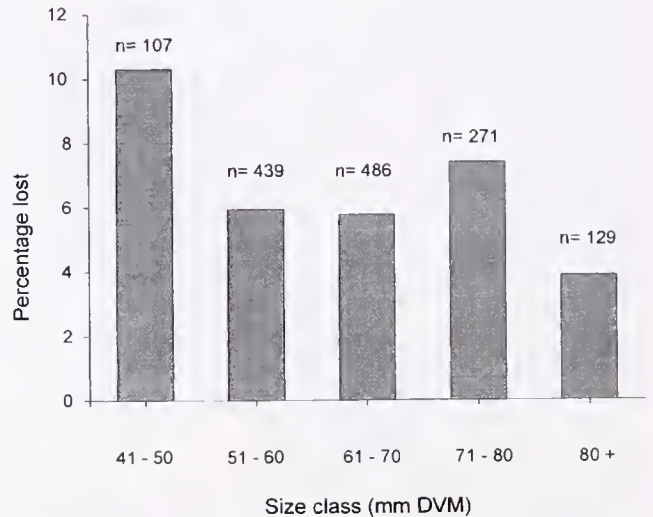


Figure 7. Percentage of *P. margaritifera* drilled at different sizes (November to December 1996) lost from chaplets by September 1997. Of the 1,440 oysters drilled and hung on chaplets, only eight dead oyster shells were recovered.

from chaplets (Fig. 7). In the experiment comparing losses of oysters held singly and in pairs, no oysters were lost through failure of the drill hole during the 4-month experiment. Oysters held on chaplets in pairs made byssal attachments to each other in  $90\% \pm 3.0$  SE of cases. In contrast, significantly fewer ( $df$  14,  $t = 6.085$ ,  $p < .001$ ) oysters held singly on chaplets made byssal attachments to the rope ( $54\% \pm 5.0$  SE). Not only were oysters more likely to make byssal attachments to other oysters than to rope, but attachments to other oysters were more secure ( $15.8 \pm 0.7$  SE byssal threads,  $n = 72$  oysters) than those of single oysters to rope ( $4.3 \pm 0.5$  SE byssal threads,  $n = 43$  oysters).

Another advantage of attaching oysters in pairs was that one valve of each oyster remained relatively free of fouling. As a result, mean cleaning times were significantly shorter ( $F_{1, 12} = 8.678$ ,  $p < .05$ ) for chaplets holding pairs of oysters ( $105 \pm 7$  SE  $s^{-1}$ ,  $n = 8$  chaplets) than for chaplets where oysters were hung singly ( $127 \pm 7$  SE  $s^{-1}$ ,  $n = 8$  chaplets). A disadvantage of attaching oysters in pairs was that growth was significantly reduced ( $p < .01$ ) by an average of 2 mm DVM over a period of 4 months (Table 2). There was no significant difference ( $p = .13$ ) in

TABLE 2.

Results of t-tests for effects on growth (DVM and wet weight) of *P. margaritifera* attached singly or in pairs to chaplets for four months. Mean growth (DVM and wet weight) is shown at the bottom of the table. Means with different superscripts are significantly different ( $P < 0.05$ ).

Source of Variation	df	t value	P
DVM	158	2.986	0.0033
Wet Weight	158	1.538	0.1261

Attachment	Single	Pairs
Mean DVM (mm $\pm$ SE)	23.1 <sup>a</sup> $\pm$ 0.50	20.85 <sup>b</sup> $\pm$ 0.56
Mean wet weight (g $\pm$ SE)	67.7 <sup>a</sup> $\pm$ 2.0	62.9 <sup>a</sup> $\pm$ 2.4

changes in wet weight of oysters between the two treatments over the 4 months (Table 2).

After byssal threads had been severed, paired oysters re-made attachments in  $96\% \pm 2$  SE of the cases after 3 weeks; whereas, only  $21\% \pm 4$  SE of single oysters made connections with the rope (Fig. 8). Again, attachments made by single oysters were composed of relatively small numbers of threads ( $2.5 \pm 0.4$  SE byssal threads,  $n = 17$ ) as compared to attachments made by oysters held in pairs ( $11.3 \pm 0.6$  SE byssal threads,  $n = 76$ ). Interestingly, oysters hung singly produced byssal threads that were seen floating, without making an attachment. The number of oysters found producing such threads was greatest ( $n = 22$  oysters, mean =  $7.4 \pm 1.0$  SE byssal threads floating) 2 days after the byssal threads had been severed and decreased as the experiment progressed.

### DISCUSSION

*P. margaritifera* cultured in the "open" reef systems of Solomon Islands were shown to be robust to the rigors of drilling, and growth of oysters on chaplets compared favorably with rates reported from the "closed" and semiclosed atoll lagoons of Polynesia. Sims (1993b) presented size at age data for *P. margaritifera* from Manihiki atoll, Cook Islands that showed that 9-month-old oysters (~81 mm DVM) were 38 mm (DVM) smaller than 2-year-old oysters (~119 mm DVM). This suggests that the mean annual growth increment for oysters of 81 mm DVM is < 38 mm DVM. Similarly, Coeroli et al. (1984) reported a growth rate of 35 mm  $y^{-1}$  for *P. margaritifera* of 70–80 mm DVM in French Polynesia. In contrast, oysters from the "open" reefs of Solomon Islands with a mean DVM of 78.7 mm grew a mean of 51 mm in 306 days (~10 months); whereas, oysters drilled at 70.4 mm DVM grew an average of 60.3 mm DVM in a year. *P. margaritifera* caught as spat (~11 mm DVM) in Solomon Islands need to be reared in intermediate culture (Friedman 1998, Friedman and Southgate 1999) and then on chaplets, for a total of ~16 months to reach 110 mm DVM; the size reported by Lintilhac (1987) and Gervis and Sims (1992) to be suitable for seeding.

Although the growout system for *P. margaritifera* in Solomon Islands resembles closely the one used in the atoll lagoons of Polynesia, the conditions found in the open reefs of Solomon Islands have more in common with those found on pearl farms

cultivating *P. maxima* in Indonesia and Australia. Whereas atoll lagoons in Polynesia are surrounded by a low-lying carbonate island (atoll) and are relatively nutrient poor (Littler et al. 1991), open reef systems are generally bordered by high islands that are the source of nutrient inputs from fresh water runoff (Chellam et al. 1987). The higher nutrient load in the lagoons of Solomon Islands may have been a factor in the good growth rates recorded in this study. Yukihiro (1998), showed that increases in food availability produced increased growth rates in *P. margaritifera* up to an optimum of  $1\text{--}2$  mg  $L^{-1}$  (ca. 10,000–20,000 cells  $mL^{-1}$ ). In the Cook Islands, Ponia (1997) found that water movement on a farm of 50,000 oysters needed to be  $> 0.01$  m  $s^{-1}$  to avoid 98% removal of microalgae by oysters hung on chaplets. His recordings of water movement in Manihiki lagoon in the Cook Islands ranged between 0–0.06 m  $s^{-1}$ . In French Polynesia, surface water movement in Takapoto atoll (closed atoll) is 0.03 m  $s^{-1}$  (Salvat 1981). In Solomon Islands, tidal water flow is greater (0–0.15 m  $s^{-1}$ ), ensuring replenishment of food to culture areas. Although measurements of food abundance were not taken in this study, we suggest that the greater nutrient loading and relatively high water movement in Solomon Islands was likely to have stimulated greater pearl oyster growth (Chellam et al. 1987).

Additional anecdotal evidence for the greater levels of nutrients in Solomon Islands than Polynesia is the fact that growth of algae on oysters and chaplet ropes was more of a problem in Solomon Islands than in the atoll lagoons of Polynesia. The noticeable difference in the level of algal fouling may have been influenced by differences in the numbers of grazers between these two regions, although this is unlikely, because there was no observable evidence that fish or invertebrate grazers were less common in Solomon Islands than Polynesia. Despite oysters in Solomon Islands becoming completely covered with algae, observations *in situ* revealed that even heavily fouled oysters were able to open their valves normally. Although oysters required regular cleaning, there was relatively little fouling by cementing organisms such as bivalves and polychaetes ("hard" fouling), which are more difficult to remove than algae. In addition, hard fouling and other byssally attached bivalves have been shown to cause shell deformity of other pearl oyster species during culture (Dharmaraj et al. 1987, Doumenge et al. 1991, Taylor et al. 1997). Such fouling may render oysters vulnerable to attack from predators such as small fish and crabs (J. Taylor, pers comm. 1998) and, in the worst cases, result in mortality (Dharmaraj et al. 1987). Hard fouling organisms present a greater problem than algal fouling, because they also compete directly with oysters for food and space (Ponia 1997). In Cook Islands, settlement of the pest species *P. maculata*, can cause longlines to sink to the substrate. This species is the dominant bivalve at pearl farms in Cook Islands, often comprising  $> 90\%$  of the total tissue biomass on culture equipment. Management of this fouling organism places a considerable burden on farm husbandry (Ponia 1997). In Solomon Islands, there was no evidence that algal fouling presented any risk of mortality to oysters. In fact, the presence of an algal covering on shell valves may have prevented successful settlement and growth of hard fouling.

Although survival was not threatened by algal fouling, growth of oysters was significantly greater when algae were brushed from chaplets on a 3–4 week cycle. This time interval is similar to that adopted for *P. maxima* culture in Indonesia and Australia (Gervis and Sims 1992, McGuinness 1994, Taylor et al. 1997), but shorter than that practiced by farmers in the atoll lagoons of Polynesia for *P. margaritifera* (J. Lyons, pers comm. 1999). Surprisingly, oysters cleaned on a 2-week schedule had some of the lowest growth

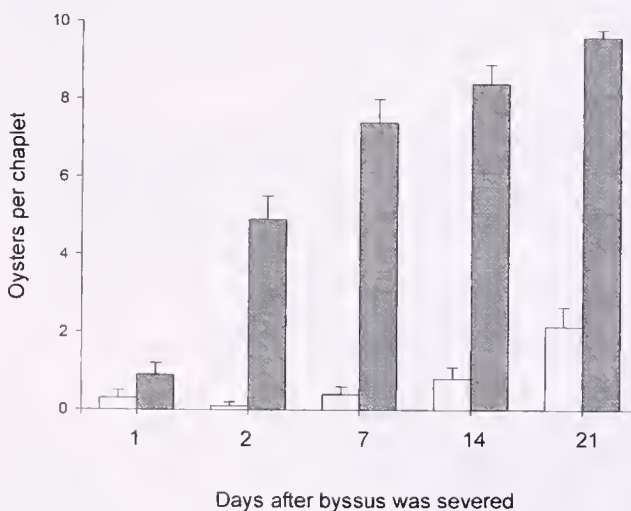


Figure 8. Mean ( $\pm$ SE) number of single (blank) and paired (solid) *P. margaritifera* per chaplet that made re-attachments after byssal threads had been severed.

rates. In addition, oysters from this treatment required re-drilling more often than oysters cleaned less frequently (K. Friedman, unpublished data 1997). In contrast, Taylor et al. (1997) showed that survival and growth of *P. maxima* in Indonesia did not differ significantly when cleaned at 2 or 4 week intervals; whereas, repeated handling results in increased mortality of scallops (Ventilla 1982, Parsons and Dadswell 1992). Anecdotal accounts suggest that the lower growth rates at more frequent cleanings in Solomon islands are attributable to "stressing" of the oysters, although the precise causes are unclear.

The number of oysters that became detached from chaplets was a concern, because the deep water (40 m) did not allow easy retrieval of lost oysters, and the sandy substrates in Gizo lagoon made the oysters difficult to find. Another notable difference between atoll lagoons and the open reefs in Solomon Islands was the presence of relatively fast water movement in the growout area (up to  $0.15 \text{ m s}^{-1}$ ). Pearl oyster culture in this relatively high energy environment and regular brushing of chaplets to remove algae may have exacerbated losses from chaplets. Also, smaller sized (< 51 mm DVM) oysters with thinner shells detached from chaplets in greater numbers than larger, thicker shelled oysters.

Oysters tied to chaplets in pairs made strong byssal attachments to one another, as is their habit in the wild (Herdman 1903, Gervis and Sims 1992). This behavior secures oysters to the chaplet even if one oyster becomes detached from the monofilament line. *Pinctada margaritifera* differ from their close relative *P. maxima* in this regard, because byssal attachment persists in adults (Doumenge et al. 1991). Examination of re-attachment after byssal threads were severed showed that oysters attempted to make attachments even when hung singly, but that successful connections to polypropylene chaplet rope were less common than to other

oysters. Because there was evidence that attaching oysters in pairs affected growth negatively, further experiments to find an alternative material for chaplet rope, which is both hard wearing and suitable for byssal attachment, would allow oysters hung singly to make attachments to the rope.

In conclusion:

1. Few mortalities resulted from drilling and attaching *P. margaritifera* to chaplets.
2. Growth of *P. margaritifera* in Solomon Islands compared well to that reported from lagoons in Polynesia; oysters caught as spat ( $\pm 11 \text{ mm DVM}$ ) reached acceptable size for seeding in 16 months.
3. Oysters on chaplets were fouled quickly by algae. However, the algae did not cause mortalities and may have been advantageous in preventing fouling by cementing bivalves and tubular polychaetes. A 3–4 week cleaning cycle resulted in significantly greater growth of oysters than more or less frequent cleaning regimes.
4. Oysters detached from chaplets in significant numbers; this problem was greatest for the smallest oysters drilled.

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## EFFECT OF SPERM DENSITY ON HYBRIDIZATION BETWEEN *CRASSOSTREA VIRGINICA*, GMELIN AND *C. GIGAS* (THUNBERG)

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**ABSTRACT** In this study, effects of sperm density on hybridization between *Crassostrea gigas* and *C. virginica* were studied. Two-by-two crosses, *C. gigas* × *C. gigas* (GG), *C. gigas* × *C. virginica* (GV), *C. virginica* × *C. gigas* (VG), *C. virginica* × *C. virginica* (VV), were conducted. Five levels of sperm density, which were measured by the relative number of attached sperm per egg (AS/egg), were produced and tested in 3 replicates. At each of the 5 levels (2–3, 5, 10, 20, and 30 AS/egg), the affinity between sperm and eggs, fertilization success, and survival and yield at 48 h were examined and determined. Reciprocal differences in fertilization in GV and VG crosses were found, with GV less successful than VG. In GV crosses, 20 or 30 AS/egg could not be attained, while in VG, GG, and VV crosses all 5 AS/egg levels were accomplished. More sperm were needed to attain a given AS/egg level in GV crosses than in VG, VV, and GG, and likewise, in VG crosses than in VV and GG at more than 10 AS/egg. The affinity between *C. virginica* sperm and *C. gigas* eggs was significantly weaker than between *C. gigas* sperm and *C. virginica* eggs. The ratio of sperm to eggs at 10 AS/egg was significantly higher than at 2–3 or 5 AS/egg. Survival and yield at 48 h in GV crosses were significantly lower than in GG, VV, and VG crosses. There was no significant difference among the first 3 AS/egg levels in fertilization, survival and yield at 48 h, but embryo deformation was found from higher than 10 AS/egg (> 10%). The results showed that the affinity between heterologous gametes was weaker compared to homologous gametes. We suggest that 2–5 AS/egg was a safe and effective range for both pure crosses and hybrid trials between these 2 species, although more sperm is needed for hybrids.

**KEY WORDS:** *Crassostrea virginica*, *C. gigas*, sperm density, fertilization, hybridization

### INTRODUCTION

Hybridization has been used widely in plant, animal, and fish breeding (Briggs 1967, Tave 1986). In oysters, the first hybridization attempt was made between the Portuguese, *Crassostrea angulata* and the European flat oyster, *Ostrea edulis*, by Bouchon-Brandely in 1882 (cited in Davis 1950). Since then, many hybridizations have been investigated between different species of oysters (Menzel 1987; Gaffney and Allen 1993). While it seems obvious that sperm density can affect fertilization, there is scant information on its effect on hybridization success. For example, to what degree is sperm density responsible for asymmetrical fertilization rates in reciprocal crosses of *C. gigas* and *C. virginica*? Fertilization success is the first concern in hybridization. Some experiments found normal fertilization in crosses between *C. gigas* (Thunberg, 1793) and *C. virginica* (Gmelin, 1791) (Galtsoff and Smith 1932, Numachi 1977, Allen et al. 1993), versus reciprocal differences in fertilization between *C. gigas* with *C. sikamea* (Numachi 1977, Banks et al. 1991, Banks et al. 1994) and *C. gigas* and *C. rivularis* (Allen and Gaffney 1993). Apparently sperm density can also affect early survival. In homologous crosses of *C. gigas*, higher ratios of sperm to oocytes could increase the fertilization rate from 75% to 100%, but decreased the larval survival (Staeger and Horton 1976). Studies reporting the effect of varying densities of heterologous sperm on fertilization and embryo survival among interspecific hybrids are lacking.

The affinity between heterologous gametes—by our definition, the propensity for adhesion of the sperm to the egg—is probably determined by a number of factors. For mollusks, sperm appear to

move in a random path to the egg without apparent attraction. As the sperm encounters the egg, it becomes oriented perpendicular to the ovum surface, then quickly binds with the vitelline layer in a process referred to as gamete binding (Hylander and Summers 1977), mediated by a substance called bindin. Bindin has been isolated from oyster sperm as a glycoprotein that attaches to glycoprotein of the ovum surface (Brandriff et al. 1978). Since gamete binding in bivalves appears to be specific (Hylander and Summers 1977), it is reasonable to expect that gamete binding between heterologous gametes vary more than in homologous crosses.

Successful fertilization during hybridization is likely determined by other factors after binding, when the acrosome is ejected from the sperm head. The acrosomal reaction consists of 2 processes: (1) the opening of the acrosomal vesicle and the release of lytic enzymes; and (2) the exposure of the acrosomal vesicle process, a rodlike structure instrumental in gamete fusion. The lytic enzymes are responsible for lysing extracellular moieties surrounding the egg and assisting the sperm in its movements to the surface of the egg, and to facilitate gamete fusion.

In associate with continuing trials on interspecific hybridization between *C. gigas* and *C. virginica*, experiments were designed to answer the following questions: (1) Should more sperm be added to fertilize eggs in hybrid crosses than in the pure crosses? (2) What is the optimal sperm density and how should this be assessed during fertilization trials?

### MATERIALS AND METHODS

#### *Oysters and Gametes*

*Crassostrea gigas* brood stock were obtained from Washington in 1988, spawned, and destroyed. Progeny were held under quarantine conditions at the Cape Shore hatchery. Oysters used in this work were an F<sub>3</sub> generation made in 1992. Sexually mature adult

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*C. virginica* were chosen from Delaware Bay native stocks. Gametes were obtained by dissection as follows. All surfaces and instruments contacting the oysters were cleaned and rinsed with hot freshwater between handling and opening of different individuals. Each oyster was opened and a gonad biopsy was taken for determining the sex of the individual by light microscopy. Gametes from each oyster were dissected directly into individual beakers.

Oocytes were separated from gonadal tissue and other debris by passing the suspension through a 60- $\mu\text{m}$  nylon screen. The oocytes were caught on a 25- $\mu\text{m}$  nylon screen (20- $\mu\text{m}$  for *C. virginica*) and resuspended in seawater. The density of the eggs was checked under the microscope, and then estimated by counting aliquots of an appropriate dilution. The eggs of the same female were divided into separate groups of  $150 \times 10^3$  eggs.

Sperm were separated from debris by passing the suspension through a 15- $\mu\text{m}$  nylon screen. The density of sperm suspensions was first counted with a hemocytometer under a microscope and varied with each dissection. Controlling sperm density in fertilization was not practical; instead, the number of sperm used in fertilization (density of sperm suspension  $\times$  volume added into 1 beaker of eggs) was recorded. This number was then divided by the amount of eggs in that beaker ( $150 \times 10^3$ ), and was called the ratio of sperm to eggs. The movement of sperm around eggs was also observed and recorded.

Fertilizations were made between 30 and 90 min after dissection so as to confirm that eggs were not self-fertilized and to reduce the susceptibility of oocytes to polyspermy. Stephano and Gould (1988) reported that when oocytes were inseminated within 15 min of their mechanical removal from the gonad, they were highly susceptible to polyspermy, even at low sperm density.

When sperm were added to eggs, beakers were gently and evenly stirred to promote even distribution of sperm. The number of sperm observed around the perimeter of at least 10 eggs was counted within 2 min of fertilization. This number is called the relative number of attached sperm per egg (AS/egg). The reason we used relative sperm attachment as the experimental variable was 2-fold. First, during fertilization, it was more convenient (and practical) to check the amount of sperm around an egg's perimeter than to count the density of the sperm suspensions. Second, from our previous experience, we have observed considerable variability in the binding of sperm among crosses in which the same amount of sperm was added, suggesting variation in gamete affinity among matings.

In this experiment, 5 levels of AS/egg were designated: 2–3, 5 (range 4–6), 10 (range 9–11), 20 (range 18–22) and 30 (range 28–32). To achieve these AS/egg levels in each cross, usually more than 5 beakers of unfertilized eggs were prepared and stored at 4 °C. First, a small amount of sperm was added to 1 beaker of eggs, and the number of sperm that associated with the egg was counted under the microscope within 2 min. If the observed AS/egg was at the desired level, the beaker of eggs was kept for the experiment; then the next AS/egg level would be attained based on the amount of sperm used in previous trial. Sperm was added to the eggs only once. If the observed AS/egg was not at the desired level, the beaker of eggs would be withdrawn and discarded. The amount of sperm used in each AS/egg level was recorded.

The final density of eggs in each group was maintained at  $15 \times 10^3/\text{mL}$  for fertilization and  $150/\text{mL}$  for 48 h thereafter, without

aeration. The temperature for all crosses and cultures was from 23–25 °C with salinity between 25–28 ppt.

### Experimental Design

The purpose of this experiment was to quantify the differences in affinity between sperm and eggs and to measure the effect of sperm density on fertilization, 48-h embryo survival, and yield in reciprocal crosses. Abbreviations for oyster species are as follows: G = *C. gigas* and V = *C. virginica*. In our nomenclature, females are listed first. Crosses between *C. gigas* and *C. virginica* were produced by a  $2 \times 2$  factorial mating of 1 female and 1 male from each species, i.e., 4 crosses were produced, VV, VG, GV, and GG, and replicated 3 times with different parents in each replicate.

Fertilization rates at each AS/egg level in all crosses were assessed by light microscopy on at least 100 oocytes at 60 min post-fertilization (PF). Fertilization was considered successful if oocytes were at or beyond polar body I formation. To observe development, oocytes from each cross were checked before insemination and 2 min PF, up to 90 min PF. Embryo survival to straight-hinge stage (48 h) was determined by sieving cultures onto a 25- $\mu\text{m}$  mesh screen and counting the number of larvae of normal appearance. Embryo survival was estimated as the proportion of larvae produced from fertilized eggs at 48 h:

$$48\text{-h survival} = (\text{No. of straight-hinge at 48 h} \div \text{No. fertilized eggs}) \times 100$$

Yield at 48 h was estimated as the proportion of larvae at 48 h produced from the total eggs, therefore not taking into account fertilization rate. This was calculated as:

$$48\text{-h yield} = (\text{No. of straight-hinge at 48 h} \div \text{No. eggs used}) \times 100$$

### Statistical Analyses

In GV crosses, treatment levels of 20 and 30 AS/egg could not be obtained, and data were not available. Therefore, only data from the first 3 levels were analyzed in a 4-way mixed factorial ANOVA model, Dam (fixed)  $\times$  Sire (fixed)  $\times$  AS/egg level (fixed)  $\times$  Replicate (random), using the computer program SPSS version 7.5. Fisher's Least Significant Difference (LSD) was used for multiple comparisons among the first 3 AS/egg levels, when significant differences ( $P < .05$ ) were indicated by ANOVA. Data from 20 and 30 AS/egg are only presented in figures.

## RESULTS

### Affinity Between Sperm and Eggs

When sperm were added to egg suspensions, they swam randomly to the eggs. When sperm encountered an egg, they attached to the outer membrane rapidly and remained bound, if the gametes were homologous. In contrast, *C. virginica* sperm attached less readily to the surface of *C. gigas* eggs, with most of the sperm only bouncing off the surface of the eggs. *C. gigas* sperm could easily attach to the eggs of *C. virginica*.

In VG crosses, when additional *C. gigas* sperm were added to *C. virginica* eggs, more sperm attached to the surfaces, so that all 5 AS/egg levels were accomplished, including higher AS/egg levels of 20 and 30. In contrast, in GV crosses, no matter how many *C. virginica* sperm were added, *C. gigas* eggs could not attain 20 or 30 AS/egg. The highest level attained was 18 AS/egg.

Results from the 4-way ANOVA showed that Sire ( $P = .022$ ), AS/egg level ( $P = .01$ ) and their interaction ( $P = .021$ ) had significant effects on the ratio of sperm to eggs (Table 1, Figs. 1 and 2). There was no significant difference among Dams, and no significant interaction between or among any other 2 or 3 factors ( $P = .05$ ).

Fisher's LSD test showed that the ratio of sperm to eggs in 10 AS/egg was significantly higher than in 2–3 AS/egg ( $P = .002$ ) or 5 AS/egg ( $P = .007$ ), while there was no significant difference between 2–3 and 5 AS/egg ( $P > .05$ ) (Fig. 1).

#### Fertilization

For fertilization rates, no significant effects were found from any main factors (Dam, Sire, Level and Replicate) or interactions ( $P = .05$ ) (Table 1 and Fig. 3).

#### Survival to 48 Hours (Embryo Survival)

Embryo deformation (< 10%) was found from 10 to 30 AS/egg levels in all 4 crosses and consisted of irregularly shaped embryos and trochophores. Results from ANOVA showed that there was a significant interaction between Dam and Sire ( $P = 0.010$ , and the average survival of each cross in the first 3 AS levels, VV = 48%, VG = 41%, GG = 42%, and GV = 12%), but no significant effects from Dam, Sire, Level, Replicate, or interaction ( $P = .05$ ) (Table 1 and Fig. 4).

#### Yield at 48 Hours

As with the 48-h survival, the 48-h yield was significantly affected by Dam  $\times$  Sire interaction ( $P = 0.026$ , ANOVA, and the average yield of each cross in the first 3 AS levels, VV = 44%, VG = 33%, GG = 44%, and GV = 12%), but not other factors ( $P = .05$ ) (Table 1 and Fig. 5).

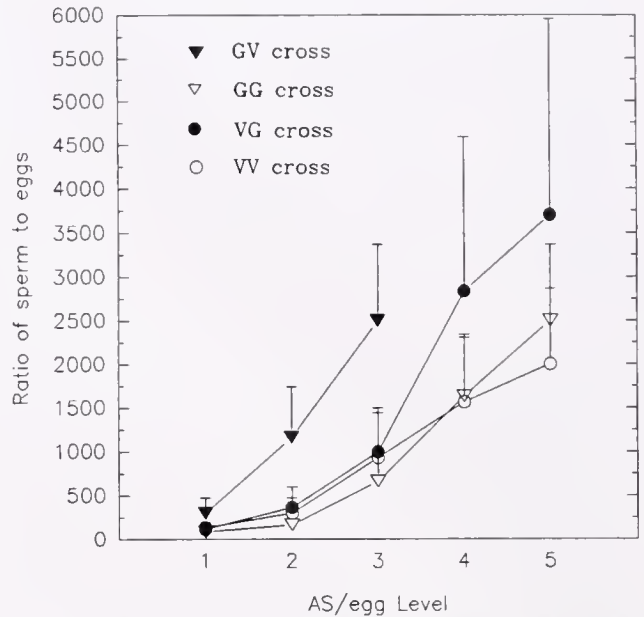


Figure 1. Mean ratio of sperm to eggs at different AS/egg levels in reciprocal crosses of *Crassostrea virginica* (V)  $\times$  *C. gigas* (G). Positive error bars represent standard deviation.

## DISCUSSION

The results in this experiment seem to support species-specific gamete binding in oysters. Gamete binding is specific on pelecypods (Hylander and Summers 1977). A glycoprotein, bindin, has been isolated as the substance responsible for the attachment of the sperm acrosome to the glycoproteins of the egg surface (Brandriff

TABLE 1.

P-values of 4-way ANOVA, Dam (fixed)  $\times$  Sire (fixed)  $\times$  AS/egg level (fixed)  $\times$  Replicate (random), in the hybridization of *Crassostrea virginica*  $\times$  *C. gigas*.

Treatment	P-value of 4-Way ANOVA			
	Sperm Ratio	Fertilization Rate	Survival	Yield
<b>Sources</b>				
Dam-i	.141	.074	.286	.342
Sire-i	<b>.022*</b>	.231	.410	.362
Dam $\times$ Sire-ij	.184	.108	<b>.010*</b>	<b>.026*</b>
Level-k	<b>.010*</b>	.058	.054	.321
Dam $\times$ Level-ik	.3130	.140	.879	.813
Sire $\times$ Level-jk	<b>.021*</b>	.336	.849	.715
Dam $\times$ Sire $\times$ Level-ijk	.120	.306	.386	.346
Replicate-m	.423	.584	.371	.345
Dam $\times$ Replicate-im	.173	.994	.732	.586
Sire $\times$ Replicate-jm	.346	.348	.563	.314
Dam $\times$ Sire $\times$ Replicate-ijm	.098	.057	.891	.732
Level $\times$ Replicate-km	.510	.657	.926	.882
Dam $\times$ Level $\times$ Replicate-ikm	.160	.422	.412	.450
Sire $\times$ Level $\times$ Replicate-jkm	.062	.473	.376	.338
Dam $\times$ Sire $\times$ Level $\times$ Replicate-m(ijk)	as error	as error	as error	as error

Bold P-values with an asterisk indicate significant difference ( $P < .05$ ).

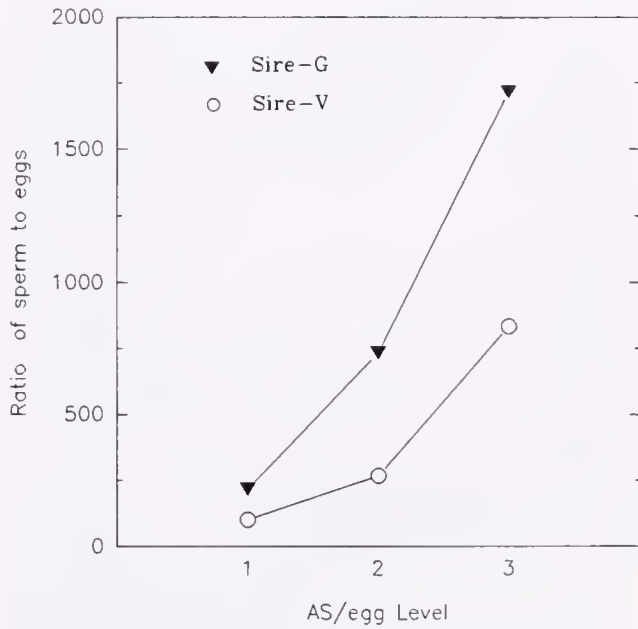


Figure 2. Interaction between Sire and AS/egg level in the ratio of sperm to eggs in reciprocal crosses of *Crassostrea virginica* (V) × *C. gigas* (G). Sire V represents the average of VV and GV crosses, and Sire G, average of GG and VG crosses.

et al. 1978). Our observations and results show that sperm could more easily attach to homologous eggs than to heterologous ones, suggesting the binding of the acrosome or the glycoprotein of the eggs could be different in *C. virginica* and *C. gigas*.

We also noticed a significant difference in gamete binding between VG and GV crosses. First, the movement of *C. virginica* and *C. gigas* sperm was quite different on the surface of foreign

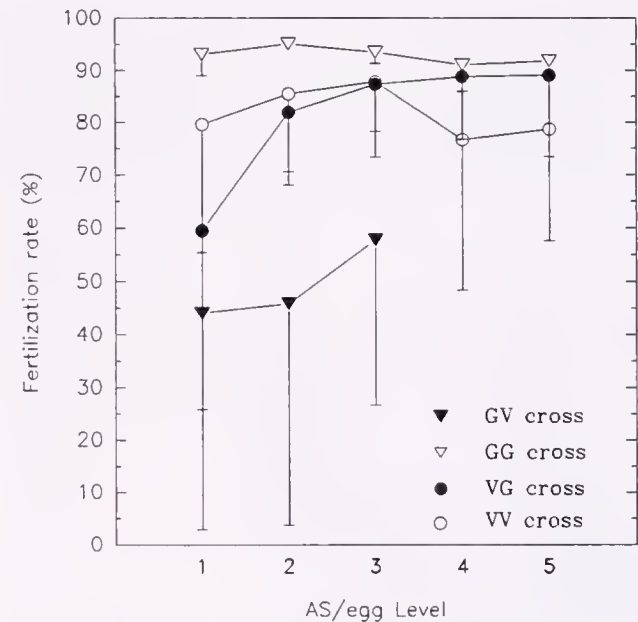


Figure 3. Mean fertilization rate (%) at different levels of AS/egg in reciprocal crosses of *Crassostrea virginica* (V) × *C. gigas* (G). Negative error bars represent standard deviation.

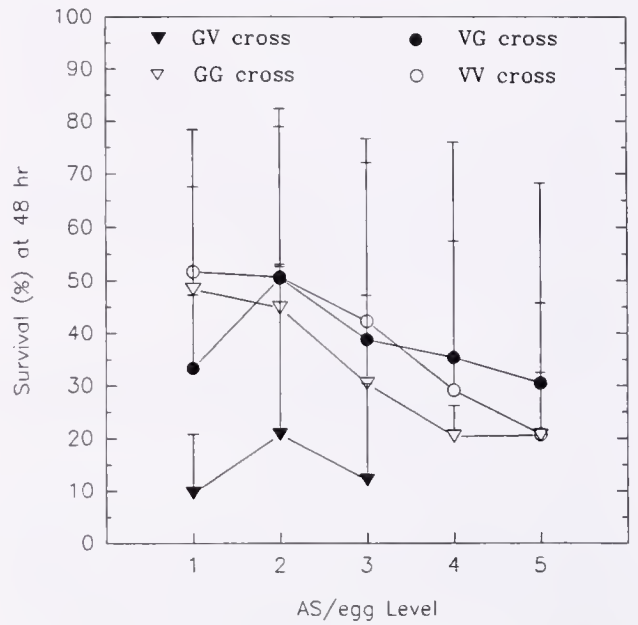


Figure 4. Mean survival rate (%) at 48-h at different levels of AS/egg in reciprocal crosses of *Crassostrea virginica* (V) × *C. gigas* (G). Positive error bars represent standard deviation.

eggs: *C. virginica* sperm seemed to be unable to attach tightly onto the surface of *C. gigas* eggs, but rather bounced around them. In contrast, sperm of *C. gigas* could bind on the surface of *C. virginica* eggs relatively quickly. Second, in VG crosses all AS/egg levels, including 20 and 30, were attained, while in GV crosses, only low AS/egg levels obtained with even large amounts of sperm addition. Therefore, hybridization was easier in the direction of VG crosses. Third, a significant interaction was found between

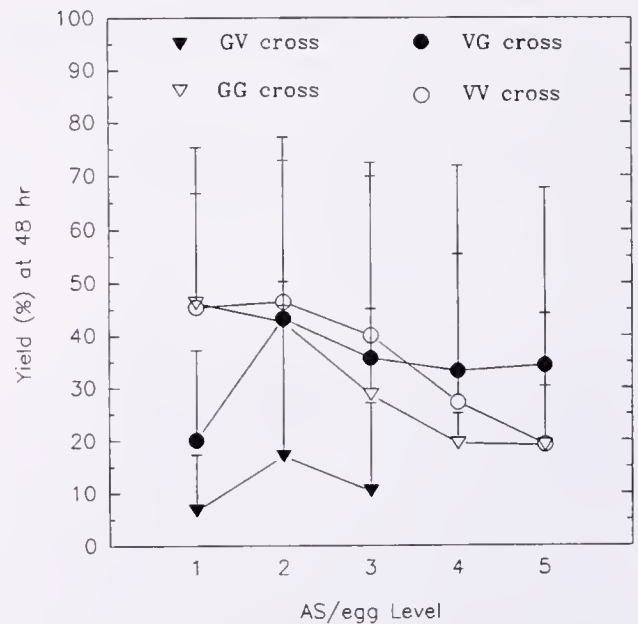


Figure 5. Mean yield (%) at 48-h at different levels of AS/egg in reciprocal crosses of *Crassostrea virginica* (V) × *C. gigas* (G). Positive error bars represent standard deviation.



Sire and AS/egg level on the ratio of sperm to eggs. The interaction between Sire and AS/egg level (Fig. 2) suggests that the effect of V and G sperm on the ratio of sperm to egg varies as AS/egg levels go from low to high. From Figure 1, it is clear that ratios of sperm to eggs in VV and GG were the same, while VG and GV were quite different. The real cause for significant Sire  $\times$  AS/egg level interaction is probably that different amounts of sperm were needed in GV and VG to attain a given AS/egg.

Reciprocal differences in fertilization of hybrid oyster crosses have been reported previously. In the study of gametic incompatibility and genetic divergence in Pacific and Kumamoto (*C. sikamea*) oysters, Banks et al. (1994) reported one-way genetic incompatibility. *C. sikamea* eggs  $\times$  *C. gigas* sperm formed viable hybrid offspring, but *C. sikamea* sperm do not fertilize *C. gigas* eggs. Epifluorescent staining revealed internal sperm pronuclei in the cross of *C. sikamea* eggs  $\times$  *C. gigas* sperm, but not *C. gigas* eggs  $\times$  *C. sikamea* sperm, and the sperm of *C. sikamea* were easily removed by washing *C. gigas* eggs. In contrast, sperm remained bound to eggs after washing in all other crosses. Banks et al. concluded that the complete fertilization failure for *C. gigas* female  $\times$  *C. sikamea* male indicated a discrete block on the molecular mechanism of sperm-egg interaction and fertilization—the apparent failure of *C. sikamea* sperm to undergo the acrosome reaction at the *C. gigas* egg surface. In our study, the reciprocal difference in sperm and egg binding between *C. gigas* and *C. virginica* might be explained also by the reciprocal differences in acrosome reaction.

Even though the fertilization rates in GV crosses were lower than in VG crosses at the lowest 3 levels of AS/egg, there was no significant effect from the main factors. For the same species, Downing (1989) reported reciprocal differences in fertilization in GV and VG crosses, with GV less successful than VG. Allen et al. (1993) reported that fertilization rates in GV and VG crosses were similar, 56% and 62%. Numachi (1977) and Galtsoff and Smith (1932) also attained similar fertilization in their GV and VG crosses. These inconsistent results could be caused by different quality of gametes or non-optimum sperm densities. No sperm density or sperm ratio to eggs were reported.

Reciprocal differences in 48-h survival and yield were also apparent between GV and VG crosses, as revealed by the significant Dam  $\times$  Sire interaction. Survival and yield in GV crosses were lower than in VG, while GG and VV crosses were similar. In the hybridization experiment of Allen et al. (1993) of *C. virginica* with *C. gigas*, survival of 48-h embryos was 42% in GG crosses and 38% in VV crosses. Their results were close to our optimum results: 48% (survival at 48-h) in GG, 51% in VV. But the result of their hybridizations were different from our experiment. Their

survival in reciprocal GV and VG crosses were about the same, 45% for GV cross and 35% for VG. In this experiment, reciprocals behaved dissimilarly, with optimum 48-h survival (at 5 AS/egg) in GV of 21% and VG, 51%. Optimum yields in the 2 pure crosses were obtained when sperm density was at 2–3 and 5 AS/egg. Yields of our pure crosses were higher than the results of Allen et al. (1993). Yields of our hybrid crosses at 48 h were also different from theirs. We had reciprocal differences in yield between GV and VG crosses, 10% and 41%, respectively. Their reciprocal yield rates were similar, 25% in GV and 22% in VG. The difference between these 2 experiments could be caused by the quality of gametes or the density of sperm.

In this experiment, deformed embryos (< 10%) were found at higher sperm densities (10–30 AS/egg) in both pure and hybrid crosses. Polyspermy or poor quality seawater resulting from high sperm density could be reasons. In a study of polyspermy in the oyster *C. gigas* (Stephano and Gould 1988), the incidence of polyspermy in naturally spawned eggs and in eggs artificially removed from the gonad but incubated in seawater for 1.5 h was much lower than the eggs removed from the gonads and immediately fertilized. They proposed that a polyspermy block is weak or absent in ovarian oocytes when they are removed from the gonad but develops during residence in seawater. They found that polyspermy of eggs incubated for 1–1.5 h was 5% using a ratio of 35 sperm/egg; 7% in 250 sperm/egg; 23% in 5,000 sperm/egg, and 69% in 50,000 sperm/egg. Eggs of our experiment were incubated for 0.5 to 1.5 h, and the ratio of sperm to oocyte was from 50 to 12,500. According to their results, polyspermy could have occurred in this experiment.

What is the optimum sperm density? We suggest 2–5 AS/egg as a safe and effective range for fertilization within and between *C. gigas* and *C. virginica*, since no deformation was found at these levels. The amount of sperm should be judged by checking the number of sperm around the eggs under the microscope, not the number of sperm added. To attain lower levels of AS/egg, more sperm will be needed in GV crosses than in VG, VV, and GG; to attain higher levels of AS/egg (> 10), more sperm will be needed in VG crosses than in VV and GG. The affinity between heterologous gametes seemed weaker than with conspecific gametes.

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## COMPARATIVE FIELD STUDY OF *CRASSOSTREA GIGAS* (THUNBERG, 1793) AND *CRASSOSTREA VIRGINICA* (GMELIN, 1791) IN RELATION TO SALINITY IN VIRGINIA

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**ABSTRACT** To evaluate and compare the performance of triploid juvenile *C. gigas* (mean shell height = 19.2 mm) and triploid juvenile *Crassostrea virginica* (mean shell height = 31.7 mm), 600 oysters of each species were deployed for 1 year in floating mesh cages at three replicate sites within low, medium, and high salinity regimes (respectively, <15‰, 15–25‰, > 25‰) in the Chesapeake Bay and the Atlantic Coast of Virginia. The comparative performance of the two oyster species varied with salinity. At low salinity sites, cumulative mortality of *C. virginica* (10%) was significantly ( $P < .05$ ) lower than that of *C. gigas* (63%), and over-all mean growth rate of *C. virginica* (2.9 mm mo<sup>-1</sup>) was significantly ( $P < .05$ ) higher than that of *C. gigas* (1.6 mm mo<sup>-1</sup>). At medium salinity sites, survival and growth rate of *C. virginica* and *C. gigas* were not significantly ( $P > .05$ ) different. Both species experienced moderately high cumulative mortality at the medium salinity sites—35% for *C. virginica* and 53% for *C. gigas*—but considerable variation among sites was observed. At high salinity sites, mean cumulative mortality was similarly low (<11%) for both species; whereas, over-all mean growth rate of *C. gigas* (7.1 mm mo<sup>-1</sup>) was significantly ( $P < .05$ ) higher than that of *C. virginica* (3.6 mm mo<sup>-1</sup>). At all sites, *C. gigas* was less susceptible than *C. virginica* to *Perkinsus marinus* infections. Infections by *Haplosporidium nelsoni* were present in *C. virginica* and absent in *C. gigas*. Infestations by mud-worm *Polydora* spp. were more prevalent and severe for *C. gigas* than for *C. virginica* at low and medium salinity sites in October 1997, but similar for both species at other times and locations. Condition index was significantly ( $P < .05$ ) higher for *C. virginica* than for *C. gigas* at low salinity in May 1998, but similar for both species for other times and locations. *Crassostrea virginica* outperformed *C. gigas* in low salinity sites in the Chesapeake Bay, *C. gigas* outperformed *C. virginica* at high salinity sites in the Atlantic Coast, and performance was similar for both species at medium salinity sites in the Chesapeake Bay.

**KEY WORDS:** *Crassostrea gigas*, triploid, growth, survival, disease susceptibility, Virginia

### INTRODUCTION

As native eastern oyster, *Crassostrea virginica* (Gmelin, 1791) stocks have declined throughout much of the mid-Atlantic seaboard of the United States through overharvesting, disease, and water quality deterioration, interest in the potential of non-native oyster species to restore the fishery and ecological functions has grown. This has been particularly apparent in the Chesapeake Bay region, where standing stocks of eastern oysters have been reduced in the last decade to 1% of late nineteenth-century levels (Newell 1988). Given that much of this decline has been caused by devastating Dermo and MSX epizootics resulting from, respectively, the protozoan parasites *Perkinsus marinus* and *Haplosporidium nelsoni* (Burreson and Ragone Calvo 1996), strategies aimed at rehabilitation of stocks largely depend upon the use of disease-resistant oysters. Although development of eastern oyster lines with resistance to MSX has been achieved (Ford and Haskin 1987) and development of lines with resistance to both Dermo and MSX is in progress (Ragone Calvo et al. 1997), applicability of selective breeding programs is mostly limited to aquaculture. Use of disease-resistant eastern oysters for fishery enhancement or ecological restoration is constrained by dilution of their gene pool with that of susceptible oysters in the wild. Furthermore, the gene flow from relatively uninfected and highly susceptible populations in low salinity areas may limit the evolution of resistance in eastern oysters (Gaffney and Bushek 1996).

The Pacific oyster, *Crassostrea gigas* (Thunberg, 1793), has been the species of choice to substitute for depleted local oyster populations decimated by disease and other factors in many countries (Mann et al. 1991, Shatkin et al. 1997). *Crassostrea gigas* is

the primary oyster species supporting shellfish industries around the globe, accounting for an estimated 80% of the world oyster production (Chew 1990). Shatkin and collaborators (1997) reviewed the worldwide experience with introductions of *C. gigas* and presented an analysis of economic, legal, and ecological factors relevant for introductions into the Gulf of Maine. Experience with the transfer of *C. gigas* beyond its native range in the Indo-Pacific coast of Asia, particularly in Japan, has been considered both successful and problematic. For example, transfer of *C. gigas* to the Pacific Northwest region of the United States has restored the shellfish industry that used to rely on the native oyster *Ostrea lurida* (Chew 1990). Transfer of *C. gigas* to France has rehabilitated the industry by substituting for *Crassostrea angulata*, which was decimated by a viral disease (Grizel and Héral 1991). Problems with the transfer of exotic oysters include parallel transfer of pests and disease agents and undesired competition of exotic species with their native counterparts. For example, spread of the viral disease affecting *C. angulata* in France has been correlated with the introduction of *C. gigas*, which was conducted in bulk and without proper measures for disease prevention (Andrews 1980, Grizel and Héral 1991). Following transplantation into southeastern Australia, *C. gigas* successfully reproduced and displaced the native oyster, *Saccostrea commercialis*, from some of its habitat (Chew 1990).

During the last decade, the possible introduction of *C. gigas* into the Chesapeake Bay has received considerable attention. Mann and collaborators (1991) developed the rationale and analyzed the risks associated with such an introduction. Gottlieb and Schweighofer (1996) further discussed the potential of *C. gigas* for restoring the Chesapeake Bay ecosystem. In Virginia, a program to

examine the suitability of nonindigenous oyster species to local conditions was established, while efforts to restore native oysters continued (VIMS 1996). Based upon ecological requirements and disease tolerance, two candidate nonindigenous oyster species within the genus *Crassostrea*, *C. gigas* and the Suminoe oyster, *C. ariakensis* (= *rivularis*) (Fujita, 1913) were initially selected for testing in the Chesapeake Bay (Mann et al. 1991, VIMS 1996). In this paper, we address field studies with *C. gigas*. No growth or disease challenge studies are available for *C. ariakensis* in the region; however, for locations on the West Coast of the United States, Langdon and Robinson (1991) reported growth rates similar to that of *C. gigas*. Studies with *C. ariakensis*, currently underway at Virginia Institute of Marine Science (VIMS), will be the object of a future report.

Both Mann et al. (1991) and Gottlieb and Schweighoffer (1996) have suggested that *C. gigas* has considerable potential for restoration in part of the Chesapeake Bay, but both indicated the need for more research. The need for field studies was particularly emphasized to assess the performance of exotic oysters under local conditions, and because there was no alternative way for challenge against MSX. Prior studies at VIMS indicated that *C. gigas* was more resistant to protozoan pathogens than the native oyster, at least under some environmental conditions. In laboratory disease challenge experiments with *P. marinus*, *C. gigas* exhibited lower disease prevalence and intensity and had lower mortality than *C. virginica* (Meyers et al. 1991, Barber and Mann 1994). A field challenge experiment conducted in the York River using triploid oysters also indicated that *C. gigas* had reduced susceptibility to *P. marinus* and *H. nelsoni* as compared to the native oyster (Burreson et al. 1994). In this field study, which lasted only 5 months, *C. gigas* had comparable shell growth rates to the native oysters, but became heavily infested by the polychaete *Polydora websteri*, resulting in poor meat quality. However, these studies were limited in duration and spatial extent, and more extensive field experiments were necessary to evaluate the performance of *C. gigas* better within a broader range of salinity and other environmental conditions. The present study was designed to (1) test the hypothesis that comparative performance of *C. gigas* and *C. virginica* would vary with salinity, (2) compare disease susceptibility in the same two species across salinity regimes, and (3) compare infestations by shell-boring organisms (e.g., mud worms and boring sponges).

## METHODS

### Study Sites

Nine sites were selected on the basis of several criteria, including salinity regime, geographic location, available information on oyster growing conditions and water quality, safety, logistics, and relevance for the oyster industry. Sites were established at triplicate locations within low salinity (<15‰), medium salinity (15–25‰), and high salinity (>25‰) areas (Fig 1). Low and medium salinity sites were established near the margins of rivers (Corrotoman, Great Wicomico, Coan, and York); or in shallow creeks surrounded by marshes (Woodas Creek, a tributary of the East River, and Nandua Creek). High salinity sites were located in well-flushed narrow channels surrounded by marshes and mudflats in the coastal lagoon system of the Atlantic Coast of Virginia.

Temperature and salinity were measured during monthly site visits with a stem thermometer and a refractometer. To characterize environmental variables further, hourly temperature, salinity,



Figure 1. Location of study sites in the Chesapeake Bay and the Atlantic Coast of Virginia. ▲ Low salinity (<15 ppt) sites, ● medium salinity (15–25 ppt) sites, ■ high salinity (>25 ppt) sites.

and turbidity were measured with Hydrolab-Minisonde® dataloggers deployed at various sites for weekly to monthly intervals.

### Oyster Groups

To ensure that this study resulted in neither the unintended reproduction of *C. gigas* nor the introduction of potential exotic pathogens, we used triploid oysters produced from progeny of quarantined brood stocks, in accordance with protocols developed by the International Council for the Exploration of the Seas (ICES). Triploid *C. gigas* (3CG) and triploid *C. virginica* (3CV) were produced for this study by Haskin Shellfish Research Laboratory (HSRL) during June to July 1996 (Table 1). Brood stock for 3CG was Miyagi strain *C. gigas* originating from the Pacific Northwest Coast of the United States and maintained in quarantine at HSRL for several generations. Triploid *C. gigas* were produced by mating tetraploid and diploid parent stocks, an approach that results in complete triploidy of progeny (Guo et al. 1996). Brood stock for 3CV was a Delaware Bay strain naturally selected against *P. marinus* and *H. nelsoni* in Delaware Bay. Triploidy in *C. virginica* was chemically induced by treatment of fertilized eggs with

TABLE 1.  
Oyster groups used.

Species	Group Code	Hatchery	Date Spawned	Size in May 1997 <sup>a</sup>
<i>C. gigas</i>	3CG	HSRL	16 July 96	19.2 mm
<i>C. virginica</i>	3CV	HSRL	11 June 96	31.7 mm

Key to group codes: 3 = triploid. CG = *C. gigas*, CV = *C. virginica*.  
<sup>a</sup> Mean shell height at the time of deployment.

TABLE 2.

Percentage market size (>76.2 mm) oysters in May 1998, based on the legal size for wild harvested oysters in Virginia.

Salinity Regime	Oyster Group	
	3CV	3CG
Low	14% (38/268)	0% (0/69)
Medium	41% (65/159)	11% (10/91)
High	52% (131/252)	100% (260/261)

Oyster group codes described in Table 1. In parenthesis, number of market size oysters/total number of live oysters.

cytochalasin-B using the methods described by Downing and Allen (1987) and Allen et al. (1989).

#### Experimental Design

Until field deployment in May 1997, juvenile 3CG were maintained first in flow-through tanks with ambient Delaware Bay water and quarantined effluents at HSRL Cape Shore, NJ, and then with York River ambient water and quarantined effluents at VIMS Gloucester Point, VA. Juvenile 3CV were also maintained first at HSRL Cape Shore, NJ, and then at Gloucester Point, VA in flow-through tanks without quarantined effluents. Between 28 April and 16 May 1997, oysters were dispensed into triplicate 3.2-mm mesh bags and placed within individual floating trays at selected sites as described below. There were 200 oysters per bag and 600 oysters per floating tray. Floating trays (2.3 m × 0.5 m × 0.3 m) were constructed by fitting wire mesh trays (25-mm square 16-gauge mesh) into floating frames built with 4-inch (10.16 cm) PVC pipe, following the design of Luckenbach and Taylor (1997). Floating trays were cleaned of fouling organisms at least once a month during regular site visits and more often if necessary. All sites were visited monthly ( $\pm 10$  days). As oysters grew, they were transferred from 3.2-mm mesh bags to 9.5-mm mesh bags in July 1997. In March 1998, when 3CG at high salinity sites approached space limitation within bags, all oyster groups at high salinity sites were split by placing half of the oysters into new bags. Oysters in the new bags were placed in a float adjacent to the original one.

A full factorial design, with three replicate sites within each of the three salinity regimes, was employed to examine the effects of triploid *C. virginica* and *C. gigas* (species), salinity regime, and time on final cumulative mortality, final condition index, prevalence and weighted prevalence of *P. marinus*, and weighted prevalence of *Polydora* spp. Differences in mean variables, between species within salinity regime, between salinity regimes within species, and between times where appropriate, were further examined by Newman-Keuls test (Zar 1974). Data were examined for compliance with analysis of variance (ANOVA) assumptions using Bartlett chi-square test for homogeneity of variance and plots of means versus standard deviations. Arcsine and logarithmic transformations were used where appropriate (Zar 1974).

#### Mortality, Growth, and Condition

All live and dead oysters within each float were counted monthly to determine survival. Monthly mortality for each oyster group was calculated as the number of oysters that died during each month interval divided by the number of live oysters at the beginning of the interval, corrected for oysters removed by sam-

pling. Cumulative mortality of each oyster group was calculated as the sum of interval mortality (Barber and Mann 1994, Krebs 1972).

To follow growth, 100 oysters within each float were individually labeled, and shell height was repeatedly measured to the nearest 0.1 mm, using calipers, once monthly, except January and February 1998. Mean monthly growth rates for individual oysters were calculated as the over-all shell height increment divided by the deployment time in days standardized for 30 days. To provide a measure of production potential, the proportion of individually labeled oysters that attained Virginia legal market size for wild stocks (3 in = 76.2 mm), within each salinity regime, was calculated at the end of the experiment.

Whole weight, shell weight, and tissue wet and dry weights were measured on the same oysters ( $n = 25$ ) collected for disease diagnoses in October 1997 and May 1998. Following Lawrence and Scott (1982), condition index (CI) was calculated, by the formula:

$$CI = \text{tissue dry weight}/(\text{total weight} - \text{shell weight}). \quad (1)$$

Oysters were allowed to air dry for 15–20 min before weighing, and whole oyster weight was recorded to the nearest 0.01g. Oysters were then shucked, shells weighed to the nearest 0.01g, and wet tissues were gently rolled on a paper towel and weighed on pre-tared vessels to the nearest 0.001g. Wet tissues were dried at 80 °C overnight, and tissue dry weight was measured the next day to the nearest 0.001g.

#### Diseases and Polydora

A baseline sample ( $n = 25$ ) was taken to assess the disease status of oyster groups before deployment in the spring of 1997. Subsequent disease samples ( $n = 25$ ) were collected, depending upon group and site, during the summer and fall of 1997 and the spring of 1998. *Perkinsus marinus* was diagnosed using Ray's fluid Thioglycollate medium (RFTM) assays (Ray 1952) on combined mantle, gill, and rectum tissue. Infection intensity was rated based on Ray (1954) and Mackin (1962), and for the calculation of weighted prevalence, the following numerical values were assigned to intensity categories: (1) light; (3) moderate; and (5) heavy. Weighted prevalence was calculated by the formula.

$$\text{Weighted prevalence} = ((n_1 * 1) + (n_2 * 3) + (n_3 * 5))/N, \quad (2)$$

where  $n_i$  = number of cases rated as ( $i$ ),

$N$  = total number of oysters examined in the sample.

*Haplosporidium nelsoni* was diagnosed using standard paraffin histology procedures with oysters preserved in Davidson's AFA and 6- $\mu$ m tissue sections stained with Harris' hematoxylin and eosin (Burrison et al. 1988). Infection intensity was rated as light, moderate, and heavy based on Burrison et al. (1988). Histological sections were also used to document the presence of other parasites and to examine development of oyster gonads. All disease and histology analyses were performed by VIMS Shellfish Pathology Laboratory.

The spionid polychaetes *Polydora websteri* and *P. ligni* are commensal with bivalves, including oysters. These suspension-feeding worms do not feed on the oyster, but the mechanical irritation caused by their presence causes the oyster to lay down additional layers of conchiolin over the worm's tube in what are often termed mud-blisters. At sufficiently high levels of infestation, this can severely limit the growth of oysters and reduce their

TABLE 3.  
Percentage genetic mosaics among *C. gigas* by salinity regime and date.

Date/Salinity	Low	Medium	High	Row Total
2–10 June 97	0.0% (0/105)	0.0% (0/105)	0.0% (0/105)	0.0% (0/315)
30 June–9 July 97	0.0% (0/105)	2.8% (3/105)	0.0% (0/105)	0.9% (3/315)
28 July–5 August 97	4.7% (5/105)	0.9% (1/105)	0.0% (0/105)	1.9% (6/315)
6–15 April 98	5.0% (3/60)	8.3% (8/96)	4.8% (5/105)	6.1% (16/261)
4–7 May 98	6.1% (20/325)	1.7% (4/233)	2.5% (9/358)	3.6% (33/916)
Column total	4.0% (28/700)	2.5% (16/644)	1.8% (14/778)	2.7% (58/2122)

In parenthesis number of mosaics/number of oysters examined.

condition index. Examination for mud-blisters associated with *Polydora* spp. was conducted on the same oysters collected for disease diagnoses in October 1997 and May 1998. Worms were not identified to species, but *Polydora websteri* is the most common species affecting oysters in the northeast coast of the United States (Blake and Evans 1972, Wargo and Ford 1993). The internal surface of right valve shells was visually inspected and rated according to the presence and extent of mud-blisters. Examination was restricted to right valves as in Wargo and Ford (1993), who reported that infestations by *Polydora* spp. were equally found in right and left valves. Following the methods of Handley and Bergquist (1997), infestation was rated as: (0) no visible mud-blisters or any evidence of boring by *Polydora* spp.; (1) mud-blisters affecting less than 25% of the valve; (2) 25–50% of the valve affected; (3) 50–75% of the valve affected; or (4) more than 75% of the valve affected. Weighted prevalence was calculated by the following formula.

$$\text{Weighted prevalence} = \frac{((n_1 * 1) + (n_2 * 2) + (n_3 * 3) + (n_4 * 4))}{N}, \quad (3)$$

where  $n_i$  = number of cases rated as ( $i$ ).

$N$  = total number of oysters examined in the sample.

#### Reproductive Status and Ploidy

Before deployment, baseline samples of 3CV ( $n = 125$  larvae) and 3CG ( $n = 35$  juveniles) were taken to confirm ploidy status. During deployment, samples of 3CG ( $n = 35$ ) were collected, depending upon site, at the beginning of the month in June, July, and August 1997 and May 1998. Only *C. gigas* was examined for ploidy during deployment, but an equal number of *C. virginica* were concurrently collected from trays to standardize the number of oysters removed by sampling. Ploidy was determined by flow cytometry of gill biopsies from individually labeled oysters. When gill tissues were found to contain any diploid cell (a condition termed mosaic), a biopsy of the gonad was examined by flow cytometry, and the remaining gonad tissue was processed by histology. Ploidy assays were conducted at HSRL and the VIMS Aquaculture Genetics and Breeding Technology Center.

## RESULTS

#### Environmental Parameters

Salinity was within the range established for low, medium, and high salinity sites for most of the monthly measures (Fig. 2). Low salinity sites experienced relatively high mean salinity ( $>15$ ‰) during September, October, and November because of drought conditions during the summer and relatively low mean salinity

( $<10$ ‰) during March, April, and May because of high rainfall during the winter. The Coan River site experienced extreme low salinity with mean daily values of 3‰ during April and May. Medium salinity sites experienced relatively low salinity ( $<15$ ‰) during March, April, and May (Fig. 2).

Temperature followed similar seasonal trends at all sites with a maximum of 27–29 °C in July and a minimum of 3–6 °C in March. High salinity sites experienced over-all cooler temperature with monthly means 2–4 °C lower than medium or low salinity sites (Fig. 2).

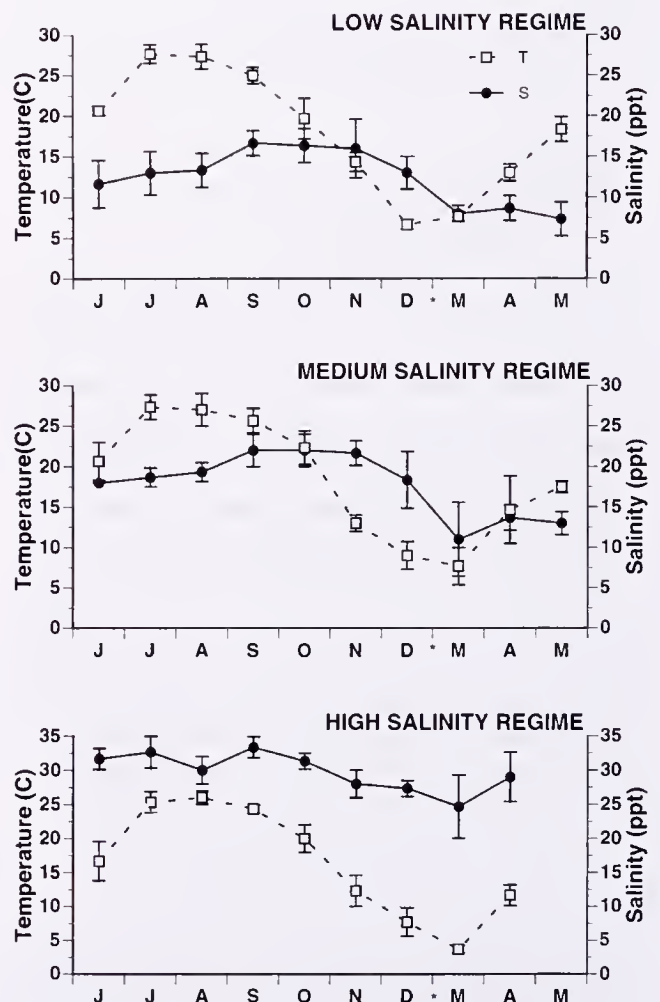


Figure 2. Mean monthly ( $\pm$  SD) temperature and salinity of three sites within low, medium, and high salinity regimes, using stem thermometer. \* Break in monthly sampling

Turbidity, measured in Nephelometric Turbidity Units (NTU), was highest at the medium salinity Nandua Creek site and Woodas Creek site. Maximum daily mean turbidity at Nandua Creek and Woodas Creek was, respectively, 436 NTU and 149 NTU, and maximum daily mean values at other sites was <38 NTU.

**Mortality**

Species, salinity regime, and their interaction had significant ( $P < .05$ ) effects on cumulative mortality. At low salinity sites, mean monthly mortality of 3CV was very low (<3%) at all times, and that of 3CG peaked at 28% in April 1998 (Fig. 3). By May 1998, mean cumulative mortality of 3CV (10%) was significantly ( $P < .05$ ) lower than that of 3CG (63%). At medium salinity sites, mean monthly mortality reached 17% for 3CV and 22% for 3CG in October 1997 (Fig. 3). By May 1998, mean cumulative mortality of 3CV (35%) was not significantly ( $P > .05$ ) different than that of 3CG (53%). High variability in mortality, for both species, among medium salinity sites was attributable to extremely high mortality at Nandua Creek. At high salinity sites, mean monthly mortality

was very low (<3%) for both species at all times (Fig. 3). In May 1998, mean cumulative mortality of 3CV (11%) was not significantly ( $P > .05$ ) different from that of 3CG (4%). Within 3CV, there were no significant ( $P > .05$ ) differences in mean cumulative mortality among salinity regimes. Within *C. gigas*, oysters at low and medium salinity experienced significantly ( $P < .05$ ) higher mortality than those at high salinity, and no significant ( $P > .05$ ) difference was detected between oysters at low and medium salinity.

**Growth**

At the initiation of the experiment, mean size of 3CV and 3CG was, respectively, 31.7 mm and 19.2 mm; subsequent growth varied with salinity regime (Table 2). At low salinity, 3CV increased its initial size advantage over 3CG, resulting in a mean shell height of 67.8 mm for 3CV and 41.1 mm for 3CG at the end of the study (Fig. 4). At medium salinity, the size differential between species was maintained throughout the study yielding a final mean shell height of 74.1 mm for 3CV and 65.1 mm for 3CG (Fig. 4). At high salinity, the initially smaller 3CG reached the same size as 3CV 3 mo after deployment, in July 1997, and continued to grow during fall and winter attaining a final mean shell height of 108.1 mm in

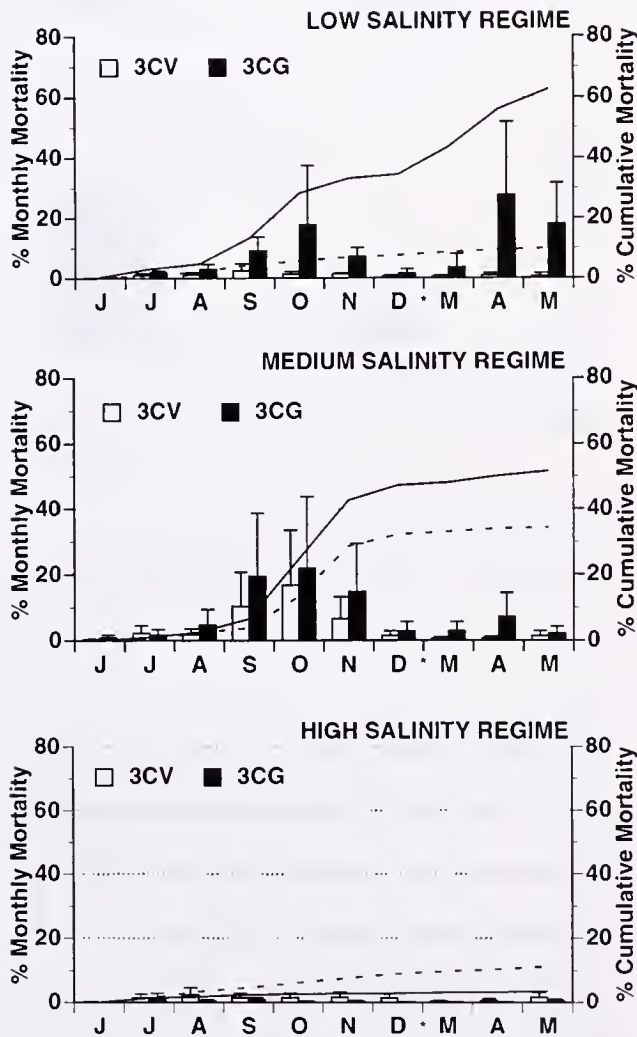


Figure 3. Monthly and cumulative mortality of triploid *C. virginica* (3CV) and triploid *C. gigas* (3CG) from June 1997 through May 1998. Bars = mean (+ SD) monthly mortality of three sites within salinity regimes. Dashed lines = mean cumulative mortality of 3CV. Solid lines = mean cumulative mortality of 3CG. \* Break in monthly sampling.

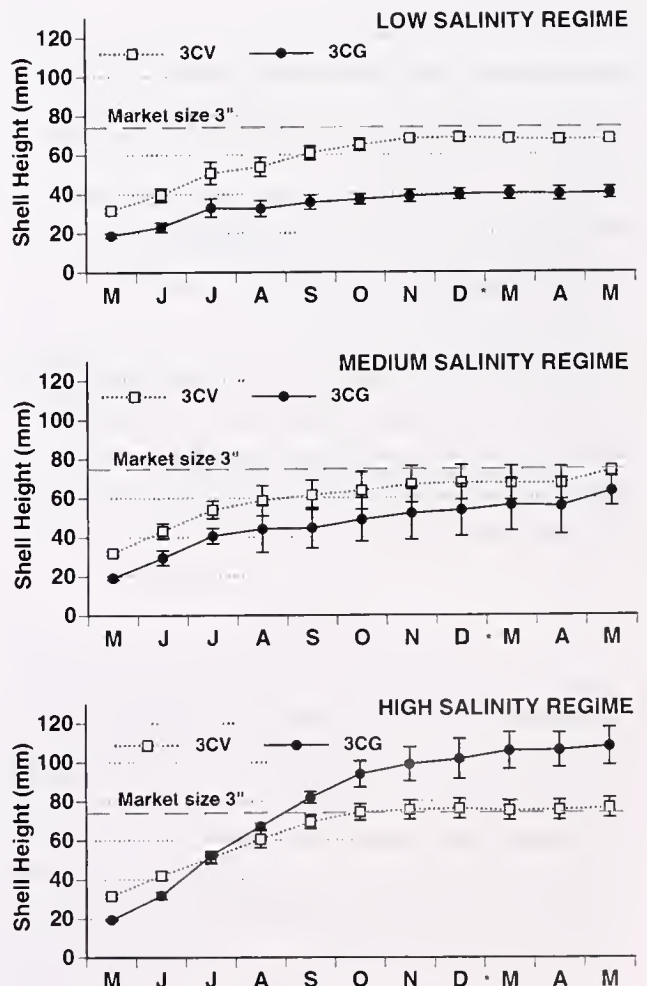


Figure 4. Monthly shell height of triploid *C. virginica* (3CV) and triploid *C. gigas* (3CG) from May 1997 to May 1998. Mean ( $\pm$  SD) of three sites within salinity regimes.

May 1998. By comparison, *C. virginica* stopped growing after October 1997 and reached 78.4 mm in May 1998 (Fig 4). Species, salinity regime, and their interactions had significant ( $P < .05$ ) effects on mean growth rate. At low salinity sites, mean overall growth rate of 3CV ( $2.9 \text{ mm mo}^{-1}$ ) was significantly ( $P < .05$ ) greater than that of 3CG ( $1.6 \text{ mm mo}^{-1}$ ), with most of the growth in *C. virginica* occurring between July and October (Fig. 4). At medium salinity sites, mean over-all growth rate for both species ( $3.0 \text{ mm mo}^{-1}$ ) was not significantly ( $P > .05$ ) different, and the monthly pattern of growth was similar. At high salinity sites, mean over-all growth rate of 3CV ( $3.6 \text{ mm mo}^{-1}$ ) was significantly ( $P < .05$ ) lower and nearly half that of 3CG ( $7.1 \text{ mm mo}^{-1}$ ). Within 3CV, growth rate did not significantly ( $P < .05$ ) differ between salinity regimes. Within *C. gigas*, growth rate at high salinity was significantly ( $P < .05$ ) higher than that at medium and low salinity regimes, and growth rate did not significantly ( $P > .05$ ) differ between medium and low salinity regimes.

#### Condition Index

Salinity regime, time, and the interactions of salinity and species and salinity and time had significant ( $P < .0005$ ) effects on final oyster condition. In October 1997, there were no significant ( $P > .05$ ) differences in condition index between species within any salinity, or between salinities within a species (Fig. 5). In May 1998, at low salinity, mean condition index of 3CV (16.2%) was significantly ( $P < .05$ ) higher than that of 3CG (8.7%); at other salinities, no significant ( $P > .05$ ) differences were detected between species. Within species, condition index increased significantly ( $P < .05$ ) with salinity, except for *C. gigas* between medium and high salinity in May 1998. For both species within any salinity, except for *C. gigas* within low salinity, condition index increased with time. Mean condition indices for oysters at Nandua Creek and Woodas Creek were lower than those of oysters at the third medium salinity site (York River).

Relative to whole oyster weight, shells of *C. virginica* were heavier than shells of *C. gigas*. For all samples combined, the percentage of shell weight relative to whole weight was 66% in 3CV and 57% in 3CG. Proportional shell weight remained fairly constant for 3CV at low, medium, and high salinity, between October 1997 and May 1998, while it decreased in 3CG at low and medium salinity and increased in 3CG at high salinity.

#### Disease

Species, salinity regime, time, and the interaction of species and time had significant ( $P < .05$ ) effects on prevalence and weighted prevalence of *P. marinus* infections. Higher prevalence and intensity of infections were observed in *C. virginica* and occurred at medium salinity during fall as compared to *C. gigas* and to other salinity regimes and times (Fig. 6). Infections in *C. virginica* were low in prevalence and intensity during the first spring and summer of deployment and subsequently increased in the fall (Fig. 6). Infections in *C. gigas* were generally of low magnitude at most sites and times; however, infections at the Nandua Creek site in fall reached 67% prevalence with two heavy intensity infections. Maximum mean weighted prevalence for *C. gigas* (0.4) was significantly ( $P < .05$ ) lower than that for *C. virginica* (1.4). At medium salinity sites, infections remained high in *C. virginica* during spring 1998 (prevalence >62%, weighted prevalence = 0.9), whereas, at low and high salinity sites, infections subsided in

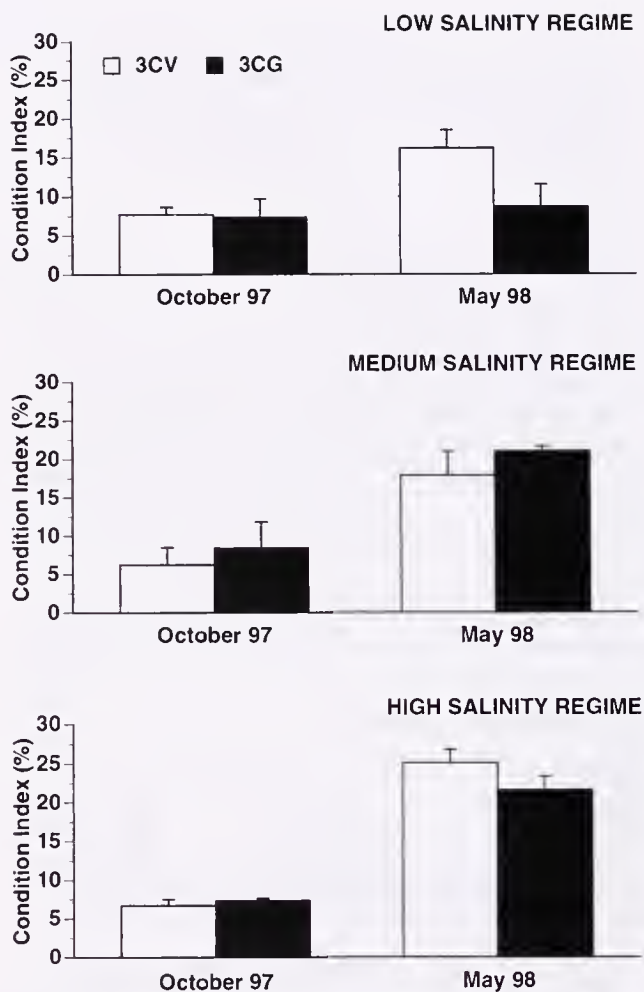


Figure 5. Condition index in triploid *C. virginica* (3CV) and triploid *C. gigas* (3CG). Mean (+ SD) of three sites within salinity regimes.

spring 1998 (mean prevalence < 23%, mean weighted prevalence = 0.1–0.3) (Fig 6).

*Haplosporidium nelsoni* was absent in *C. gigas* but was present at low prevalence (< 16%) in 3CV at medium and high salinity sites. At low salinity, no infections were detected in any of the samples.

#### Polydora

Mean prevalence of infestations by *Polydora* spp. was high (>95%) for 3CV and 3CG at low and medium salinity sites regardless of time. At high salinity sites, however, although mean prevalence for 3CV remained at 64%, it decreased for *C. gigas* from 52% in October 1997 to 12% in May 1998. Differences in weighted prevalence between oyster species were more pronounced than differences in prevalence.

Species, salinity regime, time, and the interaction of salinity regime and species had significant ( $P < .0005$ ) effects on mean weighted prevalence. Triploid *C. virginica* had significantly ( $P < .05$ ) lower weighted prevalence than *C. gigas* at medium and low salinity sites in October and similar levels of *Polydora* spp. infestation at all other times and locations (Fig. 7). For 3CV, within any salinity, mean weighted prevalence was not significantly ( $P > .05$ ) different between October and May, whereas, for 3CG at low and



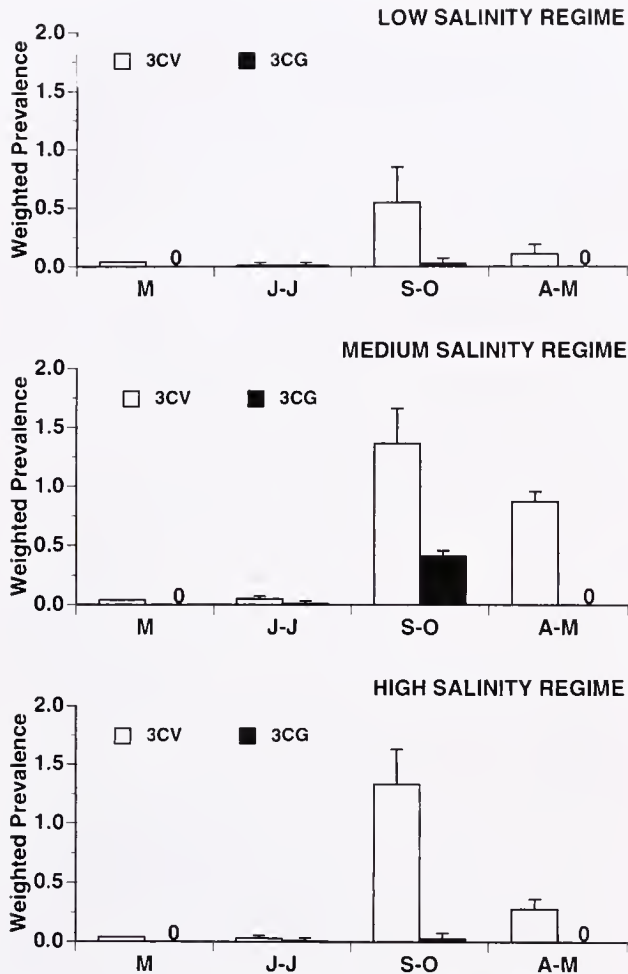


Figure 6. Intensity of *P. marinus* in triploid *C. virginica* (3CV) and *C. gigas* (3CG) from April 1997 through May 1998. Mean (+ SD) of three sites salinity regimes.

medium salinity, mean weighted prevalence significantly ( $P < .05$ ) decreased from October to May. Within 3CG, at high salinity, mean weighted prevalence was not significantly ( $P > .05$ ) different between October and May.

#### Ploidy

Baseline samples confirmed 100% triploidy among naturally induced triploid *C. gigas* and revealed 85% triploidy among chemically induced triploid *C. virginica*. The proportion of *C. gigas* gill samples in which combinations of diploid and triploid cells (mosaics) were detected by flow-cytometry varied with time and salinity (Table 3). The proportion of mosaics, pooled for all salinity regimes, increased from 0.0% in June 1997 to 6.1% in April 1998, and then decreased to 3.6% in May 1998. The proportion of mosaics, pooled for all times within low, medium, and high salinity, was respectively, 4.0%, 2.5%, and 1.8%. For all samples collected during the study combined, regardless of salinity, the over-all proportion of mosaics was 2.7%.

Examination of 23 oysters with mosaic gill cells revealed that 5 were females, 15 were males, and 3 were undifferentiated. Among oysters with mosaic gill cells, there was one individual in which haploid cells were detected in a gonad biopsy (a male collected in Bogue Bay on 14 April 1998). Concerns over the po-

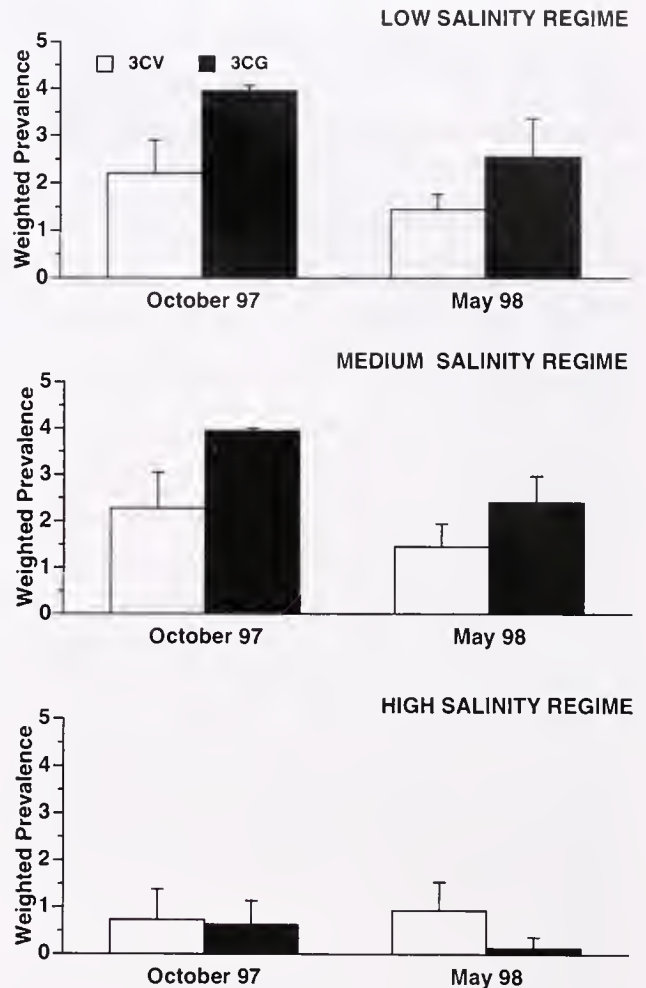


Figure 7. Intensity of *Polydora* spp. infestations in triploid *C. virginica* (3CV) and triploid *C. gigas* (3CG). Mean (+ SD) of three sites within salinity regimes.

tential reproduction of *C. gigas* following the finding of an individual oyster with potentially haploid gametes, resulted in termination of the experiment. By 6 May 1998, all *C. gigas* were removed from the water and maintained in quarantine conditions at VIMS.

#### DISCUSSION

This study demonstrated that the comparative performance of *C. virginica* and *C. gigas* in the Chesapeake Bay and the Atlantic Coast of Virginia varied with salinity regime. At low salinity, survival, growth rate, final condition index, and resistance to infestations by *Polydora* spp. were significantly greater for *C. virginica* than for *C. gigas*. However, *C. virginica* was more susceptible than *C. gigas* to *P. marinus* infections. High mortality (63%) and poor growth ( $1.6 \text{ mm mo}^{-1}$ ) observed for *C. gigas* at low salinity sites were not surprising considering the previously reported optimal salinity of 35‰ for growth in this species (Mann et al. 1991). High mortality of *C. gigas* at the low salinity Coan River site in April (56%) can probably be attributed to a prolonged period of extreme low mean daily salinity (3‰ for 1 month). Most of the growth for *C. virginica* and *C. gigas* occurred in the spring subsequent to deployment.

At low and medium salinity, shells of *C. gigas* with severe *Polydora* spp. infestations were very fragile and often disintegrated during monthly inspections of labeled individuals for growth. The decrease in the severity of *Polydora* spp. infestations between October 1997 and May 1998, primarily for medium and high salinity sites, can be attributed to oyster shell repair. In May 1998 nacre shell deposits were often observed to cover blisters. Comparing shell weight for oysters of similar size, Barber and Mann (1994) found that shell weight was significantly ( $P < .05$ ) greater for similar sized *C. virginica* than *C. gigas*. Similarly, in the present study, *C. virginica* had heavier shells proportional to whole oyster weight relative to *C. gigas*. It is possible that the relatively thinner shells of *C. gigas* made it more susceptible to heavy *Polydora* spp. infestations.

At medium salinity sites, mean cumulative mortality, growth rate, and final condition index of *C. virginica* were not significantly different than that of *C. gigas*. *Crassostrea gigas* was more susceptible to infestations by *Polydora* spp. and less susceptible to *P. marinus* than *C. virginica* in this salinity regime. Both *C. virginica* and *C. gigas* experienced a high variability in mortality and growth rate because of extremely poor performance at Nandua Creek, relative to the other two medium salinity sites. High mortality and poor condition of *C. virginica* and *C. gigas* at Nandua Creek can be attributed to prevalent and severe *P. marinus* infections. Oysters at Nandua Creek, and to a large extent at Woodas Creek, experienced the most prevalent and severe *P. marinus* infections recorded in this study. We speculate that high density of other oyster lots present in the immediate vicinity of the experimental oysters, coupled with relatively poor water exchange and high turbidity, resulted in high disease pressure and environmental stress at those sites.

Barber and Mann (1994) reported greater growth rates for *C. gigas* than *C. virginica* at the York River site, although this study did not find significant differences in growth of the two species at the site. This incongruity may arise from different environmental conditions at the site between years or from differences in the timing of spawns and handling of oysters between the studies. Furthermore, the experiment of Barber and Mann (1994) involved exposing diploid oysters to unfiltered York River water in quarantined tanks, while our study was conducted *in situ* with triploid oysters deployed within mesh cages.

Growth rate of *C. gigas* at high salinity in the present study was higher than that reported in other studies for high salinity environments. In a study of *C. gigas* growth at Seto Inland Sea in southern Japan where temperature ranged from 8–30 °C (Kobayashi et al. 1997), oyster shell height increased from 27.0 to 93.1 mm between May 1990 and January 1991. Studies with *C. gigas* in Canada and Korea reviewed by Kobayashi et al. (1997), reported similar growth rates. By comparison at high salinity sites in the present study, where temperature ranged from 4–27 °C, shell height of *C. gigas* increased from 19.2 to 101.6 mm between May and December 1997. Higher growth rates of *C. gigas* in the present study may be attributed to the use of triploid oysters; whereas, diploid oysters were used in the other studies cited above. In general, because gametogenesis is restricted in triploid oysters, more energy is available for somatic growth. Allen and Downing (1986) and Davis (1989) indicated that increased growth in triploid *C. gigas* mostly occurred during the normal reproductive season. Additional factors that would explain the difference in growth

among *C. gigas* between studies may include different environmental conditions among study areas and times.

In summary, during the course of the study *C. gigas* performed no better than *C. virginica* at low and medium salinity sites in the Chesapeake Bay. However, considering the large variability in performance between the two oyster species among medium salinity sites and given the wide temporal salinity fluctuations in the Chesapeake Bay, caution should be exercised in extrapolating performance of *C. gigas* at these sites over longer periods of time. In contrast, performance of *C. gigas* at high salinity sites in the Atlantic Coast of Virginia was clearly superior to that of *C. virginica*.

The results of this study, however, are not sufficient to conclude that *C. gigas* is or is not an appropriate species for introduction or use in these environments. Before reaching a decision concerning introduction of exotic species, ICES, as well as the European Inland Fisheries Advisory Commission (EIFAC) and the American Fisheries Society (AFS), have recommended that appropriate authorities, including fishery managers, examine the candidate species to: (1) assess the justification for the introduction; (2) assess its relationship with other members of the ecosystem and the possibility of introducing associated pathogens and parasites; and (3) examine the probable effects including a prediction of the range for the establishment of the species (Turner 1988). Use of reproductively capable diploid *C. gigas* would likely result in its introduction into some regions within the waters of Virginia and neighboring states. An important determinant of the extent to which this species might spread if introduced is the interactive effects of temperature and salinity on reproduction and larval development. Based on the review by Mann et al. (1991) and other reports indicating that optimal temperature and salinity ranges for *C. gigas* larvae are, respectively, 18–35 °C and 19–35‰, Gottlieb and Schweighofer (1996) postulated that, if introduced, *C. gigas* would likely reproduce and establish resident populations in the lower portion of the Chesapeake Bay. Spreading would likely occur, via larval dispersal, into other areas of the Mid-Atlantic coast of North America. Interactions with other species—such as competitive interactions with *C. virginica* and predator–prey interactions may further influence the possible range extension. Additional investigations into environmental constraints on reproduction, competitive interactions with native species and predator–prey dynamics would enhance our predictive capability to determine the potential range for establishment of *C. gigas* in habitats in the Mid-Atlantic region.

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## MODELING THE MSX PARASITE IN EASTERN OYSTER (*CRASSOSTREA VIRGINICA*) POPULATIONS. I. MODEL DEVELOPMENT, IMPLEMENTATION, AND VERIFICATION

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**ABSTRACT** A mathematical model simulating the host-parasite-environmental interactions of eastern oysters (*Crassostrea virginica*) and the pathogen, *Haplosporidium nelsoni*, which causes MSX disease, has been developed. The model has 2 components. One replicates the infection process within the oyster and the other simulates transmission. The infection-development component relies on basic physiological processes of both host and parasite, modified by the environment, to reproduce the observed annual prevalence cycle of *H. nelsoni*. Equations describing these rates were constructed using data from long-term field observations, and field and laboratory experiments. In the model, salinity and temperature have direct effects upon *in vivo* parasite survival and proliferation as well as on transmission rates. Cold winters depress transmission rates for 1 or 2 years after the event, even if temperatures return to normal. Warm winters have no effect on transmission in subsequent years. Hemocyte activity, parasite density, and the overall environmental quality provided to the parasite by the host also influence the modeled infection process. Hemocytes scavenge and eliminate parasites that die over the winter or that degenerate as a result of failed sporulation. Replication rates of *H. nelsoni* are slowed at high parasite densities. The environmental quality provided by the host, which is a function of oyster food availability and the oyster's potential growth efficiency, affects doubling times and also determines whether the parasite completes its life cycle by forming spores. Spore production is related to a threshold environmental quality, which occurs only in small oysters because of their high growth efficiency. Simulations that use environmental conditions characteristic of Delaware Bay reproduce the observed seasonal *H. nelsoni* cycle, consequent oyster mortality, and spore production in juvenile oysters. The oyster-*H. nelsoni* model provides a quantitative framework for guiding future laboratory and field studies as well as management efforts.

**KEY WORDS:** *Haplosporidium nelsoni*, numerical modeling, MSX disease, marine pathogen, host-parasite environment

### INTRODUCTION

Among the most important influences on population dynamics of eastern oysters, *Crassostrea virginica*, in the United States over the past half century has been disease. Two major diseases, both caused by water-borne protistan parasites, have severely diminished the abundance of natural oyster populations, particularly in the middle Atlantic states (Ford and Tripp 1996). The first to be recognized was Dermo disease, caused by *Perkinsus marinus*. Although it was discovered in the late 1940s in the Gulf of Mexico, it had probably been present throughout the southeastern United States and Gulf of Mexico for many decades (Ray 1996). Between its discovery and 1990, Dermo disease was prevalent only in waters south of Delaware Bay; since then however, epizootic outbreaks have been recorded as far north as Massachusetts (Ford 1996). The second, MSX disease, is caused by *Haplosporidium nelsoni*, a parasite believed to have been introduced to the east coast of the United States, where it began causing epizootic mortalities in Delaware and Chesapeake Bays in the late 1950s and early 1960s. *H. nelsoni* is now present along the entire east coast, although its major impact has been from Virginia north to Maine (Ford and Tripp 1996, Barber et al. 1997).

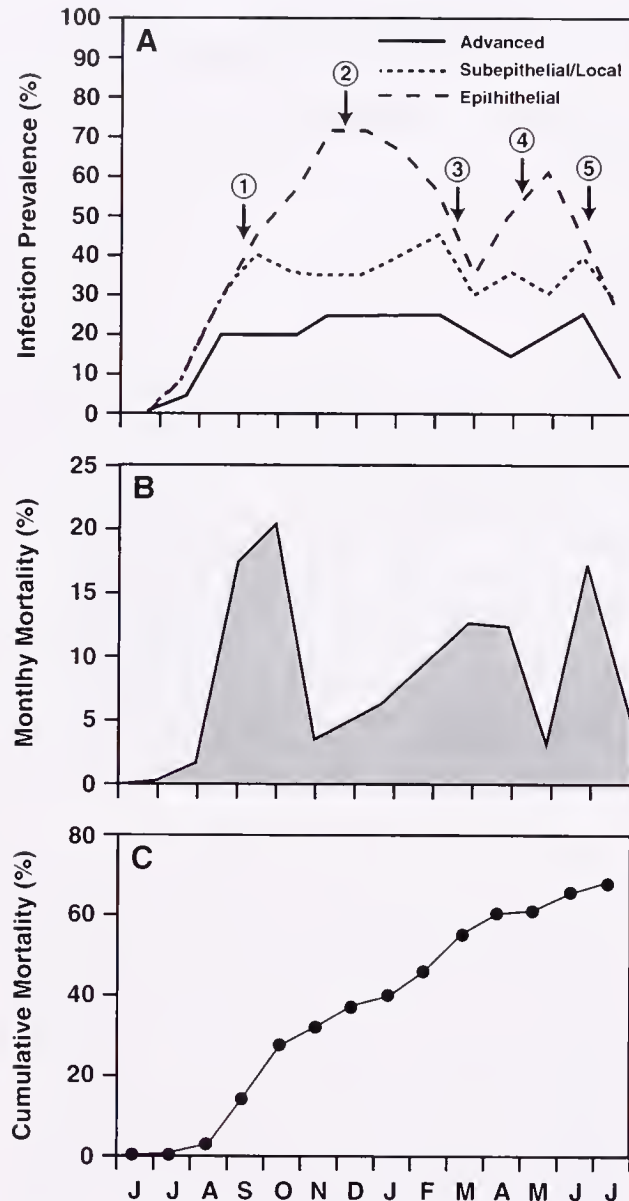
To synthesize available data and to investigate the factors influencing the interactions of host, parasite, and environment in Dermo disease, mathematical models for *P. marinus* and *C. virginica* were developed (Hofmann et al. 1992, Hofmann et al. 1994, Hofmann et al. 1995). The individual models were then coupled to examine the effects of temperature, salinity, total seston, and food availability on the integrated host-parasite system (Powell et al. 1994, Powell et al. 1996). Simulations indicated that temperature controls on both host and parasite growth rates, and food avail-

ability to the oyster, were the major elements influencing the interaction. High oyster reproduction and growth rates in southern latitudes allows populations to withstand Dermo disease pressure much better than in mid-latitudes, where both fecundity and growth rate are lower. Simulations also indicated that an important survival mechanism for the oyster is simply to increase body mass (i.e., growth) at a higher rate than the parasite can proliferate and thus to keep *P. marinus* densities from reaching lethal levels.

In many of the locations where *P. marinus* is present, *H. nelsoni* is also. Particularly in the mid-Atlantic states and along the northeastern coast, both parasites cause major, recurring epizootics. Therefore, to understand the effects of disease on oyster populations in this region, it is necessary to consider the actions and interactions of both parasites on hosts at the individual and population level. Both parasites display distinct seasonal and inter-annual cycles of infection onset, development, and impact on the host. These cycles are largely a function of environmental factors, primarily temperature and salinity, to which both parasites and the oyster are sensitive. However, the ability to tolerate environmental extremes, or to profit from favorable conditions, is specific to each species. A numerical model offers an effective way to synthesize the many data available for the parasites and their host in a mechanism for understanding the complex interactions among these organisms and their environment.

The objective of this paper is to describe a model developed for *Haplosporidium nelsoni* in oysters. Like that for *P. marinus*, it is a physiological model structured around proliferation and death rates of *H. nelsoni* under different environmental conditions. Equations describing these rates were constructed using data from long-term field observations, and field and laboratory experiments.

The *H. nelsoni* model is described in the following section. The



**Figure 1.** (A) Annual prevalence cycle for *Haplosporidium nelsoni* infections in eastern oysters in Delaware Bay, NJ, (first year of infection) showing relative contributions of epithelial (BFU = 1), subepithelial/local (BFU = 2), and systemic (BFU = 3 & 4) infections to the overall prevalence (see text for definitions). The arrows and numbers indicate different phases of the infection cycle as described in the text. (B) Monthly and (C) cumulative nonpredation mortality for oysters undergoing first year exposure in the same location. Adapted from Ford and Haskin (1982).

succeeding section presents a series of model outputs that illustrate its ability to simulate the seasonal cycle of *H. nelsoni* prevalence and intensity, and consequent oyster mortality, in a high-salinity enzootic area. The model described in the current paper is the basis for the studies presented in two subsequent papers: the effects of varying salinity on MSX disease development (Paraso et al. this volume) and a comparison of the disease in Delaware and Chesapeake Bays with a discussion of the transmission issue (Powell et al. this volume).

## METHODS AND MODEL DESCRIPTION

### Model Overview

#### Conceptual Framework

*Haplosporidium nelsoni* is classified in the phylum Haplosporidia (Corliss 1984, Perkins 1990). In the oyster, it occurs primarily as a multinucleated plasmodium (Ford and Tripp 1996). A second life form, the spore, is found rarely in adult oysters, but can be common in juveniles (R. D. Barber et al. 1991, Burreson 1994). The method of transmission is unknown and may involve another host (Burreson 1988, Haskin and Andrews 1988). To replicate the oyster-*H. nelsoni* interactions, the mathematical model was divided into two principal components. One simulates the infection process within the oyster, including the formation of spores. The second simulates the transmission process, which occurs outside, and independent of, the oyster (Powell et al. this volume).

Within the oyster, observed prevalence and intensity of *H. nelsoni* follows a defined seasonal pattern in all areas where it has been followed closely (Fig. 1a) (Andrews 1966, Farley 1975, Ford and Haskin 1982, Matthiessen et al. 1990). In the mid-Atlantic, infections are acquired from late May/early June through early fall. The earliest recognized stages are plasmodia confined to the gill epithelium. Once established in the epithelium, parasites proliferate, penetrate the basal lamina, and move into the circulatory system where they are carried to all tissues. Acquisition of new infections and *in vivo* parasite proliferation result in rising prevalence and intensity levels throughout the summer and fall (Fig. 1A, point 1), and result in host mortality during late summer and fall. High infection prevalence and intensity occur in the autumn and into the winter, when low water temperatures have slowed the activity of both host and parasite (Fig. 1A, point 2). In late winter and early spring, infection prevalence and intensity decrease, presumably from the degeneration of *H. nelsoni* plasmodia as well as from the deaths of heavily infected oysters (Fig. 1A, point 3). In early spring, infection prevalence and intensity again increase, coincident with rising water temperature, reaching a peak in late May or early June (Fig. 1A, point 4). This peak, which can be the most intense of the annual cycle, is often followed by a dramatic decrease in the number of infected oysters, again linked with the death of heavily infected oysters, but more so with the disappearance of parasites from live oysters (Fig. 1A, point 5). When sporulation occurs, it coincides with both the spring and the fall prevalence/intensity peaks. The *in vivo* component of the *H. nelsoni* model is designed to replicate the above pattern.

To simulate the *in vivo* relationship, the model relies on basic physiological processes of host and parasite to reproduce the complex, bimodal, annual prevalence cycle observed in nature (Andrews 1966, Ford and Haskin 1982). Parasite proliferation, stage transition, and death rates, which are modified by environmental variables both external and internal to the host, form the basis of the model. Salinity and temperature have direct effects upon *in vivo* parasite survival and proliferation (Paraso et al. this volume). They also have both local and regional effects upon transmission. Hemocyte activity, parasite density, and the overall environmental quality provided by the host are additional factors that influence the parasite. The last affects not only parasite doubling times, but whether or not *H. nelsoni* completes its life cycle by producing spores. The environmental quality experienced by the parasite de-

TABLE 1.

Relationship of *Haplosporidium nelsoni* infection categories (see Ford and Haskin, 1982) to range and mean abundance of parasites, expressed in parasites (unit area)<sup>-1</sup>, in tissue selections of infected oysters. Counts were made from oysters with Little Ford Unit (LFU) ratings of 1 to 6 in either gill tissue, visceral mass tissue, or both. In each location, parasites were counted in a total area equalling 64,000  $\mu\text{m}^2$ . The intensity ratings are indicated as Rare (R), Very Light (VL), Light (L), Moderate (M), and Heavy (H). The correspondence between LFUs and Big Ford Units (BFU) is also shown.

INFECTION CATEGORIES			GILL				VISCERAL MASS					
Distribution	Intensity	BFU	LFU	Epithelial		Subepithelial		Epithelial		Subepithelial		
				Range	Mean	Range	Mean	LFU	Range	Mean	Range	Mean
Epithelial	R, VL, L	I	1	0.1–5.4	0.9	0	0	1	0	0	0	0
Subepith/Local	VL, L	II	2	0.5–3.9	1.4	0.1–1.1	0.5	2	0	0	0–0.2	0.1
Systemic	R, VL	III	3	0–1.2	0.3	0.2–0.5	0.3	3	0	0	0.1–0.6	0.4
Systemic	L	III	4	0.2–0.4	0.2	0.2–2.1	1.1	4	0–0.1	0.1	0.5–2.9	1.7
Systemic	M	IV	5	0.4–13.9	5.4	3.0–18.9	8.2	5	0–1.2	0.2	0.6–7.4	3.9
Systemic	H	IV	6	0.3–9.9	3.2	11.9–36.5	20.8	6	0–1.6	0.5	6.8–28.5	17.5

pend, in turn, upon the quantity of food available to the oyster and its potential growth efficiency. Finally, the model simulates deaths of oysters as a consequence of parasitism.

The transmission component of the model is discussed fully by Powell et al. (this volume). Unlike most disease transmission models, including that for *P. marinus*, it does not rely on the density of nearby oysters as a measure of infective parasite concentration. In fact, there is no direct link between spore formation and transmission in the model. Although spores are assumed to be an important element in the life cycle of *H. nelsoni*, it is not known if they are directly infective to other oysters. The infective stage is unknown, but histological observations of infected oysters suggest it is waterborne (Farley 1968, Ford and Haskin 1982). In the model, the relative abundance of these particles is influenced by salinity, on both local and estuary-wide scales, and long-term temperature fluctuations. The infection rate is a function of the abundance of infective particles and the filtration rate of oysters.

This paper focuses on the *in vivo* model, which was constructed by applying rate functions developed from experimental and field data to an overall governing equation that controls the movement of oysters among infection classes according to the parasite load that they have at any time during a simulation. At each step in the construction of this model, output was compared with actual data and modifications implemented, if needed, to fit the model to field observations. To model the cycle in the absence of complete data on host-parasite interactions, and especially transmission, certain assumptions had to be made. The background and biological basis for these assumptions are stated briefly, along with the particular mathematical relationship, and are considered more fully in the Discussion.

#### Model Units

The first step in developing the *H. nelsoni* model was to define the units that provide the basic reference frame and that allow the model calculations and output to be consistent with measurements and to be compared with observations. The majority of the observations on MSX disease are made by tissue-section histology and make use of scales that categorize *H. nelsoni* infection level according to parasite distribution (local or diffuse) and abundance in the oyster tissue. The scale reflects disease progression in the

oyster as infections move from initial light lesions in the gill epithelium to heavy systemic (whole body) infections.

The infection rating system used for the model is based on one developed for studies in Delaware Bay (Ford and Haskin 1982). This semi-quantitative scale involves 3 levels of distribution in the tissue (epithelial, subepithelial/local, and systemic) and 5 levels of abundance (see Ford and Haskin 1982 for details), resulting in a scale of 0 to 15 when the location and intensity for each oyster are multiplied. For reporting and statistical purposes, however, these 15 categories are reduced to 6 or 4, depending on need (Table 1). Similar ratings systems are used in Chesapeake Bay and elsewhere (Farley 1968, Y. Bobo, pers. comm., E. Burreson, pers. comm., R. Smolowitz, pers. comm.).

In contrast to the rating systems in which most observations are reported, the oyster-*H. nelsoni* model is based on the number of parasites per oyster. It was therefore necessary to establish, at the outset, a relationship between parasites per oyster and the semi-quantitative scale. The 0–6 point scale (referred to as Little Ford Units [LFU]) was used as the basis for this relationship because it provided more precision than the 0–4 point scale. The 0–4 point scale was chosen as the final output from the model, however, because it is the simplest, because it can readily be compared with previous publications, and because it is most easily comparable to systems employed by other researchers. These units are referred to as Big Ford Units (BFU) (Table 1). Conversion between the scales simply involves combining the 4 highest LFUs into 2 BFUs for observational use (Table 1); however, the identical mathematical treatment results in a more complex conversion formula. A complete presentation of the conversion system is given below.

#### Conversion of Infection Categories to Parasite Density

The conversion of the LFU rating system into parasites per oyster was made by selecting archived slides with tissue sections in each of the categories (total  $n = 50$ , approximately equally distributed among the 6 categories of infected oyster). Each slide was then re-analyzed using a gridded ocular. All parasites were counted in 40 ( $40 \mu\text{m} \times 40 \mu\text{m}$ ) grids, 20 placed randomly over gill tissue and 20 over the remaining visceral mass. Resulting counts showed that the mean number of parasites per grid in each LFU category was similar for both the gill and the visceral mass (Table 1). The resulting empirical relationship between *H. nelsoni*

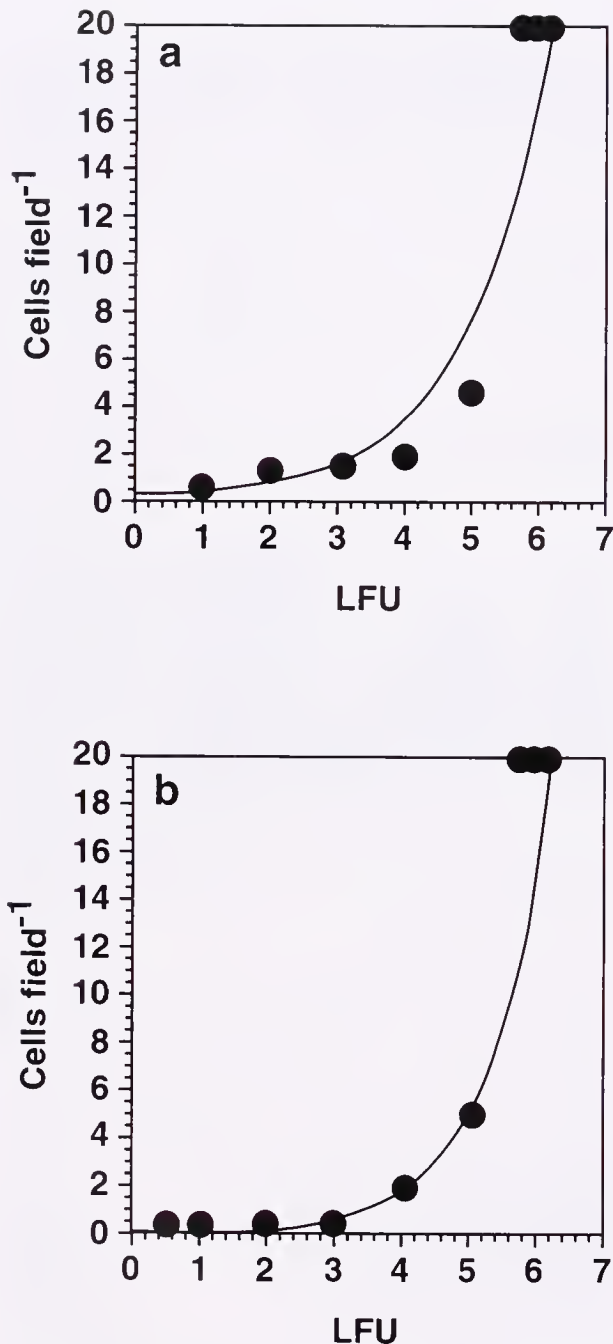


Figure 2. *Haplosporidium nelsoni* plasmidia per 64,000 μm<sup>2</sup> tissue-section field versus infection intensity categories expressed as Little Ford Units (LFU) for a) epithelial and b) systemic tissues.

infection category and the number of parasites in the oyster was exponential, with a rapid increase in numbers per grid as infections became systemic (LFU 4–6) (Fig. 2a, b). The location of the *H. nelsoni* cells in either epithelial or systemic tissue is included in the final relationship, which is based on a logarithmic scale of the form:

$$LFU = a_{e/s} \ln \left( \frac{C_{e/s}}{bc_{e/s}W_0 \text{frac}_{e/s}} \right) \quad (1)$$

where  $a_{e/s}$  is a constant that differs for the epithelial ( $e$ ) and sys-

temic ( $s$ ) tissue (the notation  $e/s$  will be used to denote constants that have different values for epithelial and systemic tissue),  $C_{e/s}$  is the number of *H. nelsoni* cells in the epithelial or systemic tissue,  $b$  is a scaling in grids per gram wet weight (gwt<sup>-1</sup>) of oyster tissue, and  $c_{e/s}$  is a constant. The coefficients represent the total dry weight in grams (gdwt<sup>-1</sup>) of the oyster tissue ( $W_0$ ) and the fraction of epithelial or systemic tissue ( $\text{frac}_{e/s}$ ) in the animal. The values of the coefficients in equation (1) are given in Table 2.

The method described above for quantifying infection intensity introduced a bias when infections were confined to the epithelium, e.g., LFU 1 (Table 1), because gill epithelium comprised only about 20% of the tissue in each section. Thus, the values obtained in these instances were multiplied by a value of 5 so that the number of parasites per gram tissue was consistent with the values obtained for systemic infections. As a result, the constants  $a$  and  $c$  vary between the epithelial and systemic conversions and the number of parasites per gram in the epithelial tissue is higher at an equivalent infection intensity than in the systemic tissue (Fig. 2).

The constant  $b$  in equation (1) is a conversion from the number of grids counted in a microscope field to the biomass of the tissue counted. In essence, this yields the weight of fixed tissue per grid. The conversion is based on the area of a grid (40 μm × 40 μm), the number of grids counted (40), and the thickness of a tissue cross-section (6 μm). Included in the conversion is a factor of 0.5 to account for the expectation that, on average, an *H. nelsoni* plasmodium would be observed in 2 consecutive cross-sections. The calculation of  $b$  also assumes a 10% shrinkage in tissue volume during fixation, thus correcting from fixed to wet tissue weight.

The values for  $\text{frac}_{e/s}$  are obtained from weights of dissected oysters that show gill tissue to comprise about 20% of the total wet weight (Table 3). Half of this weight was estimated to be epithelium, based on point count stereology of tissue sections. The value for  $\text{frac}_{e/s}$  was therefore given a value of 0.1.

Rare or very light epithelial infections (LFU = 1) may be identified by as few as 1 or 2 parasites in the gill epithelium in a standard tissue cross-sectional analysis. With this method, however, it is likely that too few parasites are present in some oysters to be detected. Thus, some oysters diagnosed as having no infections (LFU = 0), are undoubtedly infected (Stokes et al. 1995). The model is constructed to reflect this circumstance. The distinction between an uninfected oyster (LFU = 0) and one in the very lightest infection category (LFU = 1) is based on a presumed detection limit and not on the absolute absence of infection. The detection limit, which differs for epithelial and systemic tissue, was obtained from the grid counts described above that were used to convert the infection scale to parasite densities. The lowest level of detection for the conversion counts was 1 *H. nelsoni* cell per 20 grids, with an average value of 0.05 parasite per grid. However, tissue sections are routinely completely scanned for *H. nelsoni* to obtain observed prevalence. Twenty grids (64 × 10<sup>3</sup> μm<sup>2</sup>) represented only an estimated 20% of the gill tissue and 10% of the visceral mass tissue present in a typical section. Therefore, the true detection limit of 1 parasite in either the gill or visceral mass after a complete search of the section would be 1 in 100 grids (= 0.01 grid<sup>-1</sup>) and 1 in 200 grids (= 0.005 grid<sup>-1</sup>), respectively. This translates into 1.3 × 10<sup>4</sup> and 6.5 × 10<sup>3</sup> parasites per gram wet weight for gill and visceral mass tissue, respectively.

#### Model Equations

The model is structured as a two-dimensional array (Figs. 3, 4) with 55 epithelial and 55 systemic infection categories. The infec-



tion level in each category is defined by the average number of *H. nelsoni* in it, with the maximum difference between adjacent classes being 1 population doubling. The difference between infection classes at the higher parasite densities is less than 1 population doubling, because of the nonlinear distribution of LFUs with respect to parasite number (Fig. 2). The nonlinear arrangement was required to provide multiple infection classes within each LFU infection category and, consequently, necessitated scaling the transfers between infection categories by the ratio of the parasite cell number (*C*) between adjacent classes as:

- for transfers up in epithelial tissue:  $C_e/(C_{e+1} - C_e)$
- for transfers down in epithelial tissue:  $C_e/(C_e - C_{e-1})$
- for transfers up in systemic tissue:  $C_s/(C_{s+1} - C_s)$
- for transfers down in systemic tissue:  $C_s/(C_s - C_{s-1})$ .

For simplicity, these scalings are not explicitly stated in the equations given below. In this array, only the [0,0] infection class is truly uninfected; however, a larger portion of the array contains infections not detectable by the tissue-section diagnostic method in which the model output is reported. To establish the boundaries of the patently uninfected class, LFU = 0, in the  $e \times s$  array, the limits of detection described earlier were used to solve equation (1) and the array steps characterized by parasite densities below that value were defined as uninfected. For example, for a 1-g oyster, epithelial classes with LFUs  $\leq 0.8$  and systemic classes with LFUs  $< -1.6$  contained parasites below the detection limit. The lower LFU limit for systemic tissue originates from the much larger tissue cross-section area searched for the parasite, as discussed previously.

The governing equation for determining the prevalence and intensity of *H. nelsoni* infections in the epithelial (*e*) and systemic (*s*) tissue of oysters (*O*) is given by:

$$\begin{aligned} \frac{dO_{e,s}}{dt} = & -\alpha_{e,s}O_{e,s} - \beta_{e,s}O_{e,s} + \alpha_{e,e-1}O_{e,s-1} + \beta_{e-1,s}O_{e-1,s} \\ & + \alpha_{e,s+1}O_{e,s+1} + \beta_{e+1,s}O_{e+1,s} - M_{e,s}O_{e,s} - \gamma_{e,s+4}O_{e,s+4} \\ & + \delta_{e,1}\delta_{s,0}\frac{1}{2}\sum_{s=3,4}^N\sum_{e=0}^N\gamma_{e,s}O_{e,s} \end{aligned} \quad (2)$$

where the first 6 terms represent the movement of oysters between infection intensity classes through gains or losses of *H. nelsoni* cells in the epithelial and systemic tissue (Figs. 3, 4). The coefficients,  $\alpha$  and  $\beta$ , determine the rate at which parasites are gained or lost. The parameterizations used to determine these coefficients are given in the following sections. The seventh term in equation (2) represents the loss of parasites through oyster mortality from lethal infections as determined by the rate of mortality, *M*. The final 2 terms in equation (2) represent the transfer of oysters from heavy infection classes to lower infection classes due to the formation or attempted formation of spores by *H. nelsoni* in the oysters with advanced infections, which results in a loss of oysters from BFU category 4 ( $s_4$  in equation 2), and a gain of infected oysters into infection class [1,0]. The  $\delta$  functions represent a step-function process in which the oysters are introduced into the [1,0] infection class only (Fig. 3). The coefficient  $\gamma$  determines the rate of this transfer process.

The establishment of infection in uninfected oysters ([0,0] class) is determined by the equation:

$$\frac{dO_{0,0}}{dt} = -\beta_{0,0}O_{0,0} + \frac{1}{2}\delta_{e,0}\delta_{s,0}\sum_{s=3,4}^N\sum_{e=0}^N\gamma_{e,s}O_{e,s} \quad (3)$$

where the first term represents the acquisition of *H. nelsoni* infective particles at a rate determined by  $\beta_{0,0}$ . The second term represents addition of oysters to the uninfected class after hypothesized abortive *H. nelsoni* sporulation events (see Section e), as given by the eighth term in equation (2). These oysters are divided evenly between the [0,0] and [1,0] infection classes, as given by the last terms in equations (2) and (3).

For the sake of simplicity and broad application, the 0–4 category BFU scale was chosen for the model output. Parasite numbers are converted into BFUs (Fig. 4) and model simulations report the proportion of oysters in each of the BFU categories. Certain rules apply to the movement of oysters among infection categories, all based on histological observations of the disease process (Farley 1968, Ford and Haskin 1982). To reflect the fact that infections are initiated in the gill epithelium, uninfected oysters [0,0 class] must move first into an epithelial class before entering a systemic class (Figs. 3, 4). Oysters never reach high epithelial infections, LFU<sub>e</sub> > 6.5, without developing systemic infections, and this is modeled by an appropriately calibrated transfer function as discussed later. Oysters in systemic classes  $\geq 7.0$  are automatically placed in the dead oyster category because parasite densities represented by these classes are higher than those found in live oysters. Additional mortality processes will be discussed later.

The diagonal line separating BFU 2 and BFU 3 (Fig. 4) is based on the observation that BFU category 2 is normally reached when advancing epithelial infections (BFU 1) give rise to local systemic infections (BFU 2), which then expand into BFU 3 and then BFU 4. BFU category 3 also includes infections decreasing in intensity. In the latter, parasite burdens diminish simultaneously in epithelial and systemic tissues, hence oysters move in a diagonal toward the undetectable infection category (BFU 0) rather than back through BFU 2 to BFU 1.

**Proliferation of *H. nelsoni***

Transfers of oysters to different infection intensity classes are assumed to be due to proliferation and death of *H. nelsoni* cells, except in two cases. First, the acquisition of initial infections is determined by an external factor termed “transmission” ( $\beta_{0,0}$  in equation 3). Second, the development of an epithelial into a systemic infection is determined by an invasion rate that is not simply a function of cell division. These transfers are discussed in the following section and a schematic showing the many linkages in the oyster-*H. nelsoni* model is given in Fig. 5. To more easily describe the sequence of processes involved, the model is described as it simulates the yearly infection cycle (Fig. 1A) beginning with the onset of infection in June.

**Temperature-dependent proliferation of *H. nelsoni*.** After the acquisition of *H. nelsoni* infections in early June (Fig. 1A), the proliferation of plasmodia in the epithelial and systemic tissue ( $g_{e/s}(T)$ ) is assumed to be exponential with a doubling rate that is modified by temperature as:

$$g_{e/s}(T) = b_{0_{e/s}}e^{d(T-T_0)} \quad (4)$$

where  $b_{0_{e/s}}$  is the proliferation rate of the parasite in epithelial or systemic tissue based on a doubling time at a reference temperature,  $T_0$ . The reference temperature was taken as 15 °C instead of the standard 20 °C, because 20 °C did not produce adequate parasite division rates in the summer as compared to field observations.

TABLE 2.

Definition, units, and values for the variables used in the oyster-*H. nelsoni* model equations. Delaware Bay and Chesapeake Bay are abbreviated DB and CB, respectively.

Variable	Definition	Units	Value
$a_e$	constant	none	1.244
$a_s$	constant	none	0.919
$C_{e/s}$	epithelial or systemic <i>H. nelsoni</i> cells	number of cells	calculated
$b$	scale factor	grids (g wet wt) <sup>-1</sup>	$1.3 \times 10^6$
$c_e$	constant	cells (grid) <sup>-1</sup>	0.135
$c_s$	constant	cells(grid) <sup>-1</sup>	0.022
$W_0$	oyster dry weight	g	chosen
$frac_e$	fraction of $W_0$ that is epithelial tissue	none	0.1
$frac_s$	fraction of $W_0$ that is systemic tissue	none	0.9
$\alpha$	growth rate	d <sup>-1</sup>	calculated
$\beta$	growth rate	d <sup>-1</sup>	calculated
$g_e(T)$	temp dependent parasite growth rate in epithelium	d <sup>-1</sup>	calculated
$g_s(T)$	temp dependent parasite growth rate in systemic tissue	d <sup>-1</sup>	calculated
$b_{0e}$	doubling time of parasite in epithelial tissue	d <sup>-1</sup>	0.23105
$b_{0s}$	doubling time of parasite in systemic tissue	d <sup>-1</sup>	0.69315
$d$	temperature effect on growth rate	°C <sup>-1</sup>	0.04
$T_0$	parasite growth rate reference temperature	°C	15
$crowd_{e/s}$	density-dependent control on growth	none	calculated
$I$ Factor	oyster ingestion factor	none	calculated
$ccrowd_e$	epithelial cell threshold for crowding	number of cells (g dry wt) <sup>-1</sup>	$2.5 \times 10^6$
$ccrowd_s$	systemic cell threshold for crowding	number of cells (g dry wt) <sup>-1</sup>	$3.3 \times 10^5$
$cp$	rate of increase of crowding effect	none	1.5
$d_0$	base cell diffusion rate	d <sup>-1</sup>	0.138
$DD$	degree days	°C d	calculated
$\Delta^\circ$	temperature differential	°C	calculated
$SM_e$	maximum rate of cold susceptibility in epithelial tissue	none	2.0
$SM_s$	maximum rate of cold susceptibility in systemic tissue	none	8.0
$k_e$	$DD$ value at which reach one-half $SM_e$	°C d	20.0
$k_s$	$DD$ value at which reach one-half $SM_s$	°C d	10.0
$SD_e$	susceptibility decay factor in epithelial tissue	(°C d) <sup>-1</sup>	0.2
$SD_s$	susceptibility decay factor in systemic tissue	(°C d) <sup>-1</sup>	0.1

TABLE 2.

continued

Variable	Definition	Units	Value
$HR(T)$	temperature dependent hemocyte rate	d <sup>-1</sup>	calculated
$hr_0$	hemocyte activity base rate	d <sup>-1</sup>	0.278
$\theta$	hemocyte activity temperature rate	(°C) <sup>-1</sup>	0.08155
$Th_0$	hemocyte activity base temperature	°C	20.0
$crowd_{eh}$	epithelial cell threshold for crowding for hemocytes	cells (g dry wt) <sup>-1</sup>	$3.0 \times 10^6$
$ehcrowd$	hemocyte crowding threshold in epithelial tissue	none	calculated
$NGER_0$	threshold value for modified net production	(g dry wt) <sup>-1</sup>	0.25
$NGER_{d0}$	minimum potential growth efficiency	none	0.25
$NGER_{q1}$	minimum accumulated potential growth efficiency needed for sporulation	(g dry wt) <sup>-1</sup>	100
$NGER_{q2}$	minimum accumulated potential growth efficiency needed for attempted sporulation	(g dry wt) <sup>-1</sup>	10
$SSR$	spore susceptibility decay rate	d <sup>-1</sup>	0.1151
$SporeS$	sporulation rate	d <sup>-1</sup>	calculated
$SporeS_d$	sporulation rate modifier	none	set at 1.0 or calculated
$SST_0$	spore temperature susceptibility	°C	15
$SST_{sp}$	susceptibility temperature switch	°C	2.64
$TempSS$	spore temperature susceptibility factor	d	none
$SpFrac$	fraction of <i>H. nelsoni</i> cells undergoing	(oyster) <sup>-1</sup>	0.25
$TotalS$	spores released	cells	calculated
$SporeN$	number of spores formed per plasmodium	number (cell) <sup>-1</sup>	25
$Spore_k$	oyster death rate from sporulation	d <sup>-1</sup>	0.1733
$Smort$	salinity mortality factor	none	calculated
$SD_1$	salinity mortality factor	none	103.0
$SD_2$	salinity mortality factor	none	0.24065
$SD_3$	salinity mortality factor	(ppt) <sup>-1</sup>	0.592456
$SD_4$	salinity halving time	d	4.0
$Sdeath$	salinity mortality rate	d <sup>-1</sup>	calculated
$Sfactor$	salinity effect of growth	none	calculated
$sg$	salinity effect on growth	(ppt) <sup>-1</sup>	0.4605
$S_0$	salinity growth effect reference salinity	ppt	15.0
$Sdeath_{max}$	maximum salinity mortality rate	d <sup>-1</sup>	0.01787

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TABLE 2.  
continuedTABLE 2.  
continued

Variable	Definition	Units	Value
$S_{diff}$	salinity effect on diffusion rate	none	calculated
$SF1$	salinity diffusion constant	none	9.0
$SF2$	salinity diffusion constant	none	2.65
$SF3$	salinity diffusion constant	ppt	3.0
$MortO$	mortality rate	$d^{-1}$	calculated
$Mspan$	mortality time span	d	30
$Ma$	mortality constant	none	0.00747
$Mb$	mortality constant	$(LFU)^{-1}$	0.717
$I1$	infection constant	none	0.0231
$I2$	infection constant	none	$1 \times 10^{-4}$
$I3$	infection constant	min particle $^{-1}$	-0.9
$IPfilter$	infective particles filtered	particles min $^{-1}$	calculated
$IPconc$	infective particle concentration	particles l $^{-1}$	calculated
$filt$	oyster filtration rate (Hofmann et al. 1992)	l min $^{-1}$	calculated
$IPtemp$	infection temperature effect	none	calculated
$IPsal$	infection salinity effect	none	calculated
$IPseason$	infection seasonal effect	none	chosen
$SM_1$	salinity mortality constant	ppt	1.6
$SM_2$	salinity mortality constant	none	11.0
$SM_0$	salinity mortality reference salinity	ppt	17.0
$IP_{conc_0}$	base infective particle concentration	particles l $^{-1}$	450 (DB, 1960s) 750 (DB, 1980s) 450 (CB)
$IPsalrate$	rate of change in spore concentration	$d^{-1}$	calculated
$IPsalrate_0$	change in spore concentration reference rate	$d^{-1}$	0.038376
$IPsal_0$	change in spore concentration base salinity	ppt	15.5
$S_{IP}$	salinity values from a specified time series for salinity oscillations	ppt	chosen
$IPsal_1$	change in spore concentration salinity constant	ppt	5.0
$IPconc_{max}$	maximum conc. of infective particles	particles l $^{-1}$	900 (DB, 1960s) 1500 (DB, 1980s) 900 (CB)
$IPconc_{min}$	minimum conc. of infective particles	particles l $^{-1}$	0.001
$DD10$	transmission degree days	$^{\circ}C$	calculated
$DD_0$	transmission degree day reference level	$^{\circ}C$	700 (DB) 520 (CB)

Variable	Definition	Units	Value
$DD^1$	transmission degree day constant	$^{\circ}C$	20.0
$DD_2$	transmission degree day constant	none	1.6
$IPtemp_{est}$	estimated effect of degree day on infective particle concentration	none	calculated

The rate at which the proliferation rate is modified by temperature is given by  $d$ , which is based on a  $Q_{10}$  of 3.2. This is also derived from the requirement to obtain adequate division rates in the summer. Data taken from field observations in the lower Chesapeake Bay were used to fit the model-derived simulations (Andrews 1966).

Proliferation rates differ for parasites in the epithelial and the systemic tissue because simulations using the same base rate for both tissues did not accurately reproduce field observations. Thus, it was necessary to assume doubling times of 1 and 3 days for the plasmodia in the systemic and epithelial tissue, respectively (Fig. 6). The biological rationale for the faster reproduction of systemic parasites is that they are continuously bathed in hemolymph, which should provide better nutrition than that received by parasites in the epithelium, where parasites are lodged between cells (Myhre 1973).

**Density-dependent proliferation of *H. nelsoni*.** The *H. nelsoni* cell division rates given by equation (4) are sufficient to simulate the observed increase in infection prevalence and intensity after the initial infection in June (Fig. 1A, point 1). However, the reduced proliferation rates and plateauing of infection levels observed in late fall and early winter (Fig. 1A, point 2) could not be simulated with a simple reduction in doubling rate resulting from decreasing temperatures. Therefore, an additional mechanism was indicated and this was assumed to be a decrease in *H. nelsoni* replication rate due to parasite density-dependent effects as has been previously described for *P. marinus* (Saunders et al. 1993, Hofmann et al. 1995). During summer and fall, parasite numbers in oysters have steadily increased and this biological control on proliferation rates occurs as parasite density approaches the carrying capacity of the environment (in this case oyster tissue). The density-dependent control is related to oyster size and *H. nelsoni* cell density as:

$$crowd_{e/s} = \min \left[ 1, \left( \frac{I factor(j) ccrowd_{e/s} W_0(j) frac_{e/s}}{C_{e/s}} \right)^{cp} \right] \quad (5)$$

where  $j$  indicates oyster size in g dry weight,  $I factor(j)$  is a size-dependent factor determined by oyster ingestion rate (discussed subsequently) and  $ccrowd_{e/s}$  is the concentration of *H. nelsoni* cells in either the epithelial or systemic tissue at which crowding begins. The crowding effect in systemic tissue becomes important at parasite numbers that are about a factor of 10 lower than those in the epithelial tissue (Fig. 7) and the cell densities (Table 2) at which this effect becomes important were determined by comparison of simulated output to field observations on infection intensification.

TABLE 3.

The average wet weight (AWW), standard deviation (STD), standard error (SE), minimum and maximum wet weight ranges, and percent of the total tissue for dissected oyster tissues (n = 103 oysters).

Tissue	AWW (g)	STD (g)	SE (g)	Minimum (g)	Maximum (g)	Percent (%)
Mantle	1.666	0.539	0.053	0.7	4.0	19
Digestive Gland	1.715	0.55	0.054	0.7	4.0	19
Gill	1.56	0.382	0.038	0.6	2.5	18
Adductor Muscle	1.677	0.549	0.054	0.6	3.2	19
Remainder	2.282	1.017	0.1	0.4	6.0	26

Diffusion between Epithelial and Systemic Tissue

The crowding effect given by equation (5) modifies the temperature-dependent proliferation rates given by equation (4) to provide the final doubling times of *H. nelsoni*. These rates apply to *H. nelsoni* proliferation in all epithelial and systemic tissues, but not to the transfer of parasites from epithelial to systemic tissue. Although the mechanism by which *H. nelsoni* penetrates the basal lamina is not known, for purposes of the model, this transfer is assumed to be governed by a one-way diffusion process; i.e., plasmodia diffuse from the epithelial tissue to the systemic tissue. This process is described by an empirical equation of the form:

$$diffusion = d_0 \max \left( 0, \sqrt{\frac{C_e - \frac{C_s \text{frac}_s}{\text{frac}_e}}{C_{e=55}}} \right) \quad (6)$$

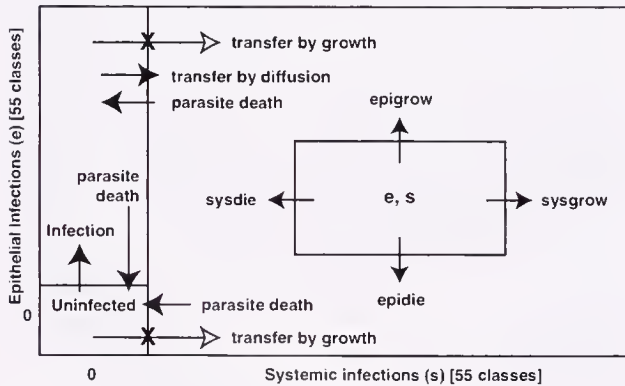


Figure 3. Conceptual model showing possible transfers of oysters through systemic (s) and epithelial (e) infection classes. The relative parasite density in each class is determined by the proliferation (epigrow and sysgrow) or death (epidie and sysdie) of *Haplosporidium nelsoni*. The [0,0] class represents uninfected oysters. Oysters cannot transfer from the uninfected class directly to a systemic infection class (open arrow with X), but must pass into an epithelial class first (i.e., initial infections are established in the epithelium), as indicated by the vertical arrow. Oysters may transfer to the uninfected class when the parasites die (horizontal and vertical arrows). Transfer of an oyster from an epithelial infection class into a systemic infection class is governed by the diffusion (horizontal arrow), not proliferation (open arrow with X), of parasites across the epithelial barrier. Oysters may transfer from systemic to epithelial infection levels by parasite death.

in which the transfer rate depends on the density of parasites in both epithelial and systemic tissues. Equation (6) results in increased diffusion (or invasion) of *H. nelsoni* to systemic tissue as the number of parasites in the epithelial tissue increases (Fig. 8). Equation (6) applies to all transfer from epithelial to systemic tissue; however, there can be no transfer from the truly uninfected compartment (0,0 class) directly to the systemic compartment. Only an epithelial infection can give rise to a systemic infection (Fig. 3).

From the above, the equations for proliferation of *H. nelsoni* in epithelial ( $G_e$ ) and systemic ( $G_s$ ) tissue become:

$$G_e = g_e(T) \text{ crowd}_e \quad (7)$$

$$G_s = g_s(T) \text{ crowd}_s \text{ diffusion.} \quad (8)$$

Thus, the basic rate of *H. nelsoni* infection development includes only temperature- and density-dependent effects on doubling time, plus a diffusive contribution of parasites to the systemic tissue.

*H. nelsoni* Mortality

*H. nelsoni* prevalence and intensity decreases in early spring (Fig. 1A, point 3). Although many of the most heavily infected oysters die at this time, it is evident from histological observations that parasites are also in poor condition and probably dying (Ford and Haskin 1982). In the model, this loss of parasites cannot be accounted for by a simple reduction in *H. nelsoni* doubling rate at low winter temperatures or by a direct effect of cold temperatures

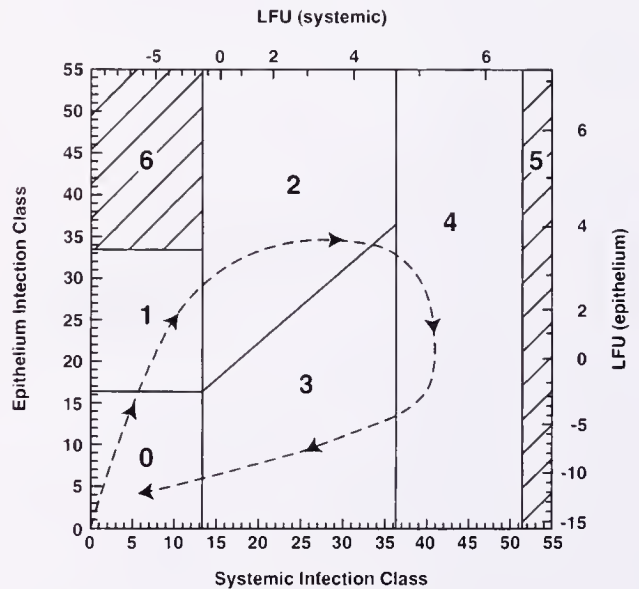


Figure 4. Schematic showing the relationship between the systemic and epithelial infection classes used in the oyster-*H. nelsoni* model and Little Ford Units (LFU). Model infection classes (0–55) for epithelial and systemic infections are shown on the left and bottom sides of the figure, respectively. Their respective LFU categories (0–6) are shown on the right and top sides. The model output is in LFUs, but these are converted to Big Ford Units (BFU) for plotting. BFUs are shown as areas within the plot, with numbers from 0–6. The cross-hatched regions indicate infection levels where the oysters are dead (BFU = 5) or epithelial infection levels that are not normally achieved in nature (BFU = 6). BFU category 0 represents infections that are below the level of detection. The dashed arrows indicate the *H. nelsoni* infection trajectory normally observed in oysters during infection proliferation and remission.

on *H. nelsoni* survival. Simulations of both conditions failed to agree with field observations. In fact, the decrease occurs at a time when temperatures are rising.

Obviously, an additional factor is operating in nature to cause the observed decrease in parasite density. Simulations agreed with observations when it was assumed that the spring decrease in parasite density is due to a combination of 2 factors: 1) *in vivo* conditions coinciding with low winter temperatures that debilitate *H. nelsoni* plasmodia and increase their susceptibility to hemocyte attack; and 2) increasing activity of oyster hemocytes in the spring that removes the damaged parasites. In addition to the apparent degeneration of parasites in late winter, the biological rationale includes experimental data showing that oyster hemocytes do not attack and phagocytose live *H. nelsoni*, but readily ingest killed parasites or those with arrested metabolism (Ford et al. 1993, Ford and Ashton-Alcox 1998). The exact mechanism that results in reduced viability of *H. nelsoni* is unknown, but for convenience, the term "cold" susceptibility is used for this factor.

**Cold susceptibility.** The susceptibility of *H. nelsoni* to low temperature was assumed to depend on the number of winter days during which the parasites are exposed to temperatures below a threshold (i.e., degree days). The number of degree days (*DD*) was determined by summing the difference between the threshold temperature and the ambient temperature ( $\Delta^\circ$ ) over time as:

$$DD = \sum_{min=0}^{max=200} \Delta^\circ dt \quad (9)$$

where *DD* ranges between 0 and 200. The threshold temperature was taken to be 5 °C, based on simulations covering a range of

temperatures between 0 °C and 10 °C. As long as  $\Delta^\circ$  was positive (ambient temperature declining), the number of degree days was related to *H. nelsoni* susceptibility to oyster-hemocyte attack in epithelial and systemic tissue (*HSus<sub>e/s</sub>*) as:

$$HSus_{e/s} = \frac{SM_{e/s} DD}{k_{e/s} + DD} \quad (10)$$

This hyperbolic saturation relationship results in increasing susceptibility of *H. nelsoni* to oyster hemocytes as cold exposure is prolonged. However, above a certain level of cold exposure, susceptibility no longer increases (Fig. 9). In the model, parasite burdens are made to diminish more rapidly in the systemic tissue than in the epithelium to match histological observations that, as infection intensity diminishes, the last parasites seen are in the epithelium (Ford and Haskin 1982, Ford 1985a). The data imply that parasites are eliminated faster from systemic locations and the biological rationale assumes that the number of hemocytes per parasite is higher in the circulation (systemic tissue) than in the epithelium and therefore the rate at which moribund parasites can be scavenged by the hemocytes is greater.

As temperatures increase in the spring, the degree-day value decreases and eventually becomes negative. During this time, the assumption is that susceptibility of *H. nelsoni* plasmodia to the oyster hemocytes decreases as parasites recover from cold exposure, or because only undamaged parasites remain. This effect is incorporated into equation (10) through the addition of a term that attenuates *H. nelsoni* cold susceptibility as temperature increases:

$$HDecay = \frac{1 + \Delta^\circ SD_{e/s}}{1 - \Delta^\circ SD_{e/s}} \quad (11)$$

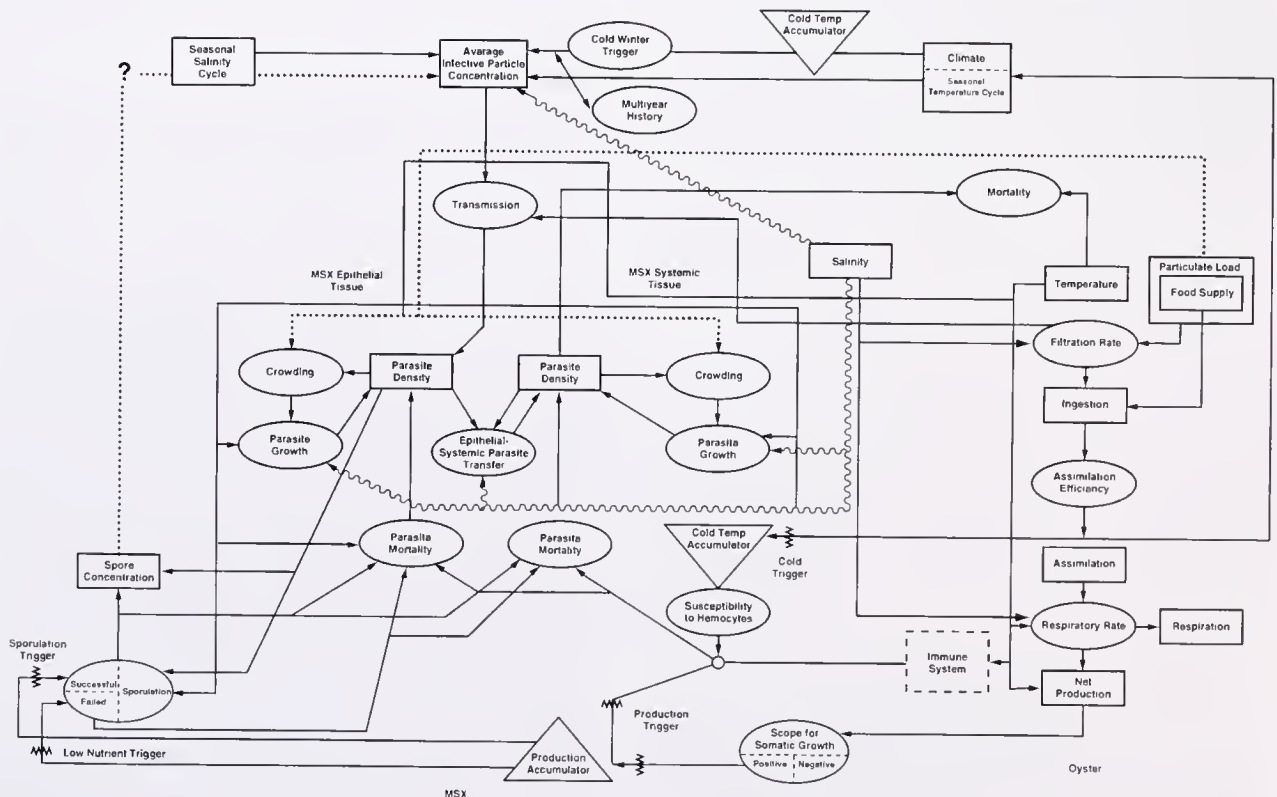


Figure 5. Schematic of the linkages and processes included in the oyster-*H. nelsoni* model.

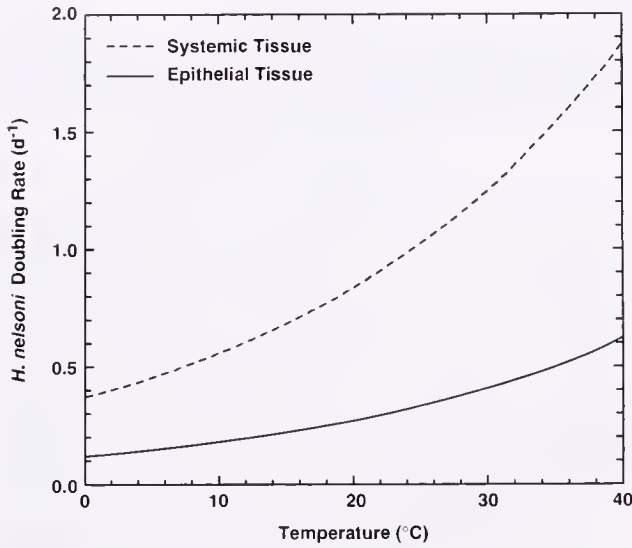


Figure 6. *Haplosporidium nelsoni* proliferation rates in epithelial and systemic tissues of oysters as a function of temperature at salinities  $\geq 15$  ppt.

where the values used to determine the rate of decay in susceptibility of *H. nelsoni* cells to hemocytes ( $SD_{e/s}$ ) differ for the epithelium and systemic tissue. Parasites in the epithelium are assumed to recover more rapidly for the same reason that they are less susceptible to the degree-day factor. As long as  $\Delta^\circ$  is negative (ambient temperature increasing) the parameter  $HSus_{e/s}$  is decreased each time step by  $H$  Decay amount.

**Hemocyte removal of damaged *H. nelsoni* plasmodia.** Once susceptible because of cold-associated damage, *H. nelsoni* plasmodia can be removed by oyster hemocytes at a rate that is dependent on temperature. The hemocytes are assumed to become maximally active at 10 °C and their activity to decrease above 10 °C (Fisher and Tamplin 1988). This is given by an equation of the form:

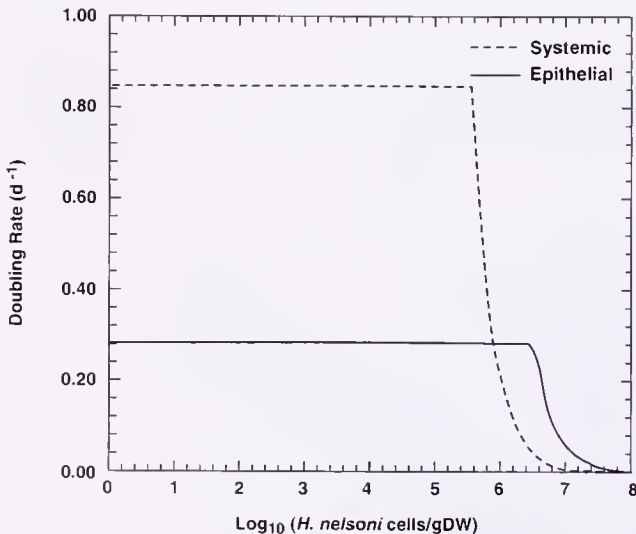


Figure 7. The relationship between the reduction in *Haplosporidium nelsoni* proliferation rates in oyster epithelial and systemic tissue as a function of increasing parasite density (self crowding) at salinities  $\geq 15$  ppt and a temperature of 20 °C.

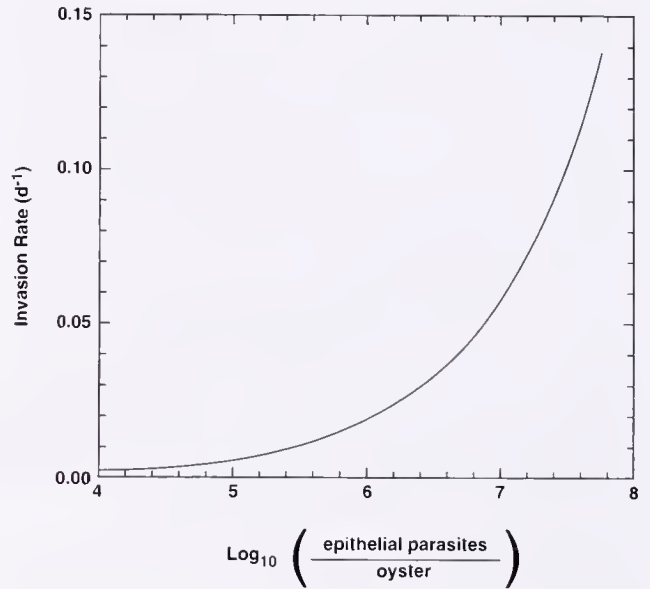


Figure 8. Relationship of the invasion (diffusion) rate of *Haplosporidium nelsoni* from oyster epithelial to systemic tissue and the number of parasites in the epithelial tissue. The rate shown is the one for the initial invasion of parasites into the systemic tissues.

$$HR(T) = hr_0 e^{\theta(\max(T, 10) - T_{h0})} \tag{12}$$

where the base hemocyte activity rate,  $hr_0$ , is related to the rate at 20 °C ( $T_{h0}$ ). The observation that the rate at which oyster hemocytes phagocytose foreign particles is reduced below 10 °C (Feng and Feng 1974, Alvarez et al. 1989) is incorporated into the model as a linear decline to zero in hemocyte activity from 10 °C down to 0 °C.

**Net Proliferation of *H. nelsoni***

From the above relationships, the net doubling time of *H. nelsoni* in the epithelial ( $NG_e$ ) and systemic ( $NG_s$ ) oyster tissue is given by:

$$NG_e = G_e - HR(T) HSus_e ehcrowd \tag{13}$$

$$NG_s = G_s - HR(T) HSus_s crowd_s \tag{14}$$

where the final terms represent parasite density effects on overall hemocyte effectiveness. For systemic infections, this term is the same as that used for parasite crowding effects in equation (5) and accounts for the fact that the increase in circulating hemocyte concentrations stimulated by *H. nelsoni* infection is relatively less than the increase in parasite density (Ford and Kanaley 1988, Ford et al. 1993). Thus, the fraction of the *H. nelsoni* population removed by hemocytes becomes progressively lower as the number of parasites increases.

The value for parasite concentration in the epithelium at which crowding occurs,  $crowd_{eh}$ , as applied to hemocyte activity, is calculated from equation (5); however, the parasite concentration at which crowding occurs is 17% larger (Table 2) than the constant for epithelial tissue used in equation (5). Once again, this value was established through comparison of simulation results and field observations, because there are no direct observations of this effect. The higher value for the coefficient,  $crowd_{eh}$ , indicates that hemocytes in the epithelial tissue remain active at proportionally higher *H. nelsoni* numbers than in the systemic tissue. In the ab-

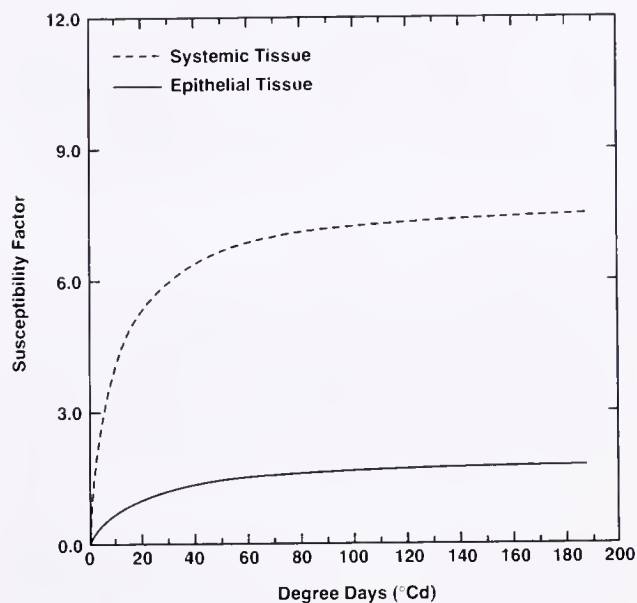


Figure 9. Relationship between the number of degree days below 5 °C and *Haplosporidium nelsoni* susceptibility to destruction by oyster hemocytes in epithelial and systemic tissues.

sence of processes discussed in subsequent sections, equations (13) and (14) determine the values of  $\alpha$  and  $\beta$  in equation (2).

#### *H. nelsoni* Proliferation in Spring

The increase in *H. nelsoni* infection prevalence and intensity in early spring shortly after the late winter die off (Fig. 1A, point 4), which coincides with rising water temperature, cannot be reproduced in the model through a simple temperature effect on doubling rate. The speed of the increase suggests that density-dependent control on parasite proliferation in the oyster has been released. The biological rationale for this argument is based on observations that, in spring, a rapid increase in oyster growth rate occurs associated with the spring bloom and rising water temperature. For purposes of the model, the environment experienced by *H. nelsoni* inside its host is assumed to improve concurrently as a consequence of an inflow of nutrients, favoring rapid parasite proliferation. It is this fastidious dependency of *H. nelsoni* on nutrients supplied to its host that will dominate the remainder of the post-infection oyster-*H. nelsoni* model.

The effect of changing nutrient supply in the spring was included in the model by relating the density-dependent control on *H. nelsoni* proliferation to food intake by the oyster through filtration and ingestion. In spring, when algal supply and oyster filtration rate are high, the density-dependent control on *H. nelsoni* proliferation is reduced, allowing the parasite to remain in the exponential phase of its growth with maximum cell division rates for a relatively long period. This effect is included in the model through a potential growth efficiency ratio (*NGER*) that is calculated as:

$$NGER = \frac{\text{assimilation} - \text{respiration} - \text{reproduction}}{\text{assimilation}} \quad (15)$$

where oyster *assimilation*, *respiration*, and *reproduction* are calculated using the relationships given in Hofmann et al. (1992, 1994). Equation (15) gives the fraction of net production available

to *H. nelsoni* after the oyster's respiratory and reproductive demands have been met, i.e., it is the oyster's potential growth efficiency. The term "potential," rather than "net," growth efficiency is used because some fraction of assimilated energy is utilized by *H. nelsoni*, rather than by the oyster, and this fraction should thus be subtracted from assimilated energy in the calculation of net growth efficiency (e.g., Hofmann et al. 1995, Eq. 1). Potential growth efficiency would be energy available for oyster growth if *H. nelsoni* were not present.

The value of *NGER* from Equation (15) is used to calculate *I factor* in the relationship that determines density-dependent crowding (equation 5) as:

$$I_{factor} = \max \left[ 1, \left( \frac{NGER}{NGER_0 W_0} \right)^{cp} \right] \quad (16)$$

where  $NGER_0$  is the threshold value above which the modified net production (*NGER*) is available to *H. nelsoni*. The threshold value was determined empirically through a series of simulations designed to reproduce the annual cycle of *H. nelsoni* infection and intensity observed in Delaware Bay (Ford and Haskin 1982). The release of the crowding effect occurs only when  $NGER \geq 0$ . The effect of *I factor* is to increase the number of *H. nelsoni* parasites that must be present before density-dependent controls on parasite proliferation become a regulating factor.

#### Sporulation of *H. nelsoni*

The factors governing spore production in *H. nelsoni*-infected oysters and the role of spores in its life cycle are among the least understood aspects of this parasite (Haskin and Andrews 1988). The parasites rarely form spores in adult oysters, but may do so regularly in juveniles in both spring and autumn (R. D. Barber et al. 1991, Burreson 1994). Spores can be shed from live oysters, but it is likely that most oysters die during or after sporulation because their infections are so heavy (R. D. Barber et al. 1991). In histological sections of adult oysters with advanced infections at the spring peak, parasites often appear degenerate, with large anomalous nuclei. These abnormal plasmodia may be evidence of a failed attempt at sporulation, after which the parasite dies without completing its life cycle.

For purposes of the model, sporulation or abortive sporulation is hypothesized to be responsible for the rapid disappearance of *H. nelsoni* from oysters in late spring to early summer (Fig. 1A, point 5). In the model, parasites in heavily infected oysters,  $LFU \geq 4$ , can attempt to sporulate, with two possible results. The first is that sporulation is successful, in which case spores are formed and released into the environment. Some oyster mortality is associated with this process. The second possibility is that sporulation is attempted, but is unsuccessful. Failed sporulation makes *H. nelsoni* more susceptible to oyster hemocytes, which remove the parasites and produce oysters with lighter infections. It may also happen that parasites in the heavily infected oysters do not attempt sporulation.

The first part of modeling sporulation required determining whether or not *H. nelsoni* should attempt sporulation; that is, to model conditions within the oyster that would, or would not, favor spore development. The reason or reasons that small oysters support sporulation whereas large oysters typically do not is unknown. The model, however, assumes that it is related to the higher growth efficiency of young oysters, which is reflected in higher *NGER*

values. The approach taken is based on the assumption that sporulation requires a period of good environmental conditions, characterized by high oyster potential growth efficiency, which provides a surplus of required nutrients or other factors to *H. nelsoni* and consequently permits sporulation. Thus, the model accumulates the value of  $NGER$  from equation 15 ( $NGER_d$ ) over time to obtain a measure of the “internal environmental quality” of the oyster in terms of its ability to support *H. nelsoni* development (Fig. 10, step 1). This was done at each time step such that:

$$NGER_d^{new} = NGER_d^{old} + \max(NGER - NGER_{d0}, 0) \Delta t \quad (17)$$

where  $\Delta t$  is the time step of the model. As  $NGER$  exceeds the value of  $NGER_{d0}$ , the quality of the parasite’s environment improves and the parasite benefits from the improved conditions, e.g.,  $NGER_d$  is positive. Equation (17) provides the basis for the remainder of the approach used to simulate sporulation (Fig. 10). Thus, the equations that control sporulation are structured around the seasonal cycle of oyster food availability (Fig. 9).

When  $NGER - NGER_{d0}$  is negative, as during periods of low food,  $NGER_d$  does not accumulate and sporulation cannot occur (Fig. 10, step 2). However, the time span of high nutrient availability required for sporulation need not be continuous so  $NGER_d$  does not decline during periods when nutrient availability is low.

Times when  $NGER_d$  is above zero have 4 possible outcomes. The first occurs if a positive  $NGER_d$  occurs during times when plasmodia are susceptible to cold (Fig. 10, step 3). It is assumed that cold-damaged plasmodia cannot take advantage of the improving quality of the internal host environment. When the sum of the cold-exposure death rates of *H. nelsoni* in the epithelial and systemic tissue (equations 10 and 12) exceeds  $0.1 \text{ d}^{-1}$ ,  $NGER_d$  is not accumulated.

The second and third possible outcomes occur when the *H. nelsoni* plasmodia are healthy and the internal quality of the host is improving (e.g.,  $NGER_d$  is positive). At these times, sporulation

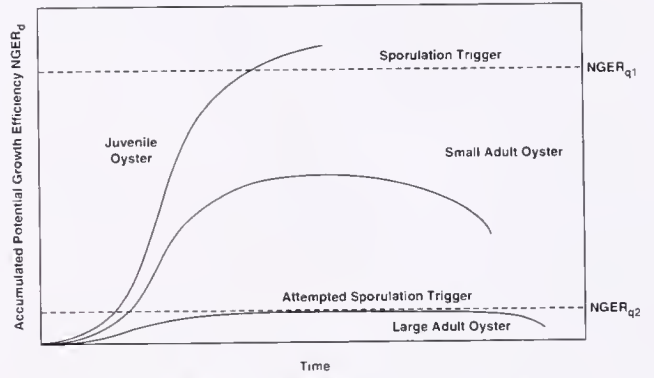


Figure 11. Relationship between  $NGER_d$ , the accumulated potential growth efficiency ratio, and the sporulation triggers for oysters of 3 sizes.

becomes a possibility. It is assumed that, as  $NGER_d$  is accumulating and the oyster quality is becoming more favorable, parasites are cued to begin the sporulation process. In the second possible outcome, sporulation is successful. For successful completion of this process, a certain level of internal host quality must be attained (Fig. 10, step 4). The quality trigger ( $NGER_{q1}$ ) for sporulation was set at  $100 \text{ gdw}^{-1}$ , a value determined empirically through the comparison of a series of simulations and field observations. As noted above, sporulation success is related to the size of the oyster host, with successful sporulation predominating in small oysters. Thus, the quality trigger is scaled by the size of the host and, when  $NGER_d$  exceeds the quality threshold ( $NGER_d \geq NGER_{q1} W_0$ ), sporulation is triggered and  $NGER_d$  is reset to zero (Fig. 11). The value of  $100 \text{ gdw}^{-1}$  permits sporulation in small (up to about 2 cm in length) oysters because of their higher potential growth efficiency, but does not permit sporulation in larger oysters.

In the third possible outcome, sporulation is unsuccessful. In larger oysters, quality also improves as  $NGER_d$  accumulates, but because of lower potential growth efficiency and, consequently, fewer resources available to the parasite, the sporulation trigger is rarely reached. In these oysters, the parasites prepare for sporulation, but the spring bloom ceases and nutrient levels decline before enough nutrients are obtained to sustain sporulation. When nutrient levels decline enough that  $NGER - NGER_{d0}$  becomes negative, abortive sporulation occurs in animals that have accumulated  $NGER_d$  above a second weight-scaled quality trigger ( $NGER_{q2} = 10 \text{ gdw}^{-1}$ ):  $NGER_d \geq NGER_{q2} W_0$  (Fig. 10, step 5). When this happens,  $NGER_d$  is reset to zero.

It is also possible that the accumulated value of  $NGER_d$  will not exceed either quality trigger ( $NGER_{q1}$ ,  $NGER_{q2}$ ). In this fourth possible outcome, sporulation is not attempted and infection intensity continues to increase as determined by the parasite doubling time (Fig. 10, step 6).

Sporulation and attempted sporulation do not occur instantaneously in all oysters meeting the nutritive requirements for the process. The rate of sporulation or attempted sporulation ( $SporeS$ ) is high immediately after the conditions of the nutritive triggers are met and decays over time. The base rate,  $SporeS$ , is defined as  $0.1 \text{ LFU}$ , which produces the desired result that sporulation and attempted sporulation events occur more frequently at higher infection intensities. This rate decreases linearly over time by first setting  $SporeS_d = 1$ , and then establishing a rate of decay.

$$SporeS_d^{new} = SporeS_d^{old} (1 - \Delta t SSR) \quad (18)$$

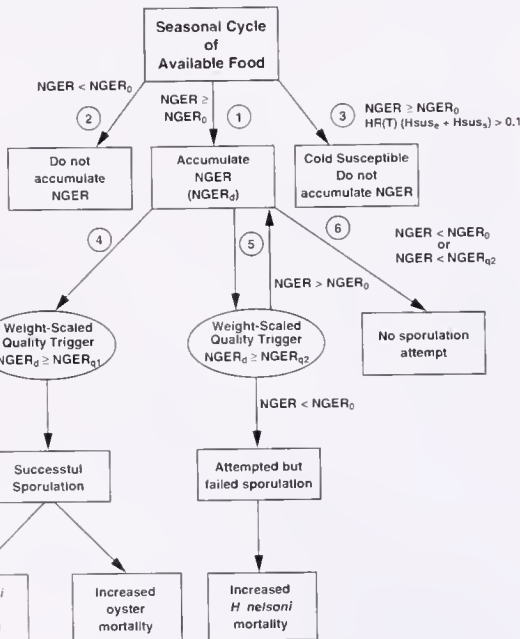


Figure 10. Schematic showing the potential pathways that may arise during sporulation and attempted sporulation by *H. nelsoni*. The numbers correspond to specific sporulation processes that are described in the text.



where  $SSR$  sets the decay rate such that sporulation or attempted sporulation ceases 60 days following the initial trigger. Sixty days provides simulations that best fit field observations of *H. nelsoni* infection intensity during the summer sporulation event. In any given time step, then, the number of oysters undergoing sporulation or attempted sporulation is:

$$O'_{e,s} = SporeS SporeS_d O_{e,s} \quad (19)$$

where  $O'_{e,s}$  are those oysters undergoing sporulation or attempted sporulation.

Spores are formed at times of rapid parasite proliferation, in the spring and late summer/early fall (R. D. Barber et al. 1991, Burserson 1994), but the marked decline in prevalence and intensity that is hypothesized to occur, at least partly as a result of failed sporulation (i.e., incomplete life cycle), occurs only in the spring as water temperatures exceed about 20 °C (Andrews 1966, Ford and Haskin 1982). This observation suggests an influence of temperature on sporulation and attempted sporulation such that neither process occurs at temperatures where *H. nelsoni* is cold susceptible and the process occurs at fastest rates above 20 °C despite adequate nutritive values ( $NGER_d$ ). Therefore, a temperature-dependent "spore susceptibility" factor ( $TempSS$ ) was used to modify equation (19). The temperature factor was defined as:

$$TempSS = \Delta t \frac{1 + \tanh\left(\frac{T - SST_0}{SST_{sp}}\right)}{2} \quad (20)$$

which allows sporulation to be set in motion at about 9 to 10 °C and reach a maximum rate at 21 °C (Fig. 12). The coefficients,  $SST_0$  and  $SST_{sp}$  determine the temperature at which  $TempSS$  is one-half its maximum rate and the temperature range over which the spore susceptibility switches from little to maximum effect (Fig. 12). The temperature effect modifies equation (19) as:

$$O'_{e,s} = TempSS SporeS Spore_d O_{e,s} \quad (21)$$

Failed sporulation results in death of *H. nelsoni*, their removal by hemocytes, and a lower intensity infection in the oyster. One-half

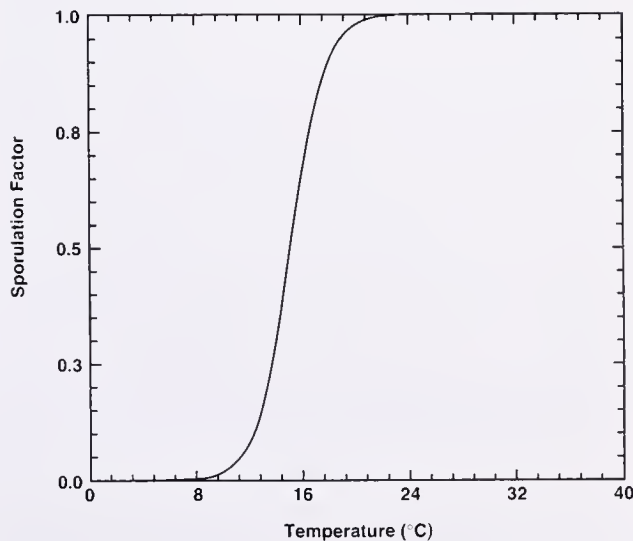


Figure 12. Relationship between the rate at which sporulation can be attempted and temperature.

of the oysters assumed to lose all parasites due to failed sporulation are placed in the uninfected oyster class ([0,0], equation 3). The remaining one-half are placed into the lowest epithelial, non-systemic infection ([1,0], equation 2) class.

Successful sporulation occurs during periods when the quality of the host's internal environment increases to the point that the weight-scaled sporulation trigger ( $NGER_{qt}$ ) is exceeded (Fig. 11). The factors that determine the number of oysters in which successful sporulation occurs are similar to those that affect the number of oysters undergoing failed sporulation, with the exception that some oyster mortality also occurs in the process. Therefore, equation (21) is used to calculate the number of oysters surviving sporulation. Successful sporulation results in the death of some fraction of the affected oysters. The number of oysters with infections in systemic LFU category 4 and all epithelial categories that die from the sporulation event is calculated as:

$$O'_{e,s} = Spore_k SporeS O_{e,s} \quad (22)$$

where the initial rate at which oysters die as a result of sporulation ( $Spore_k$ ) is assumed to be equivalent to a four-day halving time. The dead oysters are removed from subsequent calculations.

Successful sporulation releases *H. nelsoni* spores into the environment. The total number of spores released ( $TotalS$ ) can be calculated as:

$$TotalS = O_{e,s} SpFrac SporeN (frac_e cells_e + frac_s cells_s) \quad (23)$$

where  $SpFrac$  is the fraction of the parasites that undergo successful sporulation and  $SporeN$  is the number of spores formed by each *H. nelsoni* plasmodium (Table 2).

#### Salinity Effects on *H. nelsoni*

Laboratory (Sprague et al. 1969, Ford and Haskin 1988) and field observations (Farley 1975, Haskin and Ford 1982, Andrews 1983, Ford 1985b) have shown salinity to be a critical environmental factor regulating the spatial and temporal distribution of *H. nelsoni* in oyster populations. The following paper (Paraso et al. this volume) describes many of these interactions and provides detailed descriptions of how the coupled model simulates salinity-*H. nelsoni* interactions. However, a brief accounting of these parameterizations is given here for completeness in the model description. In the model, salinity affects *H. nelsoni*-oyster interactions by controlling parasite proliferation rate, mortality rate, transfer rate from epithelial into systemic tissues, and infection rate.

The basis for the effect of salinity on *H. nelsoni* proliferation *in vivo* is a relationship derived from measurements of acute *in vitro* salinity tolerance of the plasmodial stage of *H. nelsoni* (Ford and Haskin, 1988). This relationship shows that, at a salinity of less than 5 ppt, *H. nelsoni* survival is zero. Between 5 and 15 ppt, the parasites show an exponential increase in survival, and above 15 ppt little mortality occurs (see Paraso et al. this volume, Fig. 3). The salinity-caused mortality ( $Smort$ ) was modeled as:

$$Smort = \min \left( 1, \frac{0.01 SD_1}{1 + \frac{SD_1 - SD_2}{SD_2} e^{-SD_3 S}} \right) \quad (24)$$

where  $S$  is the ambient salinity in ppt and  $SD^1$ ,  $SD^2$ , and  $SD^3$  are constants. The actual salinity-induced parasite death rate is calculated as:

$$Sdeath = \frac{-\ln(Smort)}{SD_4} \quad (25)$$

where the death rate calculated from *in vitro* data is assumed to occur over four days ( $SD_4$ ) to account for the buffering effect of an *in vivo* situation (Galtsoff 1964, Shumway 1996). The salinity-caused mortality modifies the net proliferation rates in the epithelial and systemic tissues that are given by equations (7) and (8). Unlike other sources of mortality, salinity-caused mortality is assumed to be able to completely eliminate infections. This occurs at mortality rates above  $0.01787 \text{ d}^{-1}$ .

It was assumed that salinity effects on parasite proliferation rates would occur over the same salinity range as that producing parasite mortality; hence, the effect of salinities between 5 and 15 ppt on parasite doubling time was included through an exponential relationship:

$$Sfactor = e^{sg(S-S_0)} \tag{26}$$

that varied between zero ( $S \leq 5 \text{ ppt}$ ) and 1 ( $S > S_0$ ), where  $sg$  determines the rate of decrease of parasite proliferation rate with increasing salinity and  $S_0$  is 15 ppt, the salinity threshold above which no reduction in parasite proliferation rate occurs. Equation (26) modifies the temperature-dependent growth rate given in equation (4).

In the initial simulations, the frequency of systemic infection decreased with decreasing salinity. Long-term observations in Delaware Bay, however, show that, after an initial decrease from the high salinity (20–23 ppt) planting grounds to the lower-most seed beds (18 ppt), the frequency of systemic infections remains unchanged along the remainder of the salinity gradient to the upper-most seed bed (9 ppt). To simulate the observed pattern, the model increases the rate of parasite diffusion between epithelial and systemic tissue with decreasing salinity by including an additional term of the form:

$$Sdiff = 1 + SF1 \frac{\left\{ 1 - \tanh \left[ SF2 \left( \frac{S - S_0}{SF3} \right) \right] \right\}}{2} \tag{27}$$

to equation (6). This relationship allows the rate of diffusion between epithelial and systemic tissues to be maximum for salinities of 12 ppt and less, and to decrease to the base rate given by equation (6) between 12 and 18 ppt. It is presently unclear whether the biological basis of the field observations is actually tied to more rapid transfer of parasites, or whether some other mechanism is responsible. Thus, equation (14) can now be updated to its final form:

$$NG_s = G_s - HR(T) HSus_s crowd_s - Sdeath + diffusion Sdiff. \tag{28}$$

**Oyster Mortality**

The ultimate result of most *H. nelsoni* infections is the death of the oyster host. To model this effect, historical data on the intensity of infection (LFUs) in live and dead oysters was assembled. The percent of live and dead oysters in each infection category was calculated as a function of the total number of live or dead oysters, respectively, in the set of samples examined. The ratio of percent dead to percent live in each category was then computed. This ratio was considered a relative measure of the likelihood that an oyster will die with a given category of infection. Results showed that oysters in LFU categories 1 and 2 are no more likely to die than

those in category 0; in categories 3–5, the likelihood rises to between two and three; and oysters with category 6 infections are six times more likely to die than those without detectable infections. This relationship (Fig. 13) is of the form:

$$MortO = \frac{-\ln(1 - M_a e^{M_b LFU})}{M_{span}} \tag{29}$$

Abundant field observations show that infected oysters can survive better at low temperatures than at high (Andrews 1968, Ford and Haskin 1982). For instance, as temperatures approach  $7^\circ\text{C}$  in late November in Delaware Bay, the mortality rate drops to nearly zero. It is assumed that this happens because both host and parasite are quiescent at low temperature: the parasite no longer actively damaging the host and the host no longer actively feeling the effects of parasitism. It is a system “on hold” over the winter. Thus, a temperature effect was applied to the death rate given by equation (27) such that oyster mortality is reduced in a linear manner from the rate at  $7^\circ\text{C}$  to zero at  $0^\circ\text{C}$ .

The total number of dead oysters in any infection class is then calculated as:

$$O_{e,s}^d = Spore_x SporeS MortO O_{e,s} \tag{30}$$

which is a modification of equation (22). In addition, any oyster in which the infection intensity exceeds that found in live oysters automatically is placed in the dead oyster category (Fig. 4). The dead oysters are removed from subsequent calculations of infection dynamics, but they are accumulated over time to provide an estimate of mortality.

***H. nelsoni* Transmission**

Transmission is dealt with fully in the third paper in this series (Powell et al. this volume). A condensed accounting of the parameterizations used for this process is given here for completeness in the model description.

The processes by which *H. nelsoni* is transmitted to uninfected oysters, and the form of the infective particle, are not known. However, observations that the earliest infections are in the gill

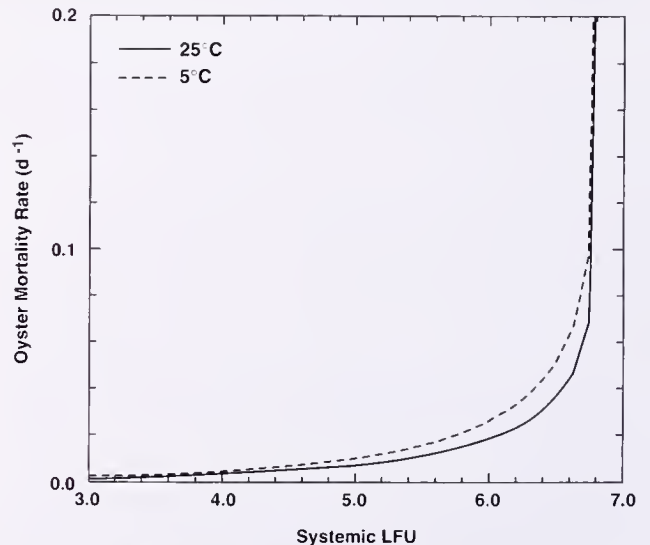


Figure 13. Oyster mortality rate as a function of systemic LFU at  $5^\circ\text{C}$  and  $25^\circ\text{C}$ , which span the range of temperature that is normally encountered in Delaware Bay.

epithelium indicate that infective particles are acquired through filtration (Farley 1968, Ford and Haskin 1982). In addition, early studies with timed imports of oysters into enzootic regions of Delaware and Chesapeake Bays clearly showed that oysters became infected only during a period from late May through early October (Andrews 1968, Ford and Haskin 1982), suggesting that there is a seasonal dependence in the ambient concentration of *H. nelsoni*. The abundance of infective particles in the water is a critical element in modeling transmission, but no measurements are available to parameterize this process. Recently, however, Barber and Ford (1992) reported finding haplosporidian spores, morphologically similar to those of *H. nelsoni*, in the digestive tract lumina of oysters in Delaware Bay and other regions enzootic for *H. nelsoni*. The spores, obviously ingested while feeding, predominated from May through October, the known infective period for *H. nelsoni*. These may not be *H. nelsoni* spores, and if they are, they may not be the stage that infects oysters. Nevertheless, these data are the only ones available on which to base a rough estimate of likely seasonal fluctuations in ambient concentrations of *H. nelsoni* infective particles. Further, both simulations and observations suggested that salinity and temperature, in addition to time of year, affect the abundance of infective particles (see below).

The actual rate at which new *H. nelsoni* infections occur in uninfected oysters ( $O_{00}$ ) is dependent upon the number of infective particles filtered out of the water. This rate ( $\beta_{0,0}$ ) is given by:

$$\beta_{0,0} = \frac{I_1}{1 + \frac{I_1 - I_2}{I_2} e^{I_3 IP_{filter}}} \quad (31)$$

where  $IP_{filter}$  is the number of infective particles filtered by the oyster. The relationship assumes a threshold dose of 8,700 infective particles filtered  $d^{-1}$  needed to generate a new infection. The rationale for using this value is given in Powell et al. (this volume). The remainder of the transmission submodel is designed to estimate  $IP_{filter}$ .

The number of infective particles filtered by the oyster was modeled as:

$$IP_{filter} = IP_{conc} \text{filt}(\text{size}) IP_{season} IP_{sal} IP_{temp} \quad (32)$$

where  $IP_{conc}$  is the ambient infective particle concentration in the water column,  $\text{filt}(\text{size})$  is oyster filtration rate,  $IP_{temp}$  and  $IP_{sal}$  are the temperature and salinity effects on infective particle abundance, respectively, and  $IP_{season}$  is the seasonal variation in infective particle availability. Oyster filtration rate is calculated using the relationships given in Hofmann et al. (1992, 1994). The relationships used to specify the seasonal, salinity and temperature dependencies of the infective particles are described below.

**Seasonal effects.** The base concentration of infective particles,  $IP_{conc}$ , was chosen by comparing results of simulations using a range of values to field observations of prevalence (discussed in Powell et al. this volume). The base concentration was then modified seasonally based on observations of ingested haplosporidian spores, which revealed that spores were present primarily during the May–October period (Barber and Ford 1992). This time series (Fig. 14) was taken to reflect the relative abundance of infective particles and was included in equation (32) as  $IP_{season}$ .

**Local salinity effects.** Initial simulations of *H. nelsoni* prevalence in low-salinity oysters showed that prevalences were

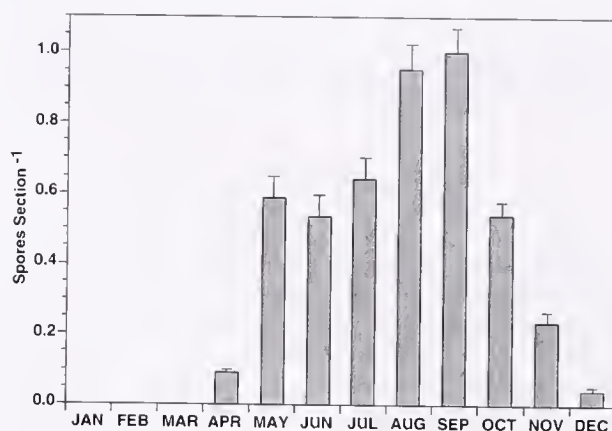


Figure 14. Time series of putative *Haplosporidium nelsoni* spores observed in sectioned *Crassostrea virginica* gut lumina as described in Barber and Ford (1992).

higher than those observed and suggested that the rate of infection, as well as the rate of proliferation within oysters, decreases with decreasing salinity (Paraso et al. this volume). A function that decreased the concentration of infective particles in low salinity water resulted in simulated prevalence levels and patterns that better match those recorded on the low-salinity Delaware Bay seed beds (Paraso et al. this volume). The function was obtained by using the model to simulate infections over a broad range of salinities in Delaware Bay and comparing these to long-term time series (Haskin and Ford 1982, Fegley et al. 1994). Based on these comparisons, the effect of local salinity on transmission rate was modeled as:

$$IP_{sal} = \frac{1 + \tanh\left(\frac{SM_1(S - SM_0)}{SM_2}\right)}{2} \quad (33)$$

The relationship makes biological sense because the salinity range affecting transmission is similar to the range affecting parasite mortality in the host and the somewhat wider range is anticipated for a potentially free-living infective particle. Whether the model simulates decreased survival of infective particles, their decreased ability to infect, or simply a dilution factor, is unknown.

**Bay-wide oscillations.** Simulations with long-term time series that were designed to test the adequacy of the transmission submodel, using the basic process of oyster filtration, infective dose, the seasonal cycle of infective particle availability, and a local effect of salinity on infectivity, showed adequate simulations for oyster populations over a wide salinity range in a specific bay, such as Delaware Bay (e.g., Paraso et al. this volume), during most years. However, the same parameterizations failed in Chesapeake Bay. Although the seasonal cycle of infective particle availability may be somewhat different, certainly the remaining processes should be equivalent in both bays. This suggested that an additional process was needed to model transmission rate.

Review of long-term time series taken simultaneously at multiple sites across the salinity gradient in both bays revealed relatively simultaneous oscillations in disease prevalence with salinity change. Addition of bay-wide salinity-dependent multi-year oscillations in infective particle availability allowed both bays to be modeled with very minor differences in the values of only 2 vari-

ables,  $IPconc_0$  and  $IPconc_{max}$  (Variations in  $IPconc_{max}$  are discussed in the third paper in this series, Powell et al. this volume). These oscillations were parameterized as follows. The rate of salinity change was calculated as:

$$IPsalrate = IPsalrate_0 \left( \frac{S_{IP} - IPsal_0}{IPsal_1} \right) \quad (34)$$

where  $IPsalrate_0$  specifies the response time of the infective particles to changes in salinity, which was taken to be 180 days. The salinity value used to specify  $S_{IP}$  can be considered representative of the salinity at which an hypothetical *H. nelsoni* secondary host lives or where some other reservoir of infective particles is found. For the simulations given in the following sections, the value of  $S_{IP}$  was taken from the most down estuary (highest salinity) site showing strong salinity excursions across the 15 ppt isohaline in both Delaware and Chesapeake Bays. Lower salinity sites failed to provide adequate simulations in either bay, as discussed in Powell et al. (this volume) and higher salinity sites were not present in the suite of available Chesapeake Bay time series. The concentration of infective particles was updated at each time step based on this rate ( $IPsalrate$ ) forced by the direction and migration of salinity change. So, for increasing salinities ( $IPsalrate \geq 0$ ),

$$\frac{dIPconc}{dt} = IPsalrate(IPconc_{max} - IPconc). \quad (35)$$

For decreasing salinities ( $IPsalrate < 0$ ),

$$\frac{dIPconc}{dt} = IPsalrate(IPconc - IPconc_{min}) \quad (36)$$

and, at model initialization,  $IPconc = IPconc_0$ . The new value of  $IPconc$  was then inserted into equation (32).

**Temperature effects.** Long-term observations from Delaware Bay show a cyclic pattern of *H. nelsoni* activity in which years of low infection prevalence follow, typically with a lag of 1 to 2 years, very cold winters (Ford and Haskin 1982). Examination of a 1989 to 1994 data set for Chesapeake Bay showed the same phenomenon. Thus, in some years, very few oysters become infected, even when appropriate salinity conditions are present (Haskin and Ford 1982, Paraso et al. this volume). This pattern suggests that, in some way, the abundance of infective particles is diminished after cold winters.

In the model, direct temperature effects on infective particle abundance were included through a calculation of degree days that is based on 10 °C ( $DD10$ ). This calculation differs from that for cold susceptibility (equation 9), which considers temperature effects on *H. nelsoni* after it has infected the oyster.

The number of days in which the temperature is below 10 °C from January to May is accumulated as:

$$DD10 = \sum_{t=1JD}^{t=150JD} 10 - T \quad (37)$$

where JD refers to Julian days. The value of  $DD10$  is then used to determine an estimated degree to which cold temperature affects the survival of infective particles as:

$$IPtemp_{est} = \frac{1}{2} \left\{ 1 - \tanh \left[ DD_2 \left( \frac{DD10 - DD_0}{DD_1} \right) \right] \right\} \quad (38)$$

where  $DD_0$  is a threshold value at which the temperature effect becomes active.

Equation (38) provides a value for the temperature effect that is based on the current degree-day calculation. To model the observed delay in the manifestation of winter temperature effects on *H. nelsoni* infective particles, the value of  $IPtemp$  determined from the current  $DD10$  value was modified based on the value calculated for the previous year. A current value of  $DD10$  less than one-half of the threshold value ( $DD_0$ ), indicates that the current year's winter is considerably warmer (an extreme difference) than that in the previous year, and the current value of  $IPtemp_{est}$  is used as  $IPtemp$ . If the current value of  $DD10$  is greater than one-half  $DD_0$  and less than the value for the previous year, such that the current year's winter is only slightly warmer than the previous year's winter, the current and previous year's values are averaged to obtain the value for  $IPtemp$ . This allows the conditions in the previous winter to affect the level of infectivity by *H. nelsoni* and thereby allows for persistence of the effects of harsh winters over a period of more than 1 year, as observed. If  $DD10$  is greater than one-half  $DD_0$  and greater than the value calculated for the previous year, then the current conditions are colder than previous year's conditions and also characteristic of a cold winter. In this case,  $IPtemp$  is specified using the current value of  $IPtemp_{est}$ .

#### Data Sets

#### Environmental Time Series

The environmental inputs to the oyster population-*H. nelsoni* model are time series of temperature, salinity, food, and total seston (total suspended solids). The time series used for simulations presented in the next section are characteristic of the environmental conditions on the lower Delaware Bay planted grounds (Fig. 1 in Paraso et al. this volume). These reference simulations are intended to reproduce the annual *H. nelsoni* cycle in high salinity.

Temperature measurements were made at a representative site, Miah Maull, by personnel from the Haskin Shellfish Research Laboratory at intervals of 1 to 3 measurements per month throughout the decade of the 1960s. These data show that the winter of 1962 and those from 1968 to 1970 were particularly cold (Fig. 3a in Powell et al. this volume). Salinity time series for the 1964 to 1968 period were derived from monthly-averaged Delaware River flow measurements taken at Trenton, New Jersey, by the U.S. Geological Survey. Salinity time series were calculated using the relationship between Delaware River flow and salinity derived by Haskin (1972) as described in Paraso et al. (this volume). This relationship accurately represents salinity conditions during the 1960s in Delaware Bay, but may be less representative of salinities thereafter because of changing river flow to salinity relationships in the estuary (Haskin 1972). The 1960s were characterized by increasingly saline conditions in the first 6 years of the decade (Fig. 4 in Powell et al. this volume), followed by a freshening trend that began in 1967. The saline conditions in 1963 to 1967 coincided with a period of average-to-relatively mild winters. The salinity during this time was optimal for the proliferation and spread of *H. nelsoni*. The intent of the oyster-*H. nelsoni* model is to simulate the basic cycle observed for *H. nelsoni* prevalence and intensity. By using the time series for 1964 to 1968, the simulations were not influenced by anomalous environmental conditions that would limit *H. nelsoni* proliferation.

Measurements of food and total seston at the Miah Maull site are not available for any time during the 1960s; however, total seston and chlorophyll measurements were made at other lower-estuary locations in Delaware Bay by Haskin Shellfish Research

Laboratory (HSRL) scientists at about monthly intervals from 1981 to 1986, with the sampling frequency increased to bi-weekly between 1982 and 1984. The chlorophyll and total seston time series given by Powell et al. (1997) were used in the reference simulations. Measurements made at a site just south of Egg Island, New Jersey, were assumed to be representative of the Miah Maull planting grounds (Fig. 1 in Paraso et al. this volume). The 6-year time series from this site was averaged to obtain a single time series of 1-year duration that was used for each year of the simulations.

Total suspended solids at the site showed variability throughout the year, with maximum values tending to occur in late spring to early autumn (Fig. 6 in Powell et al. this volume). The chlorophyll time series shows a distinctive spring bloom that occurs in March to May, with the maximum in March (Fig. 6 in Powell et al. this volume). A consistent fall bloom does not occur, although transient increases in chlorophyll concentration do occur from time to time. Chlorophyll values drop to seasonally low levels in July and remain, for the most part, at or near these levels until the next spring. Chlorophyll *a* in  $\mu\text{g L}^{-1}$  was converted to oyster food in  $\text{mg DW L}^{-1}$  using the relationship derived by Powell et al. (1997) from Soniat et al. (1998):

$$\text{food} = \alpha \times \text{chlorophyll } a + \beta \quad (39)$$

where  $\alpha = 0.088 \text{ mgdw } (\mu\text{g chl})^{-1}$  and  $\beta = 0.26 \text{ mgdw L}^{-1}$ .

#### *H. nelsoni* Prevalence and Intensity Time Series

*H. nelsoni* prevalence and intensity were measured at numerous sites in Delaware Bay from 1959 to 1992 by personnel from the Haskin Shellfish Research Laboratory (Ford and Haskin 1982,

Haskin and Ford 1982, Fegley et al. 1994). These measurements (Fig. 1A) provide the calibration and verification for the reference simulation (described in the next section) obtained from the oyster-*H. nelsoni* model for lower Delaware Bay.

#### Model Implementation

The oyster-*H. nelsoni* model was solved numerically using a 2-step pseudo-steady state approximation scheme (Verwer and van Loon 1994) with a time step of 1 hour. Each simulation begins on 1 June 1964 and extends through December 1968. The first simulation established a reference to which all other simulations were compared. The reference simulation was designed to reproduce the seasonal cycle of *H. nelsoni* prevalence and intensity as observed in a high-salinity location (Fig. 1A). Subsequent simulations were designed to show the modifications to this seasonal cycle that arise when some of the assumptions used in developing the oyster-*H. nelsoni* model were relaxed or removed (Table 4). In this regard, these simulations serve as a measure of the sensitivity of the model to the assumptions on which the model is based. Other simulations evaluate the response of the model to variations in environmental conditions.

## RESULTS

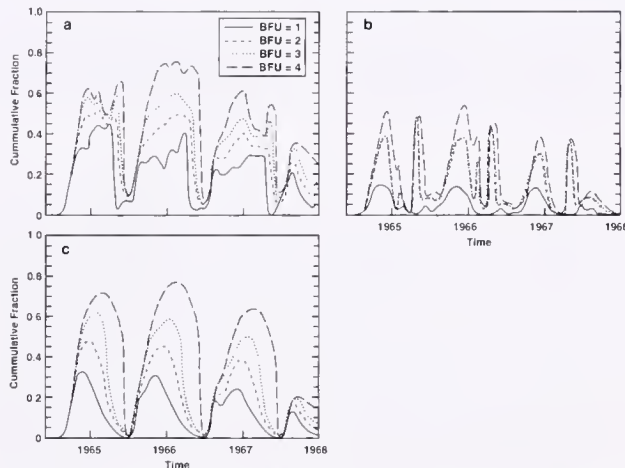
#### Reference Simulation

The simulated time-development of *H. nelsoni* infection in oysters from June 1964 to December 1968 (Fig. 15a), using the environmental time series from the Miah Maull site in Delaware Bay, reproduces the observed annual cycle (Fig. 1A). The first (June 1964 to June 1965) and third (June 1966 to June 1967) years show

TABLE 4.

Simulations done with the oyster-*H. nelsoni* model to test the effect of certain model assumptions and environmental conditions on the simulated infection prevalence and intensity. For each simulation, the changes made in the environmental conditions, oyster size, or model dynamics relative to the conditions used to produce the reference simulation are given. The figure number showing the resultant simulation is indicated.

Simulation	Environmental data set	Oyster size (g)	Model change	Figure number
Reference	Miah Maull 1964–1968	1	none	15a
Crowding effect	Miah Maull 1964–1968	1	density-dependence effect removed (equation 5)	15b
Winter temperature	Miah Maull 1964–1968	1	cold suscep. of <i>H. nelsoni</i> removed (equation 10)	15c
Food effect on sporulation	decreased food in 1965	1	none	16a
Spring food effect	no spring bloom in each year	1	none	16b
Oyster size-sporulation effect	Miah Maull 1964–1968	0.3	none	17a
Oyster size-sporulation effect	Miah Maull 1964–1968	0.1	none	17b
Winter temp-sporulation effect	Miah Maull 1964–1968	0.1	winter temperature effect on sporulation removed (equation 20)	18
Cold winter	winter 1965–1966 colder	1	none	19a
Warm winter	winter 1965–1966 warmer	1	none	19b



**Figure 15.** Simulated time-development of *Haplosporidium nelsoni* infection for 1-g AFDW oysters in Delaware Bay using a) the environmental time series from June 1964 to June 1968, which represents the high-salinity, lower Bay grounds; b) with the density-dependent control on *Haplosporidium nelsoni* growth, equation (5), removed; and c) with the cold susceptibility of *Haplosporidium nelsoni*, equation (10), removed. The term “cumulative fraction” means that the line for each BFU category represents the total prevalence of infections in that and all lower categories.

the expected pattern in disease progression, with an increase in June to early fall (Fig. 1A, point 1), a plateauing in fall (Fig. 1A, point 2), the winter decrease (Fig. 1A, point 3), an increase the following spring (Fig. 1A, point 4), and the decrease in late spring (Fig. 1A, point 5). Year 2 has a slightly modified version of this cycle, with the pattern during the late winter being less distinct. Year 4 of the simulation (Fig. 15a) shows the expected progression for the half year that is depicted. The simulated *H. nelsoni* infections are initially primarily epithelial (BFU = 1) and progress rapidly to higher infection intensities. In the first and third years, about 30% to 40% of the oyster population has systemic infections of BFU  $\geq 2$  by late summer. In the second year, over 50% of the oyster population is infected at this level. These year-to-year differences in prevalence result from the different environmental conditions in each year, as discussed in Powell et al. (this volume). The maximum total prevalences of about 60% to 80% that are attained in the early fall agree with the maximum prevalences reported for lower Delaware Bay at this time (Ford and Haskin 1982). Also, the partitioning of the disease between epithelial and systemic infections in the observed and simulated distributions is similar, with about 60% to 70% of the infections being systemic at peak prevalences (Fig. 1A). Thus, the simulated annual cycle of prevalence and intensity accurately reproduces both observed patterns and infection levels.

#### Sensitivity of Density-Dependence and Cold Susceptibility Factors

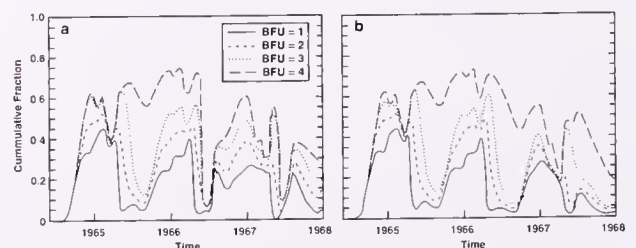
One of the assumptions made in the oyster-*H. nelsoni* model is that the plateau in disease intensity in late summer is due to self crowding by the parasites. However, since there is no direct observation of this effect, it is instructive to determine how sensitive the model is to this assumption. To do this, equation (5) was set to zero. Without the density-dependent control, *H. nelsoni* proliferates rapidly in the summer and triggers a large oyster mortality in December and January, sharply reducing prevalence by midwinter

(Fig. 15b). Neither mortality nor a drop in prevalence is observed at this time in the field (Fig. 1B). Even without the density dependent control, proliferation of *H. nelsoni* does slow in winter due to the cold temperatures, however, this reduction is not sufficient to limit oyster mortality. In particular, a second large oyster mortality event occurs in the late spring of the second, third, and fourth years of the simulation due to the very rapid increase in *H. nelsoni* cell number as temperatures increase in spring. The excessive mortalities of heavily infected oysters cause the simulated infection levels in surviving oysters to be lower than either the reference or observed values. Observed oyster mortality due to *H. nelsoni* does occur in late spring (Fig. 1B), but it is only 10% to 15% of the oyster population rather than the nearly 50% that die in this simulation.

Similarly, the removal of the cold susceptibility of *H. nelsoni* (equation 10) results in simulated disease prevalences and intensities (Fig. 15c) that do not reproduce the observed annual cycle. In the observed cycle (Fig. 1A) and the reference simulation (Fig. 15a), decrease in *H. nelsoni* prevalence and intensity does not occur in late winter. Removal of cold susceptibility predicts that the high parasite values that were present at the end of the previous summer and fall persist through the next spring. Increasing temperatures and subsequent rapid parasite proliferation result in infection prevalences (almost 80%) and intensities (nearly 80% systemic) that are higher than observed in late spring. These high disease levels are followed by a very large sporulation event and coincident oyster mortality in mid-summer, which is also not observed (Figs. 1B, 15a).

#### Sensitivity of Oyster Size and Environmental Conditions on Sporulation

Of the many assumptions made in the development of the oyster-*H. nelsoni* model, those related to sporulation are mostly based on inferences made from observations of MSX disease progression in oyster populations and corresponding changes in the host, rather than from direct observation of the process itself. One of the basic assumptions made concerns the release of density-dependent control on *H. nelsoni* growth in response to increased food levels in the spring. The sensitivity of the model to this assumption was tested by reducing the food supply in early 1965, which affects the calculation of *I* factor given by equation (16). The resulting simulation does not show an attempted sporulation event in the summer of 1965 (Fig. 16a). Rather, *H. nelsoni* prevalence remains high (BFU = 4) and about 70% of the oyster population is infected throughout the following year. In the spring of 1966, when the food levels return to the normal high values, a large attempted sporulation event occurs resulting in a sharp prevalence decline in



**Figure 16.** Simulated time-development of *Haplosporidium nelsoni* infection in BFUs (1 to 4) for a 1-g AFDW oyster in Delaware Bay after a) the oysters were exposed to low food values in 1965 and b) no spring bloom occurred in any year.

early summer. Removal of the spring bloom in all years of the simulation (Fig. 16b), disrupts the expected annual cycle completely, indicating that food supply in the spring is crucial to attempted sporulation.

Attempted sporulation events are either successful and spores are formed or unsuccessful in which case *H. nelsoni* mortality increases (Fig. 10). The difference in the two outcomes is assumed to be related to the size of the oyster. In the reference simulation (Fig. 15a), which uses a 1-g AFDW oyster, sporulation is attempted in early summer, but is unsuccessful. Parasite densities are reduced because failed sporulation leads to *H. nelsoni* death. However, *H. nelsoni* cells in a 0.3-g AFDW oyster can undergo successful sporulation and release spores (Fig. 17a). In this simulation, one successful sporulation event occurred in each of the summers of 1966 and 1967. For smaller oysters, fall sporulation is also possible (Fig. 17b), as observed (Andrews 1979, Burreson 1994). There is no *a priori* reason to expect *H. nelsoni* to attempt sporulation at only 1 or 2 times per year. In fact, when the winter temperature effect on sporulation (equation 20) is removed, small oysters can sporulate into the winter and throughout the year (Fig. 18). However, observations indicate that this does not happen and therefore some factor, such as temperature, must be restricting this process to certain times of the year.

#### Effect of Winter Temperature

Many of the relationships in the oyster-*H. nelsoni* model are dependent on winter temperature. The sensitivity of the model to these assumptions can be tested by altering the winter temperature values in the temperature time series used as input to the model. Decreasing by 50% the 1965 to 1966 winter temperatures falling below 10 °C results in a prolonged period at temperatures of 0 °C to 5 °C, which increases the number of degree days during which *H. nelsoni* is exposed to cold. The resulting simulation (Fig. 19a) shows the expected annual cycle of disease progression, although prevalence is somewhat reduced relative to the reference simulation beginning in late 1965. Because cold winters affect transmission in subsequent years (Powell et al. this volume), the major effect of the cold winter does not occur until the infection cycle beginning in the summer of 1966. Prevalences in that cycle and the following one are sharply reduced so that by the winter of 1968, only 10% of the oysters are infected. Thus, the effect of a single

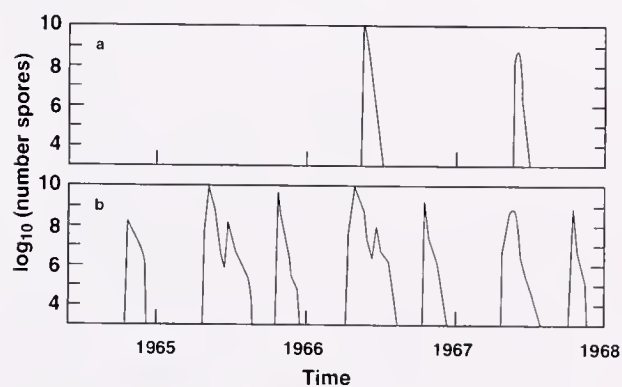


Figure 17. Simulated time-development of successful sporulation events for a) a 0.3-g AFDW oyster and b) a 0.1-g AFDW oyster in Delaware Bay using the environmental time series from June 1964 and June 1968, which represents the high-salinity, lower Bay grounds.

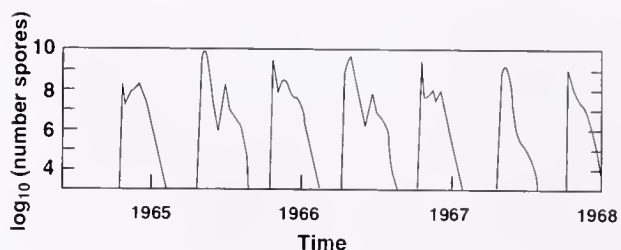


Figure 18. Simulated time-development of successful sporulation events for a 0.1-g AFDW oyster in Delaware Bay using the environmental time series from June 1964 to June 1968, representing the high-salinity lower Bay grounds. For this simulation, the winter temperature effect on *H. nelsoni* sporulation, equation (20), was removed.

cold year can persist into subsequent years, even after winter temperatures have returned to normal.

The effects of a warm winter were investigated by increasing by 50% the temperatures falling below 10 °C. In this simulation, the parasites spend little time at temperatures below 5 °C and do not experience the late-winter die off (Fig. 19b). As a consequence, parasite concentrations are already high at the start of the following spring. They increase further, resulting in heavy infections in the early summer of 1966 and consequent high oyster mortality. The return to normal winter temperatures in subsequent years results in the same annual cycle as seen in the reference simulation. Thus, the effect of a single warm winter does not persist into subsequent years.

## DISCUSSION

#### Model Characteristics

A numerical model describing relationships between the protistan parasite, *Haplosporidium nelsoni*, and its host, the Eastern oyster, *Crassostrea virginica*, has been developed. The model is unusually complex, particularly compared to that developed for the other major parasite of Eastern oysters, *Perkinsus marinus* (Hofmann et al. 1995, Powell et al. 1996). In the *P. marinus*-oyster model, *in vivo* parasite proliferation and death rates are a relatively simple function of temperature and salinity. Further, there is only a single life stage involved and transmission is dependent solely on the density of neighboring oysters and their infection level (Hofmann et al. 1995, Powell et al. 1996). The complexity of the *H. nelsoni* model derives from the need to consider epithelial and systemic tissues as separate compartments, the failure of the para-

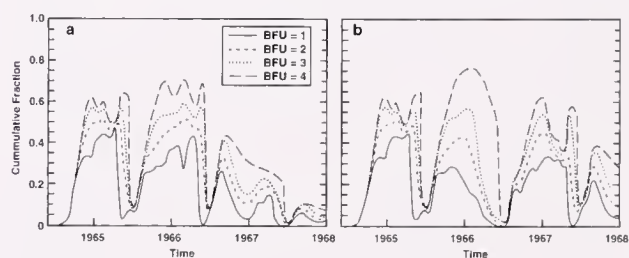


Figure 19. Simulated time-development of *Haplosporidium nelsoni* infection in BFUs (1 to 4) for a 1-g AFDW oyster in Delaware Bay with a) the winters of 1965–1966 made colder by decreasing by half the observed winter temperatures below 10 °C and b) the winter of 1965 to 1966 made warmer by increasing by half the observed winter temperatures below 10 °C.

site to respond in a straightforward way to temperature and salinity change, the need to reproduce parasite sporulation only during certain times of the year and in certain size classes of oysters, and the decoupling of transmission from host infection levels or host density. Construction of the model involved making certain assumptions about the physiological or ecological processes underlying the host-parasite relationship. Some of these assumptions are well grounded in experimental or observational data, or physiological principles; others are less so and may simply be surrogates for the true mechanism, but which happen to give the same answer. The following discussion considers these assumptions, as they occurred in the construction of the *in vivo* model. Assumptions made in the transmission component of the model are discussed in Powell et al. (this volume).

#### Quantifying Infection Categories

The model is quantitative; it uses parasites per oyster to track *H. nelsoni* infection development and decay. In contrast, the data used to construct and verify the model consist of semi-quantitative categories (LFU and BFU), which were converted into parasite densities by counting parasites in tissue section. Thus, a crucial assumption is that extrapolations from these counts adequately estimate total parasite burden, and that the conversion from LFUs to parasite numbers in the model is correct. In effect, the model converts from LFUs to parasite number for calculation and from parasite number back to LFUs (and then to BFUs) for data presentation. As a result, most of the constants used in the model equations are dependent upon the conversion between LFU and parasite density given by equation 1. Should that relationship change with improved quantification methods, the absolute values of most model constants would also necessarily change.

Diagnosis of *P. marinus* infections is also typically done using a semi-quantitative staging system (Mackin 1962), but a relatively accurate conversion between this system and parasite density exists and was used in construction of the *P. marinus* model (Choi et al. 1989). The *P. marinus* conversion was achieved by a process that frees the parasites from oyster tissue for counting. Plasmodial stages of *H. nelsoni* are extremely fragile and would not survive this type of manipulation. Nevertheless, some comparisons between extrapolated *H. nelsoni* densities and actual *P. marinus* counts are instructive. Estimates of *H. nelsoni* and *P. marinus* concentrations in the hemolymph of infected oysters have been made (Ford and Kanaley 1988, Ford et al. 1990, Gauthier and Fisher 1990, Bushek et al. 1994). For both parasites, maximum densities are in the range of  $5 \times 10^5$  to  $5 \times 10^6$  mL<sup>-1</sup>. Maximum densities of *P. marinus* in soft tissues are around  $10^6$  parasites gwwt<sup>-1</sup> (Choi et al. 1989, Bushek et al. 1994), and our estimate of peak *H. nelsoni* concentrations from tissue sections was about the same. Further, the lethal level,  $10^6$  parasites gwwt<sup>-1</sup>, appears to be the same for both parasites, as higher densities are rarely found in live oysters. Interestingly, the estimated detection limit for *H. nelsoni* infections using tissue section histology ( $10^3$ – $10^4$  parasites gwwt<sup>-1</sup>) is similar to the detection limit found for *P. marinus* using the standard Ray/Mackin tissue subsample method (Choi et al. 1989, Bushek et al. 1994). These values suggest fundamental similarities in the per-parasite use of nutrients from, and the damage caused to, the oyster host.

#### The Annual Infection Cycle within the Oyster

The estimated *in vivo* doubling times for *H. nelsoni* used in the model were 1 to 1.4 days in the systemic tissues, and 3 to 4 days

in the epithelium, over the 15–25 °C range. Over the same temperature range, *P. marinus* doubling times were estimated to range between 1.3 and 2.5 days (Hofmann et al. 1995). These rates fall well within the range for most free-living and symbiotic single-celled eukaryotes (Laybourn-Parry 1987, Zaika 1973).

The *in vivo* proliferation rate of *H. nelsoni* is based on a  $Q_{10}$  of 3.2. This high value, set because lower values failed to provide adequate proliferation rates at elevated temperature, suggests that *H. nelsoni* is very sensitive to temperature change. By comparison, the  $Q_{10}$  used to model *P. marinus* cell division rates is 2.0 (Hofmann et al. 1995). Under increasing temperature, then, *H. nelsoni* doubling rates should increase faster than those of *P. marinus* and under decreasing temperatures, they should decrease faster. Over the temperature range where both parasites co-exist, approximately 0 °C to 35 °C, *H. nelsoni* has the higher proliferation rate. These comparisons of modeled proliferation rates are supported by field observations: when oysters are exposed to both parasites in the field, *H. nelsoni* begins killing before *P. marinus* does (Andrews 1967, Chintala et al. 1994).

Declining autumn temperatures failed to slow the proliferation of *H. nelsoni* sufficiently to replicate the observed plateauing of infection levels at that time of year (Andrews 1966, Ford and Haskin 1982). Consequently, it was necessary to add a crowding factor such that, at high densities, proliferation is inhibited. There is no experimental evidence that this happens in *H. nelsoni* infections, but it was also necessary to include a crowding effect in the *P. marinus* model (Hofmann et al. 1995) and there is experimental evidence that a density-dependent inhibition on proliferation does occur with this oyster parasite (Saunders et al. 1993, Ford et al. 1999). Further, a crowding effect is biologically defensible because the host is a limited resource and at some point can no longer provide enough nutrients for all parasites. For both parasites, ample evidence exists that circulating and stored nutrients are diminished by infection (Ford 1986, Barber et al. 1988, Chintala and Fisher 1991, Paynter 1996). The mechanism is analogous to cells in an *in vitro* culture, which reach a stationary phase of reduced division as culture-medium nutrients are exhausted and cellular byproducts accumulate. In the *P. marinus* and *H. nelsoni* models, crowding begins at similar parasite densities: 1 to  $7 \times 10^5$  parasites gwwt<sup>-1</sup>. The *P. marinus* values were obtained from empirical data as described in Hofmann et al. (1995); those for *H. nelsoni* were determined by fitting model simulations to observed MSX disease prevalence and intensity. The similarities in the threshold values for the two parasites further supports evidence presented earlier, of fundamental similarities in the amount of nutrients and the damage produced by each parasite, be it a *P. marinus* or a *H. nelsoni* cell.

The epithelium is one of the most important barriers to infection encountered by an endoparasite. Although *H. nelsoni* readily enter the epithelium, it is truly a barrier because plasmodia proliferate along the base of epithelial cells, obviously prevented from immediate entry into the circulation and often accumulating considerable parasite loads in this layer before the first subepithelial parasites are observed (Farley 1968, Ford and Haskin 1982). Infections confined to the epithelial layer are not lethal and often have few measurable effects on the oyster; further the ability to restrict parasites to the epithelium is one manifestation of resistance to MSX disease (Ford 1988, Ford and Tripp 1996). Consequently, the epithelium and the systemic tissues were considered as separate compartments in the model and the parasites behave somewhat differently in each. For instance, systemic parasites have faster division rates than do epithelial parasites, but become



crowded at lower cell densities. It was necessary to assign different proliferation rates in order to fit the model to observed infection patterns, but there is good biological rationale based on histological observation and reasoning. Myhre (1973) pointed out that in the epithelium, plasmodia are located between oyster cells. Once they have become systemic, they are continuously bathed by hemolymph. Even though the shell cavity fluid of bivalves contains dissolved proteins, indicating the availability of nutrients to a parasite lodged in this compartment, levels are approximately half that in the hemolymph (Allam and Paillard 1998, Ford unpublished). Consequently, it seems reasonable to infer that the hemolymph should provide more nutrients than the epithelium, and should allow faster multiplication. Why the crowding effects seems to run counter to this argument remains unclear, but without a higher crowding threshold in the epithelium, parasites rarely reached densities great enough to allow transfer into the systemic compartment. Although the crowding factor is based on the very plausible hypothesis of food limitation at high parasite densities, there may be another, less obvious, mechanism operating in the case of epithelial crowding.

The mechanism by which plasmodia transverse the basal lamina and enter the circulatory system is not known, although structures known as haplosporosomes, which are common in the Haplosporidia, have been postulated to contain lytic enzymes that may aid in penetration of host tissues, including the basal lamina (Perkins 1968, Scro and Ford 1990). Nevertheless, it is clear that movement of plasmodia across the basal lamina is not a simple function of parasite replication; otherwise one would not expect to see an accumulation of parasites in this layer before they appear in the subepithelial space. The approach used to model the transfer was a simple diffusion equation that depends on the concentration of parasites in both compartments. This is admittedly an artificial mechanism for transporting an organism across a membrane; however, the fact that it provided good results indicates that the true mechanism may have a similar basis. That is, the presence of large numbers of parasites is more likely to allow transfer, perhaps by weakening the basal lamina through the excretion of proteases, than is the presence of just a few plasmodia. In contrast, the *P. marinus* model does not consider the epithelium and systemic tissues as separate compartments and consequently the transfer of *P. marinus* across the epithelial barrier is a simple matter of parasite replication. The fact that this strategy works for *P. marinus*, but not for *H. nelsoni*, indicates an important difference in the way the two pathogens actually cross the barrier. In fact, it is likely that *P. marinus* is carried across within hemocytes, which routinely move between the epithelium and the circulatory system (Mackin and Boswell 1955, Alvarez et al. 1992). Thus, the chances of a phagocytosed *P. marinus* cell being carried across the basal lamina is likely to be the same for a single parasite as it is for one of many in an assemblage of parasites.

In late winter, the observed infection cycle shows a marked prevalence and intensity decline, which is considered to be a combination of the deaths of heavily infected oysters and the mortality of *H. nelsoni* plasmodia in surviving oysters (Andrews 1966, Ford and Haskin 1982). The latter is concluded from the histological appearance of plasmodia at the time. They become dense, so that it is progressively more difficult to distinguish intracellular details, then begin to stain poorly, and finally are difficult to distinguish at all. Frequently they are inside hemocytes. It is not clear what the killing mechanism is. Low temperature is an obvious candidate, but enough parasites survive to initiate a new round of infection

proliferation when temperatures begin to rise in the spring (Ford 1985a). Those parasites that do survive this period apparently are lodged in the epithelium, as that is the focus of renewed proliferation activity in spring.

The initial attempt to model this observation was, in fact, to make *H. nelsoni* die as a direct result of exposure to low temperature. This strategy failed to diminish the parasite burden fast enough, as did the use of an accumulator of low temperature, degree days. The addition of host hemocyte activity against parasites made "susceptible" by prolonged (i.e., degree day) cold, reproduced, in the model, the same infection decline recorded in nature. The use of degree days does not imply that low temperature alone is causing parasite deaths. Temperature could simply be a correlate for some other condition that the parasite experiences over the winter. Ford and Haskin (1982) hypothesized that a long period of anaerobiosis with a buildup of metabolic byproducts, rather than a direct cold effect, might be deleterious to *H. nelsoni*. In fact, the presence of abundant mitochondria in the plasmodia (Scro and Ford 1990) suggested a dependence on oxidative metabolism. Whereas the mechanism causing parasite degeneration over winter is unclear, the behavior of hemocytes toward them is explainable from experimental results. Hemocytes are becoming increasingly active with rising temperatures (Fisher and Tamplin 1988). Oyster hemocytes fail to attack and phagocytose live *H. nelsoni*, but they readily ingest and eliminate parasites in the post-winter period because the plasmodia are dead or damaged (Ford et al. 1993, Ford and Ashton-Alcox 1998). Thus, the need to add to the model, for the first time, an element of host activity is entirely in accord with both observed and experimental evidence.

To fit the model to observations that declining infections persist longer in the epithelium than in the systemic tissues (Ford and Haskin 1982), systemic parasites made "susceptible" by cold are eliminated faster than those in the epithelium. Similarly, to reflect the observation that infections proliferate again from epithelial foci once temperatures begin to rise, the model sets faster recovery rates for the epithelial parasite population. This may reflect recovery of individual parasites or simply the component of undamaged parasites that remain. A possible biological explanation for the observed differences in epithelial and systemic locations is that there are probably more phagocytes per parasite in the hemolymph than in the epithelium so that the rate at which moribund parasites can be eliminated is consequently higher. Hemocyte numbers can become very high in epithelial lesions; however, they are frequently degenerate in appearance and being shed, along with parasites, into the gill cavity (Farley 1968, Ford and Tripp 1996). Differences in hemocyte-to-parasite ratios appear plausible, but there is no evidence for this hypothesis and the actual reason may be quite different.

The rate at which heavy infections decrease in late winter was observed to be slower than that for lighter infections. To model this event, it was necessary to have the overall effectiveness of the hemocyte population respond to parasite density, such that the response was relatively less effective at removing parasites at high *H. nelsoni* densities. It is reasonable that this could occur because of changing parasite-to-hemocyte ratios as infections intensify. The number of hemocytes in circulation and in tissues increases with increasing *H. nelsoni* infection intensity, but the change is relatively small (about 1.5-fold for circulating hemocytes, from a mean of  $3.1 \times 10^6$  cells  $\text{mL}^{-1}$  in an uninfected oyster to a mean of  $4.5 \times 10^6$   $\text{mL}^{-1}$  in a heavily infected oyster) compared to the change in parasite concentration (from none to  $>10^5$   $\text{mL}^{-1}$ ) (Ford

and Kanaley 1988, Ford et al. 1993). The disproportionate increase in parasites means that the number of *H. nelsoni* cells removed by hemocytes becomes a progressively lower proportion of the total parasite population as the number of parasites increases. Once again, it was necessary to model different rates for the systemic and epithelial tissues to reproduce observed differences. Thus, in relation to their number, epithelial hemocytes remove more parasites than do systemic hemocytes. There is no observational or experimental evidence for this model function other than the need for simulation to fit field observations of the *H. nelsoni* seasonal cycle.

Up to this point in the annual infection cycle, late winter/early spring, the model relies on temperature, parasite-density, and hemocyte activity to replicate the observed seasonal changes in parasite loads. A new element was needed, however, to explain the rapid spring infection increase from pre-existing foci, and subsequent sporulation. That element is oyster food, which remains of paramount importance throughout the remainder of the modeled annual cycle. Proliferation rates naturally increase with rising spring temperature, but the effect of temperature on parasite doubling time was inadequate to reproduce the observed, very rapid infection development in April and May. Particularly evident in field observations was the development of very heavy infections, indicating that high parasite division rates continued at densities where proliferation was otherwise restricted by self crowding. In addition to a rise in temperature in spring, the parasite experiences other changes inside the host. The oyster becomes active again after several months of quiescence over the winter. Oxygen availability rises and the accumulation of end products from anaerobic metabolism ceases. A spring bloom typically occurs, and as oyster food consumption increases, the quantity of nutrients transported in the hemolymph rises (Fisher and Newell 1986). All of these changes should provide an increasingly favorable environment for *H. nelsoni* proliferation. Further, the fact that metabolic activity and nutritional status of the oyster is increasing in the spring should provide more or better resources for the parasite, and permit higher parasite densities before crowding interferes with replication, than in late autumn when oyster metabolism is shutting down, even though nutrient reserves are generally high. Following this biological argument, the model eases the crowding effect so that higher parasite densities can be achieved rapidly in the spring. With this modification, simulations show the rapid infection intensification that occurs in the late spring and which culminates in what are often the highest parasite burdens of the year (Ford and Haskin 1982).

Nutritional status, as modeled by oyster potential growth efficiency, is equally important in the next and last phase of the annual cycle, which is the production or attempted production of spores. It is also the most complex aspect of the annual cycle model. The observation that the model needed to fit was that the late May/early June prevalence peak is relatively brief, in contrast to the winter peak, and is followed by a rapid decline in prevalence (Andrews 1966, Ford and Haskin 1982). Like the loss of infections in late winter, part of this decline is due to the deaths of heavily infected oysters and part to the loss of parasites from live oysters. To simulate this event, a second life stage, the spore, was introduced into the model. In other members of the phylum Haplosporidia, plasmodia regularly form spores (Perkins 1990), which presumably allow them to survive outside the host and are an important element in transmission. *Haplosporidium nelsoni* does form spores in adult oysters, but very rarely (Couch et al. 1966). Recent re-

ports, however, suggest that spores are regularly formed in juvenile oysters with advanced infections (R. D. Barber et al. 1991, Burrenson 1994). Spore production coincides with the May/June infection peak and also occurs as infections intensify in the fall. Sporulation takes place in the epithelium of the digestive tubules and mature spores can be shed from live oysters; however, most oysters probably die during or after the sporulation process because the overall infections are so heavy (R. D. Barber et al. 1991).

Although spores are rare in adult oysters, histological observations at the late May/early June infection peak suggest that some parasites may begin the sporulation process in adults. Oysters with advanced infections often have plasmodia in digestive tubule epithelia, sometimes with large, anomalous nuclei and a generally deteriorating appearance. We hypothesize that these plasmodia are evidence of failed sporulation, after which parasites die without completing their life cycle in the oyster. Their death consequently results in the post May/June drop in prevalence.

Observational evidence, then, suggests a difference in the environment experienced by *H. nelsoni* in young/small oysters, which allows the parasite to form spores, and that in larger/older hosts, which does not. This difference is not a question of differential susceptibility or resistance because adult oysters of both types do not support spore formation. For purposes of the model, the internal environmental quality needed for sporulation was related directly to the potential growth efficiency of the host and indirectly to food availability. Growth efficiency is an index to the amount of energy available after the host's basic metabolic requirements are met. This energy should be available to the parasite in the form of nutritional resources and relatively more of it should be available in younger oysters because of their higher growth efficiency.

Spore formation, in the model, begins with the accumulation of nutritional reserves and the accompanying intensification of infections. The parallel field observation is the movement of parasites into the digestive tubule epithelium, where they begin to undergo the many changes that accompany sporulation (Perkins 1969). The initial stages of simulated sporulation can happen regardless of oyster size, but to inhibit completion of the process in large oysters, the model establishes a threshold quantity of reserves that must be exceeded for spore production to occur. If that threshold is not reached, the process is not completed. Because of their higher growth efficiency, the threshold is exceeded only in small oysters, which consequently are the only oysters in which spores are formed. If the threshold is reached, sporulation is successful. Spores are shed from live oysters or after the host dies. The model considers that parasites that fail to sporulate are no longer viable. They become susceptible to hemocyte attack and are eliminated. In either case, resulting model simulations show a dramatic reduction in prevalence, as is seen in field observations.

The growth-efficiency basis for sporulation used by the model is hypothetical, as is failed sporulation, to explain the early summer prevalence decline in adult oysters. Some other factor, perhaps a chemical or physical "cue" having nothing to do with growth efficiency or nutritional status, may well trigger sporulation. Or, there may be a suite of elements involved that occur in juveniles only. Nevertheless, the concept of a necessary threshold of some factor or factors remains a biologically defensible generalization for the fact that *H. nelsoni* can complete its life cycle in small oysters, but rarely in large ones.

Modeling of the sporulation process needed to take into account the observation that spores are formed in juveniles in the

autumn, as well as in the spring (Andrews 1979, Burreson 1994). The process is probably set in motion in adults, too, but is rarely successful. In the fall, however, there is no abrupt prevalence decline. The model achieves this result in two ways. First, food supply is lower in the fall so only the smallest oysters have a potential growth efficiency adequate to trigger sporulation. Second, the model contains a temperature dependency on the loss of viability of plasmodia that have failed to sporulate. Thus, if sporulation fails at relatively low temperature, plasmodia become less susceptible to hemocyte attack than those failing at relatively high temperature. Oysters remain infected and eventually die in late winter. In fact, if the "internal environment cue" hypothesis is correct and is related to the accumulation of nutrients, the slower reserve build up in adults compared to juveniles may simply retard the spore-formation process until the temperature is too low for parasite activity, so the plasmodia are never damaged.

### Salinity Effects

Temperature is undoubtedly the most important environmental variable influencing the seasonal infection cycle, both directly and indirectly, and in the field and in the model. Salinity is also important, but its effect is more obvious when considered on spatial or long-term temporal scales (Paraso et al. this volume, Powell et al. this volume). In the model, salinity affects *H. nelsoni* inside the oyster by affecting both survival and proliferation rates. Both are parameterized from *in vitro* experiments describing survival of plasmodia after acute salinity change (Ford and Haskin 1988). Results of these trials showed that survival was very low below about 9 ppt and very high above about 15 ppt, which roughly approximates its distribution in nature (Ford and Tripp 1996). Between those ranges, the parasite is highly sensitive to small salinity change. The model also considers that inside the oyster, parasites are buffered from rapid changes in salinity by the behavior of oysters themselves. When exposed to a large salinity change, bivalves typically close their valves and thereafter open them only briefly so as to allow entry of only small amounts of ambient water (Schoffeniels and Gilles 1972, Davenport 1979). The salt content of their body fluid thus changes more slowly than does the external water. Consequently, the model extends the *in vitro* death rate over a period of 4 days. In the absence of data on the effect of salinity on *in vivo* doubling times, it seems reasonable to assume that the salinity range over which it occurs is roughly the same as for survival, and that within this range, the response pattern is similar.

In the model, salinity also affects the rate at which parasites move into the systemic tissue from the epithelium: at low salinity, the rate increases. This was a way to maintain the constant ratio of systemic to local infections observed along the salinity gradient (Haskin and Ford 1982, Fegley et al. 1994). Without it, the frequency of systemic infections decreased with decreasing salinity. Low salinity may, in fact, make it easier for parasites to make this transition, although the physiological mechanism is unclear. The actual reason may be quite different and this may be a case where the mathematical device provided a good approximation of observed patterns without a good biological rationale. Nevertheless, the need to include a factor that increased the proportion of systemic infections indicates that a simple salinity effect on parasite survival and growth is not sufficient to explain what is observed in field data.

### Oyster Mortality

Oysters die, in the model, when *H. nelsoni* densities exceed that which is seen in live oysters. The same is true for the *P. marinus* model, but the *H. nelsoni* model also reflects the fact that the lethal parasite density for some oysters is lower than this maximally observed level. A few individuals die with relatively light infections and an increasing proportion die as infections intensify. It is this variation in ability to tolerate infections that forms one of the bases for selective breeding: comparisons between oyster strains selected and unselected for resistance to MSX disease indicate that one measure of resistance is the ability to survive with relatively heavy infections (Ford and Haskin 1987, Ford 1988, B. J. Barber et al. 1991).

### Transmission

Incomplete knowledge of the life cycle and mechanism of transmission of *H. nelsoni* is probably the single greatest impediment to further understanding this important parasite and the disease it causes. The sparsity of information about transmission made modeling this aspect of MSX disease particularly difficult because many assumptions had to be made. Yet the exercise was both intriguing and insightful. The transmission model is a separate component of the overall *H. nelsoni*-oyster model. It differs from most transmission models in that it simulates success or failure of transmission based on external environmental factors rather than on the density and infection levels of neighboring oysters. Modeling of the transmission process is detailed and discussed by Powell et al. (this volume).

### SUMMARY

The component of the *H. nelsoni* model that describes host-parasite interactions inside the oyster is constructed using functions describing physiological rates for both organisms: proliferation, translocation, and death (or degradation) of the parasite; and hemocyte activity, filtration rate, and growth efficiency of the oyster. The rates, in turn, are controlled by four environmental variables: temperature, salinity, food, and total seston. Using only these few elements, the model is able to reproduce the bimodal annual infection cycle that includes infection intensification and remission, a life stage change of the parasite, response of the oyster's internal defense system, and, eventually, oyster death. With few exceptions, the physiological rate functions are based on experimental or observational evidence or general physiological principles. For instance, the effect of salinity on *in vivo* parasite survival, and the response of oyster hemocytes to dead or damaged parasites is well grounded with experimental, as well as observational, data (Haskin and Ford 1982, Fisher and Tamplin 1988, Ford and Haskin 1988, Ford et al. 1993, Ford and Ashton-Alcox 1998). Parasite doubling times and the relationship between oyster mortality and infection intensity were computed directly from field data (Andrews 1966, Ford and Haskin 1982). Physiologically well-reasoned arguments were made for the self-crowding effect, the release of crowding in the spring, parasite degradation over the winter, differences in parasite growth and death rates between epithelial and systemic compartments, and the "threshold" trigger for sporulation. Whether failed sporulation in adult oysters is the cause for the rapid prevalence decline after the spring infection peak, whether lower salinity facilitates the movement of parasites from the epithelium into the systemic tissues, and the increased "efficiency" of the hemocyte component in the epithelium are

highly conjectural. Because virtually nothing is known about the transmission mechanism, this component of the model includes more hypothetical elements: specifically the infective dose threshold and the concentration of infective particles and their relationship to salinity and temperature (Powell et al. this volume).

The fact that certain hypothetical mechanisms were used to fit the model to observation does not detract from its efficacy. Because the simulations reproduce observed temporal and spatial patterns, and assuming that the major biological and physical systems involved have, at some level, reasonably predictable responses, the model suggests ways in which the host-parasite system must work. For instance, the modeling exercise clearly shows that temperature effects on parasite doubling times or salinity effects on *in vivo* parasite survival, cannot by themselves, explain field observations. The model demonstrates that other factors must be involved and points to where efforts must be concentrated to gain a better understanding of the overall host-parasite relationship. Clearly, an improved knowledge of the complete system rests with a better understanding of the parasite's life cycle and mode of transmission, combined with an ability to infect oysters experimentally. Nevertheless, the fact that this very complex and detailed model works, with few modifications, in Chesapeake Bay as well

as in Delaware Bay, is a measure of its power and potential usefulness in other areas.

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## MODELING THE MSX PARASITE IN EASTERN OYSTER (*CRASSOSTREA VIRGINICA*) POPULATIONS. II. SALINITY EFFECTS

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**ABSTRACT** An oyster population model coupled with a model for *Haplosporidium nelsoni*, the causative agent of the oyster disease MSX, was used with salinity time-series constructed from Delaware River flow measurements to study environmentally-induced variations in the annual cycle of this disease in Delaware Bay oyster populations. Model simulations for the lower Bay (high salinity) site reproduced the annual cycle observed in lower Delaware Bay. Simulations at both upper Bay (low salinity) and lower Bay sites produced prevalences and intensities that were consistent with field observations. At all sites, low freshwater discharge resulted in increased disease levels, whereas high freshwater discharge produced decreased levels. At upper Bay sites, simulated changes in runoff produced high variability in disease prevalence; in the lower Bay, they produced a much lesser effect. Changes in salinity within the 10–20 ppt range produced the greatest changes in disease levels and patterns. Simulated shifts in timing of the spring runoff from March to either February or May affected the mid-Bay (13–19 ppt) only. A February runoff reduced the spring prevalence peak and caused a complete loss of systemic infections. In contrast, a May discharge occurred too late to affect parasite proliferation in the spring so that the spring peak was higher than average. Almost 100% of the infections were systemic by June, which resulted in high oyster mortality during July at this site. Model results indicate that parasite infection intensity under changing salinity is more complex than a simple function of salinity as it affects parasite proliferation and death rates within the oyster, and that the rate of infection is most likely reduced at low salinity. The simulated results demonstrate the ability of the model to reproduce field measurements and its usefulness in elucidating the association between the magnitude and timing of Delaware River discharge, its associated salinity variations, and the *H. nelsoni* annual cycle.

**KEY WORDS:** MSX, model, eastern oyster, *Crassostrea virginica*, *Haplosporidium nelsoni*, Delaware Bay, salinity

### INTRODUCTION

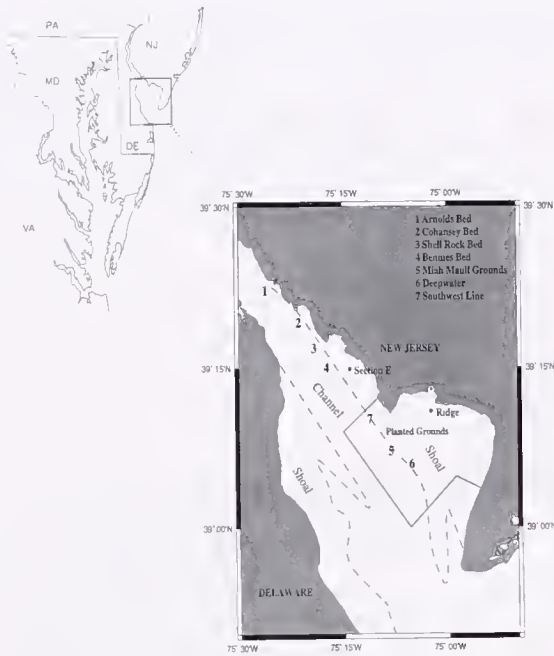
The Eastern oyster, *Crassostrea virginica*, has been an important economic resource in Delaware Bay (Fig. 1) for at least 2 centuries (Ford 1997). The practice of “farming” oysters, which began in the mid-19th century, involves the transplantation of seed oysters during May and June from natural setting areas (seed beds) in the upper regions of the Bay to privately leased sections (planted grounds) in the lower Bay (Ford and Haskin 1982). Salinity of the water covering the seed beds ranges from 9 to 18 ppt under mid-tide, mean river-flow conditions, which protects the juvenile oysters from major predators (e.g., oyster drills) that are intolerant of low salinities and hence enhances oyster survival. The planted grounds are located in areas of higher salinity (20–23 ppt) that promotes growth and fattening of the oysters. Traditionally, oysters have been marketed the fall following the spring planting or have remained on the planted grounds an additional year or more before harvesting (Ford and Haskin 1982).

In the late 1950s and early 1960s, the causative agent of the Eastern oyster disease MSX (Multinucleated Sphere Unknown), was first observed in Delaware and Chesapeake Bays (Andrews 1966, Haskin et al. 1966). It was described as a haplosporidian by Haskin et al. (1966) and named *Haplosporidium nelsoni* (Sprague 1978). By 1959, 90%–95% of the planted ground oysters and about 50% of seed bed oysters had died in Delaware Bay. Gradually, in the late 1960s and the 1970s, Delaware Bay seed beds recovered as native oysters developed some resistance to MSX disease (Haskin and Ford 1979, Ford 1997). Following 15 years of

modest success, the Delaware Bay oyster industry suffered another setback due to a resurgence of *H. nelsoni*, which made significant incursions onto the seed beds during a drought in the mid 1980s.

The annual infection cycle (Fig. 2) of *H. nelsoni* in lower Delaware Bay oysters was described by Ford and Haskin (1982). New infections are acquired beginning in early June and for the remainder of the summer, infections increase in number and intensity, reaching peak infection levels in late fall. Prevalences remain relatively stable through late winter, and then drop as heavily infected oysters die and parasites are lost from the remaining oysters. Prevalence and intensity increase again in April and May, coincident with rising temperatures, and a resurgence of infections that were suppressed by low winter temperatures (Ford 1985b). The spring peak, often having the most intense infections of the year, is typically followed by a rapid loss of infections, the reason for which is not completely understood. By this time, infective particles are once again present in the water and the cycle begins again.

Since poikilothermic and poikilosmotic animals are directly affected by the temperature and salinity of the environment in which they live, interactions between a parasite and a poikilothermic or poikilosmotic host may be strongly influenced by external environmental conditions. For example, temperature and salinity have well-documented effects on the relationship between *H. nelsoni* and *C. virginica* (e.g., Andrews 1964, Andrews 1983, Ford 1985a, Ford and Haskin 1988). Early field observations suggested that the parasite was salinity limited (Andrews 1964, Andrews

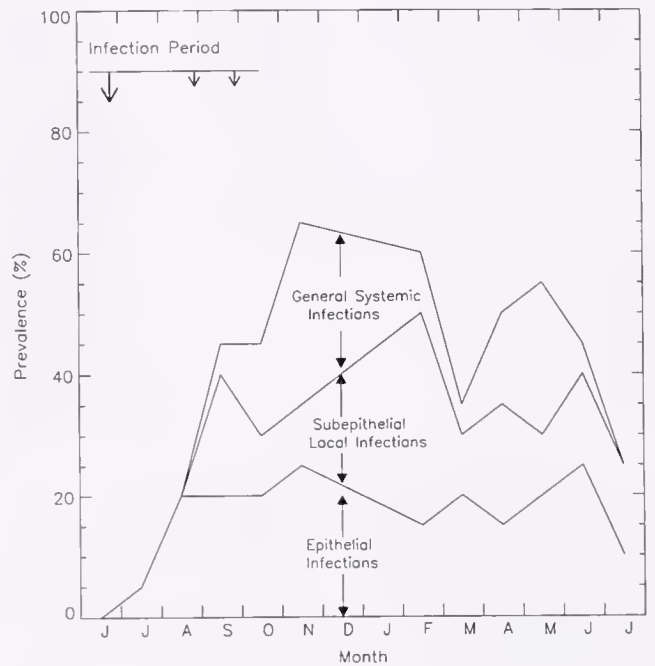


**Figure 1.** Location of Delaware Bay. Inset shows locations of the New Jersey oyster seed beds (sites 1–4) and the planted ground (sites 5–7) stations. The solid line indicates the area designated as planted grounds. The (\*) indicates the locations where time series of temperature, chlorophyll *a*, and total seston were acquired. The dashed line distinguishes between shnal and channel areas as designed by Pennoek and Sharp (1986).

1983, Farley 1975, Haskin and Ford 1982). Although oysters are capable of activity at salinities as low as 5 ppt, infected oysters are extremely rare at salinities below 10 ppt and MSX disease does not become epizootic at salinities below 15 ppt (Andrews 1964, Andrews 1983, Haskin and Ford 1982). Ford (1985a) observed a rapid loss of parasites as mid-tide salinities decreased from 15 to 5 ppt. Full parasitic activity occurs at salinities above 20 ppt (Haskin and Ford 1982, Andrews 1983).

Haskin and Ford (1982) proposed two explanations for the observed decrease in *H. nelsoni* prevalence along a salinity gradient in Delaware Bay. The first hypothesis, which does not involve a direct salinity effect, is that the principal source of infective particles is in the lower Bay. Consequently, the concentration of *H. nelsoni* infective particles in the upper Bay would be reduced due to increased distance from their source (Haskin and Ford 1982).

The second hypothesis is that reduced prevalence in low salinity areas was the result of a physiological response, either by the host or the parasite. Andrews (1983) suggested that low salinities might enhance the oysters' natural defenses, resulting in active expulsion of the parasite. Fisher and Newell (1986) found that *in vitro* rates of hemocyte locomotion were greater in low, compared to high, salinity and suggested that enhancement of hemocyte activity at low salinity might play a role in the expulsion of *H. nelsoni* from oysters in low salinity water. On the other hand, because the oyster does not osmoregulate, the parasite is exposed to salinities that mirror the ambient water. Thus, the parasite may be physiologically incapable of surviving in less than 10 ppt (Andrews 1964, Andrews 1983, Haskin and Ford 1982, Ford 1985a). Ford (1985a) proposed that a physiological limitation may restrict the distribution of *H. nelsoni* in low salinity water. Tolerance of plasmodial stages to acute *in vitro* salinity change roughly parallels



**Figure 2.** Schematic of the annual *Haplosporidium nelsoni* infection cycle reproduced from Figure 4 in Ford and Haskin 1982. Filled arrows represent the percentage of oysters in each infection level (prevalence), with epithelial infections representing the lightest oysters (BFU category 1) and general systemic infections representing the heaviest infections (BFU category 4). The infection period designates the time in which infections begin in uninfected oysters and are designated by the unfilled arrows. The length of the arrows represents the relative intensity of infection pressure. The area above the general systemic infection line represents the portion of oysters in the undetectable category.

the parasite's distribution in nature and indicates that its reduced occurrence in low salinity water is most probable due to its physiological inability to tolerate reduced salinity, rather than to enhanced effectiveness of host defense mechanisms (Ford and Haskin 1988).

To investigate the effect of environmental factors, specifically salinity, on the time and space distribution of MSX disease in Delaware Bay oyster populations, simulations were done with a time-dependent numerical model for oyster populations that was coupled with a model for *H. nelsoni*. The oyster-*H. nelsoni* model includes the physiological processes that affect the parasite-host interaction and are responsible for the predictable cycle in infection intensity and prevalence observed in Delaware Bay (Ford et al. this volume). Simulations were done to determine how seasonal variations (timing and strength) in Delaware River flow affect *H. nelsoni* prevalence and intensity at various sites within the Bay and the results from these were then compared with long-term field observations from the same sites.

**MATERIALS AND METHODS**

*The Delaware Bay Study Site*

Delaware Bay was chosen as a study site because a long-term database on MSX-disease exists for its oyster population. The Bay (Fig. 1) is a funnel-shaped estuary, extending 215 km from the head at Trenton, New Jersey, to the mouth between Cape Henlopen, Delaware, and Cape May, New Jersey (Wong 1994, Wong



1995, Ford 1997). The Delaware River, gauged at Trenton, New Jersey, contributes approximately 58% of the total freshwater input to the estuary, with an average discharge of 330 m<sup>3</sup> s<sup>-1</sup> (Sharp et al. 1986, Wong 1995). The Schuylkill River entering through Philadelphia, Pennsylvania, near the head of the Bay, contributes another 15% of the total freshwater input. No other single freshwater source provides more than 1% of the total discharge (Sharp et al. 1986). A direct, negative correlation exists between salinity in Delaware Bay and the Delaware River flow (Fegley et al. 1994, Wong 1995).

Five sites in Delaware Bay were selected for this study (Fig. 1). Arnolds, Cohansey, Shell Rock, and Bennies Beds are on natural seed beds, whereas Miah Maull Grounds are located in the planted grounds. The depth of the seed beds ranges from 5 to 7 m. The location and area of each seed bed is described in Fegley et al. (1994). Arnolds Bed is located 64.1 to 67.0 km upbay from the mouth of the Bay and 0.1 to 2.9 km off the New Jersey shore, and has an area of 2.32 km<sup>2</sup>. Cohansey Bed, located 54.4 to 58.5 km upbay and 0.1 to 3.4 km offshore, is 5.45 km<sup>2</sup>. Shell Rock Bed with an area of 4.04 km<sup>2</sup>, is located 50.5 to 53.4 km upbay and 1.4 to 3.6 km offshore. The seed bed site furthest downbay, Bennies Bed, is located 45.4 to 49.7 km upbay and 1.7 to 5.4 km offshore, and has an area of 6.36 km<sup>2</sup>. Miah Maull Grounds is the site closest to the mouth and is representative of the entire leased area. This site is located approximately 25.9–27.8 km from the mouth, 6.5 km from the New Jersey shore, and occupies an area of approximately 32.3 km<sup>2</sup>.

**Oyster Population–Haplosporidium nelsoni Model**

**General Characteristics**

A time-dependent oyster population–*H. nelsoni* model, described in Ford et al. (this volume), was used to stimulate infection prevalence and intensity along the salinity gradient in Delaware Bay. The host-parasite model contains components to simulate the dynamics of a single size-class oyster population (1 g dry wt, in this case), the proliferation and death of *H. nelsoni* in those oysters (Fig. 5 in Ford et al. this volume), and transmission of *H. nelsoni* (Powell et al. this volume). The model components are coupled by relationships that describe the infection of uninfected oysters, the removal of oyster energy by the parasite to support its metabolism, and the relationship of parasite density in host tissue to oyster mortality.

**Governing Equation**

The governing equation for the oyster-parasite model moves oysters among *H. nelsoni* infection classes as parasite densities increase or decrease in the epithelial (*e*) and systemic (*s*) tissue of the oyster (*O*) over time. It is formulated as:

$$\begin{aligned} \frac{dO_{e,s}}{dt} = & -\alpha_{e,s}O_{e,s} - \beta_{e,s}O_{e,s} + \alpha_{e,s-1}O_{e,s-1} \\ & + \beta_{e-1,s}O_{e-1,s} + \alpha_{e,s+1}O_{e,s+1} \\ & + \beta_{e+1,s}O_{e+1,s} - M_{e,s}O_{e,s} - \delta_{e,s4}O_{e,s4} \\ & + \delta_{e1}\delta_{s0} \frac{1}{2} \sum_{s=5,4}^N \sum_{e=0}^N \delta_{e,s}O_{e,s} \end{aligned} \quad (1)$$

where the first 6 terms represent the movement of oysters between infection intensity classes through gains or losses of *H. nelsoni* cells in the epithelial and systemic tissue (Fig. 3 in Ford et al. this volume). The coefficients,  $\alpha$  and  $\beta$ , determine the rate at which *H. nelsoni* cells are gained or lost. The parameterizations used to

determine these coefficients are given in Ford et al. (this volume). The seventh term in equation (1) represents the loss of parasites through oyster mortality from lethal infections as determined by the rate of mortality, *M*. The final 2 terms in equation (1) represent the transfer of oysters from heavy infection classes to lower infection classes due to the formation or attempted formation of spores by *H. nelsoni* in the oysters with advanced infections, which results in a loss of parasites from infection classes with BFU = 4 (*s*<sub>4</sub> in equation 1), and a gain of infected oysters into infection class [1,0]. The  $\delta$  functions represent a step-function process in which the oysters are introduced into the [1,0] infection class only. The coefficient  $\gamma$  determines the rate of this transfer process.

The establishment of infection in uninfected oysters ([0,0] class) is determined by the equation:

$$\frac{dO_{0,0}}{dt} = -\beta_{0,0}O_{0,0} + \frac{1}{2} \delta_{e,0} \delta_{0,s} \sum_{s=5,4}^N \sum_{e=0}^N \gamma_{e,s} O_{e,s} \quad (2)$$

where the first term represents the acquisition of *H. nelsoni* infective particles at a rate determined by  $\beta_{0,0}$ . The second term represents addition of oysters to the uninfected class after hypothesized abortive *H. nelsoni* sporulation events, as described in Ford et al. (this volume), and is given by the eighth term in equation (1). These oysters are divided evenly between the [0,0] and [1,0] infection classes, as given by the last terms in equations (1) and (2).

**The Oyster Population Model**

The model component that describes the time-dependent development of the oyster population model is that described in Powell et al. (1992, 1994, 1995, and 1996) and Hofmann et al. (1992, 1994). For the purpose of this study, only a single size class of oyster (1 g dry wt) was used. The oyster model includes parameterization for the processes that determine the production of somatic and reproductive tissue, which are based on assimilation efficiency, filtration rate, ingestion, and respiration as modified by the environmental conditions of food supply, total seston, temperature and salinity.

**The *H. nelsoni* Model**

Parameterization of the processes that were used to develop the oyster population–*H. nelsoni* model, and the biological bases for the mathematical relationships, are described and discussed by Ford et al. (this volume). Processes that influence *H. nelsoni* interactions with its host include the rate of infection as a function of oyster filtration rate and ambient infective particle concentration; a density-dependent control on parasite proliferation based on oyster accumulated potential growth efficiency; parasite sporulation or attempted sporulation related to oyster accumulated potential growth efficiency; and *H. nelsoni* mortality due to cold exposure and increased susceptibility to oyster hemocytes, which themselves are influenced by temperature. The processes by which oysters move among infection categories is described in Ford et al. (this volume).

In addition to *in vivo* processes, the host-parasite model contains a subcomponent for disease transmission, which is dependent on infective dose, the filtration rate by which infective particles reach the oyster, and seasonal and salinity-dependent processes that control the concentration of infective particles in the water column (Powell et al. this volume). As this study is directed at salinity effects on the MSX disease process, the parameterizations involving salinity are discussed in more detail below.

**Salinity Parameterizations**

The effect of salinity on *H. nelsoni* mortality was parameterized using measurements of acute *in vitro* salinity tolerance of plasmodia, the most common stage in oysters (Ford and Haskin 1988). This mortality effect begins at 15 ppt, increases in intensity from 15 to 5 ppt, and causes rapid *H. nelsoni* mortality below 5 ppt. A sigmoidal function was fit to observations of the fraction of live plasmodia remaining after exposure to a range of salinities and modified to ensure that no parasite mortality occurred above salinities of 15 ppt (Fig. 3). It is formulated by

$$Smort = \min \left( 1, \frac{0.01 SD_1}{1 + \frac{SD_1 - SD_2}{SD_2} e^{-SD_3 S}} \right) \quad (3)$$

where *Smort* is the fraction of parasites still alive after exposure,  $SD_1 = 103$ ,  $SD_2 = .240650$ ,  $SD_3 = 0.592456 \text{ ppt}^{-1}$ , and *S* is salinity in ppt. The salinity response was assumed to have a time scale of 4 days, which reflects the estimated period required for oyster hemolymph to equilibrate with external salinity after an ambient salinity change and for parasites to respond to the new conditions. The death rate of *H. nelsoni*, *Sdeath*, expressed as  $d^{-1}$  is then given by:

$$Sdeath = \frac{-\ln(Smort)}{SD_4} \quad (4)$$

where  $SD_4 = 4.0$ . Under normal circumstances it is assumed that *H. nelsoni* infections, once acquired, are never lost. Therefore, the

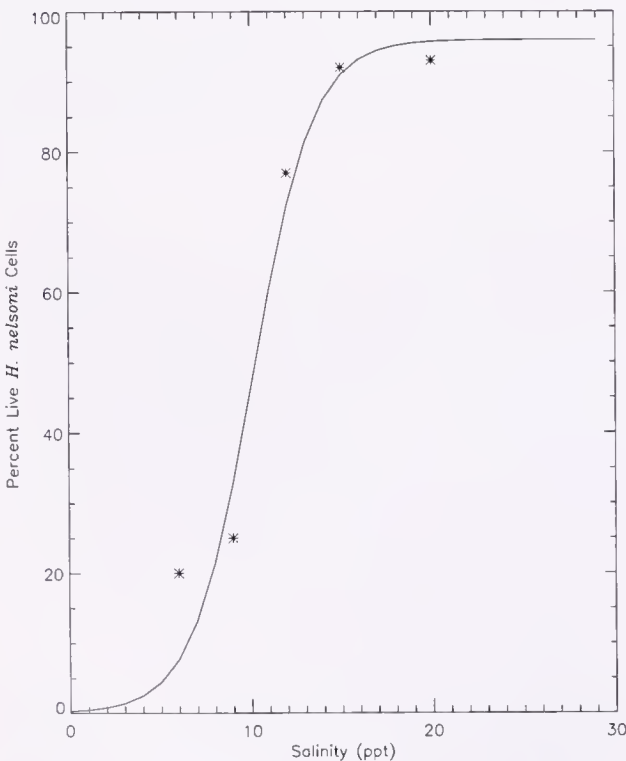


Figure 3. The relationship between salinity and acute *in vitro* *H. nelsoni* mortality based on observations (asterisks) by Ford and Haskin (1988) of the fraction of *H. nelsoni* plasmodia appearing viable (i.e., excluding Trypan Blue dye) after exposure to a range of salinities.

oysters cannot decrease through *H. nelsoni* cell mortality to a level lower than an initial epithelial infection with no systemic infection ( $e_1, s_0$ ). However, for salinity mortality, it is assumed that the infection can be lost at salinities  $\leq 14$  ppt, which is equivalent to  $Sdeath = 0.01787$ .

It was assumed that salinity effects on parasite proliferation rates occur over the same salinity range producing cell mortality. The effect of salinity on cell growth was parameterized as an exponential relationship that produces reduced growth rates at salinities less than 14 ppt and essentially no growth at salinities less than 5 ppt. The form of the salinity-dependent *H. nelsoni* growth rate, *Sfactor*, is:

$$Sfactor = e^{sg(S-S_0)} \quad (5)$$

where *sg* determines the rate of decrease of parasite proliferation rate with increasing salinity and  $S_0$  is 15 ppt, which represents the threshold above which no reduction in parasite proliferation rate occurs. The salinity effect on cell growth is such that it has the value of zero at  $S \leq 5$  ppt and the value of one at  $S > S_0$ . Equation (5) modified the temperature-dependent *H. nelsoni* growth rate that is given in equation (4) of Ford et al. (this volume).

Initial simulations with the above model showed prevalences on the seed beds that were higher than those observed (Haskin and Ford 1982, Fegley et al. 1994). Subsequent simulations indicated that the source of this mismatch was related to the rate at which oysters became infected and suggested that reduced salinity, in some way, reduced infection efficiency. The model thus contains a parameter that varies transmission rate by reducing the number of infective particles at low salinity and is of the form:

$$IPsal = \frac{1 + \tanh \left( SM_1 \frac{(S - SM_0)}{SM_2} \right)}{2} \quad (6)$$

where *IPsal* is the fractional reduction in spore number that is applied to the calculation of spores filtered given by equation (29) in Ford et al. (this volume). The constants  $SM_1, SM_2$ , and  $SM_3$  have the values 1.6, 11.0, and 7.0, respectively, and *S* is the ambient salinity. Equation (6) is biologically valid because the salinity range affecting transmission is similar to the range causing cell mortality *in vivo*. However the effect described by equation (6) has a somewhat wider range, as might be expected by direct exposure to a free-living infective particle. Whether salinity causes cell mortality or just reduced infectivity in infective particles is unknown at present.

The initial simulations for the seed bed sites also showed a decreasing infection intensity at upbay sites, which is inconsistent with observations that showed a single-step drop in infection intensity from the planted grounds to the seed beds, with little additional change related to decreasing salinity (Haskin and Ford 1982). Essentially, the simulations underestimated the systemic infections at low salinity. To include this effect the rate of *H. nelsoni* diffusion from the epithelial to systemic tissue, *Sdiff*, was increased at low salinity using the relationship:

$$Sdiff = 1. + SF_1 \frac{\left\{ 1. - \tanh \left[ SF_2 \left( \frac{S - S_0}{SF_3} \right) \right] \right\}}{2} \quad (7)$$

where the reference salinity,  $S_0$  is 15 ppt and the constants  $SF_1, SF_2$ , and  $SF_3$  have the values 9.0, 2.65, and 3.0 ppt, respectively.

The value of  $S_{diff}$  was set to be 0.955 at 12.0 ppt, 0.5 at 15.0 ppt, and 0.005 at 18.0 ppt. Equation (7) gives the fractional reduction in *H. nelsoni* cell diffusion rate that modifies equation (6) in Ford et al. (this volume). This relationship is based on the rationale that osmotic stress increases the leakiness of the basement membrane thereby permitting *H. nelsoni* cells to more easily cross to the oyster circulatory system.

#### Environmental Data Sets

The environmental factors that force the oyster population–*H. nelsoni* model are salinity, temperature, food, and total seston. Data sets for temperature, chlorophyll *a* (representing food), and total suspended solids (representing total seston), collected over a 5-year period (1981–1986), were obtained from the Haskin Shellfish Research Laboratory of Rutgers University. Collections used for this study were made at Section E (representing the seed beds) and at the Ridge (representing the planted grounds) sites (Fig. 1). Data collected in each month were averaged over all years to construct a representative 1-year time series.

#### Temperature Data Sets

Temperatures for both Section E and Ridge time series began to increase during February and reached a maximum of about 26 °C in July and August (Fig. 4A). Temperature decreased with the onset of autumn and a minimum temperature of (2 °C) occurred in February.

#### Food Data Sets

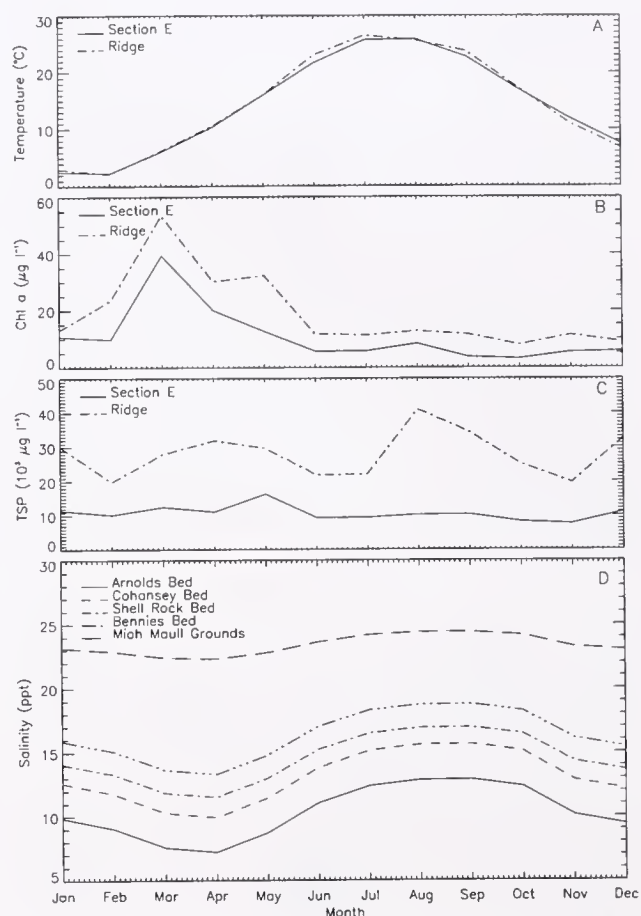
Overall, chlorophyll *a* measurements, representing food available to the oyster, were approximately 5–10  $\mu\text{g L}^{-1}$  higher at Ridge than at Section E (Fig. 4B). Chlorophyll levels began to increase earlier (January) at the Ridge site, compared to the Section E site, where the increase began in February. Maximum chlorophyll concentrations occurred during March and were approximately 55  $\mu\text{g L}^{-1}$  at Ridge and 40  $\mu\text{g L}^{-1}$  at Section E. Chlorophyll then decreased steadily at Section E, with a minimum of approximately 3  $\mu\text{g L}^{-1}$  occurring in October. Levels at Ridge remained relatively high (32  $\mu\text{g L}^{-1}$ ) through May, then decreased to a minimum of 8  $\mu\text{g L}^{-1}$  in October.

During verification studies of the oyster–*P. marinus* model in Galveston Bay, Texas (e.g., Powell et al. 1995), agreement between the simulated oyster populations and observed populations was improved if a non-chlorophyll component was added to the chlorophyll measurements to obtain total food supply. This modification to the chlorophyll time series was done using an empirical relationship between chlorophyll and total available food (Soniati et al. 1998) of the form:

$$\text{Food} = a \times \text{chlorophyll} + b \quad (8)$$

where *Food* is given in  $\text{mg DW L}^{-1}$  and chlorophyll is in  $\mu\text{g L}^{-1}$ . The constants *a* and *b* have the values of 0.088  $\text{mg DW } \mu\text{g}^{-1}$  and 0.52  $\text{mg DW L}^{-1}$ , respectively. This equation recognizes that total available food can include a significant non-chlorophyll component.

Similarly, verification studies with the oyster–*P. marinus* model for Delaware Bay (Powell et al. 1997) showed that chlorophyll alone did not provide an adequate food supply for the oysters. Therefore, the relationship between chlorophyll and total available food in Delaware Bay was assumed to be of the same



**Figure 4.** Time series of environmental data measured by the Haskin Shellfish Research Laboratory (monthly averages for 1981 to 1986), used as input to the model to simulate average conditions. Panels A, B, and C show time series of temperature, food, and total seston (TSP = total suspended particulates) data, respectively, for seed bed (Section E) and planted ground (Ridge) sites. Panel D shows mean salinity time series, estimated from freshwater inflow data, for the seed bed (Arnolds, Cohansey, Shell Rock, and Bennies Beds) and planted ground (Miah Maull Grounds) sites.

form as that for Galveston Bay and the coefficients were determined by an iterative procedure in which simulations and observations were compared (see Powell et al. 1997 for details). This procedure resulted in a conversion to food supply given by equation (7), but with *b* having the value 0.26  $\text{mg DW L}^{-1}$ .

#### Total Seston Data Sets

Total seston, as measured by total suspended solids, was relatively constant at Section E ( $10\text{--}12 \times 10^3 \mu\text{g L}^{-1}$ ) with a slight increase in May to  $16 \times 10^3 \mu\text{g L}^{-1}$  (Fig. 4C). The Ridge total seston time series was more variable than in Section E, ranging from  $20 \times 10^3 \mu\text{g L}^{-1}$  in November to  $41 \times 10^3 \mu\text{g L}^{-1}$  in August (Fig. 4C). Total seston values were greater at Ridge for all months.

#### Salinity Data Sets

Monthly-averaged Delaware River flow for the period 1913 to 1993 was measured at Trenton, New Jersey, by the United States Geological Survey. Salinity time series were then calculated for

1953 to 1993, using a relationship between Delaware River flow and salinity derived by Haskin (1972):

$$y = c + dx^e \tag{9}$$

where  $y$  is salinity in ppt calculated from the preceding mean 30-day river flow ( $x$ ) in  $\text{ft}^3 \text{s}^{-1}$  ( $1 \text{ ft}^3 \text{ s}^{-1} = 2.83 \times 10^{-2} \text{ m}^3 \text{ s}^{-1}$ ). Derived values of  $c$ ,  $d$ , and  $e$  for each of the 5 sites shown in Figure 1 are presented in Table 1. The resultant salinity time series were used as input into the oyster population-*H. nelsoni* model.

At each site, the 8 years (20%) between 1953 and 1993 with the highest calculated average monthly salinity were grouped together and a mean salinity calculated for each month at each site. The resultant time series were assumed to be representative of a year with low freshwater runoff (Fig. 5A). Similarly, the 8 years (20%) with lowest monthly mean salinities were also grouped for each site and the salinity time series was taken to represent a year with high freshwater runoff (Fig. 5B). Salinities for the remaining 24 years (60%) were averaged to create a mean freshwater runoff time series (Fig. 4D).

Although the salinity values changed (Table 2), the seasonal pattern for average and low runoff years was similar (Figs. 4D, 5A). Salinity minima occurred in March/April and the maximum values extended from July through October. The pattern for a high runoff year was only slightly different, with a distinct minimum occurring in April and a shortened period of maximum salinities extending from July through September (Fig. 5B).

To simulate a shift in the timing of spring runoff, the maximum freshwater discharge, which occurred during March/April in the mean time series (Fig. 4D), was moved to either February or May (Fig. 5C, D). For these simulations, only the timing was changed; the quantity of freshwater discharge remained that of an average year.

***H. nelsoni* Prevalence and Intensity Data**

Prevalence and intensity of *H. nelsoni* in Delaware Bay oysters was measured over the period 1959 to 1992 by personnel from the Haskin Shellfish Research Laboratory (Ford and Haskin 1982, Haskin and Ford 1982, Fegley et al. 1994). Oysters from the lower Bay planted grounds were obtained by dredge at regular intervals during the year. Seed bed oysters were typically collected during late autumn/early winter and the following late spring. These dates were chosen to coincide with peaks in the annual infection cycle (July 1 to June 30 of the following year) (Fig. 2). Stained tissue sections of each oyster were examined to determine the distribution and abundance of *H. nelsoni* in the tissues, and these were

TABLE 1.

Derived constants for the Delaware River flow-Delaware Bay salinity relationship ( $y = a + bx^c$ ) for the 5 sites displayed in Figure 1, where  $y$  is the salinity in ppt and  $x$  is river flow in  $\text{ft}^3 \text{ s}^{-1}$  (From Haskin 1972).

Site	$a$ ppt	$b$ ppt ( $\text{s ft}^{-3}$ ) <sup>c</sup>	$c$ non-dimensional
Arnolds Bed	-37.85	99.32	-0.08036
Cohansey Bed	-38.91	102.32	-0.07465
Shell Rock Bed	-45.91	104.95	-0.06083
Bennies Bed	-64.25	122.04	-0.04575
Miah Maul Grounds	19.86	152.16	-0.41722

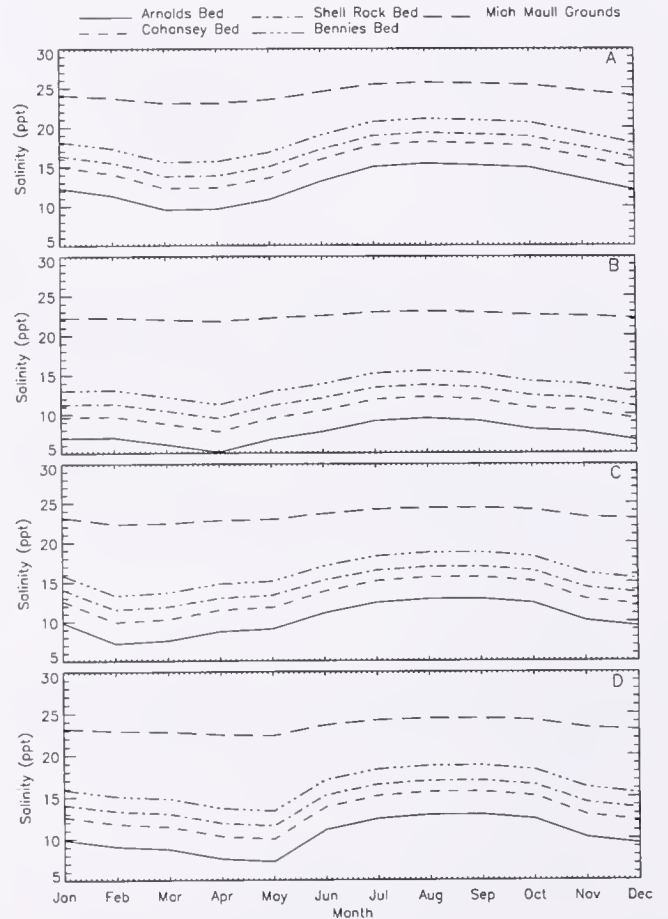


Figure 5. Idealized time series of salinity, constructed from Delaware River flow measurements, used as input to the model to simulate A) low freshwater runoff, B) high freshwater runoff, C) a year with an early spring (February) freshwater runoff period, and D) a year with a late spring (May) freshwater runoff period at seed bed (Arnolds, Cohansey, Shell Rock, and Bennies Beds) and planted ground (Miah Maul Grounds) sites.

used to develop a scale, Big Ford Units (BFU, described in Ford et al. this volume), for comparing observed and simulated MSX disease infection and intensity. The BFUs were to define intensity categories as:

BFU = 0; no infections or infections too light for detection by standard histology (hereinafter referred to as "undetectable infections").

BFU = 1; Epithelial infections - *H. nelsoni* plasmodia in the gill epithelium only.

BFU = 2; Subepithelial/local infections - *H. nelsoni* have broken through the basal lamina between the epithelium and the underlying tissues, but remain largely concentrated near that site.

BFU = 3; Light systemic infections - *H. nelsoni* have spread through the tissues via the circulatory system, but parasite abundance remains relatively low.

BFU = 4; Advanced systemic infections - *H. nelsoni* have spread throughout the tissues via the circulatory system and parasite abundance is high. These are typically lethal infections.

TABLE 2.

Salinity ranges for high freshwater runoff, average freshwater runoff, and low freshwater runoff time series (Figs. 4D, 5A, and 5B) for the sites displayed in Figure 1. The high value in each range represents the late summer values and the low value represents the spring values.

Station	High Runoff (ppt)	Average Runoff (ppt)	Low Runoff (ppt)
Arnolds Bed	5.1–9.5	7.2–12.9	9.6–15.4
Cohansey Bed	7.8–12.2	9.9–15.6	12.3–18.2
Shell Rock Bed	9.5–13.7	11.5–16.9	13.8–19.3
Bennies Bed	11.2–15.5	13.3–18.7	15.6–21.1
Miah Maul Grounds	21.8–23.0	22.4–24.4	23.0–25.7

The distribution of BFUs in relation to epithelial and systemic infections is shown in Figure 4 of Ford et al. (this volume).

Observed infection prevalences and intensities were used to verify model simulations. In the case of the planted ground simulations, comparisons were made with the complete annual cycle. For the seed beds, comparisons were made with the winter (November/December) and spring (late May/early June) prevalence peaks. Observed category 1 and 2 infections were combined to represent the prevalence of localized infections and categories 3 and 4 were combined to represent systemic infections. These data were used to construct a time series of prevalence and intensity (localized vs. systemic infections) in oyster populations at each seed bed and planted ground site (Fig. 6). Simulations for Miah Maul Grounds represent the annual *H. nelsoni* cycle and infection levels of the entire leased area, since there is relatively little variation within this area as compared to the variation among seed beds. Since measurements at Miah Maul Grounds were sparse, they were supplemented with data from Deepwater and Southwest Line planted grounds (Figs. 1, 7).

**Model Implementation**

The oyster population-*H. nelsoni* model was solved numerically using the 2-step pseudo-steady-state approximation scheme (Verwer and van Loon 1994) with a time step of 1 hour. Each simulation began on June 1 and extended for 3 years, with the first 2 years needed for the solution to reach a steady state. The same input data were used for each of the 3 years. The model assumes that the oysters have been continuously exposed to *H. nelsoni*, as is the natural stock, so that the population starts each cycle with the infection intensity and prevalence remaining at the end of the cycle.

To investigate the effect of salinity variations on the prevalence and intensity of *H. nelsoni* in Delaware Bay oyster populations, a number of stimulations were considered under various environmental conditions (Table 3). A reference simulation was run for each site using mean environmental conditions. A series of simulations to determine the influence of high or low freshwater runoff on infection levels were subsequently run and compared to the reference simulation. Finally, the effect of the timing of the freshwater runoff was investigated.

**Statistical Analysis**

To analyze variations in prevalence, intensity, and timing of *H. nelsoni* infections in Delaware Bay oyster populations as a result of the various environmental scenarios, several statistical analyses

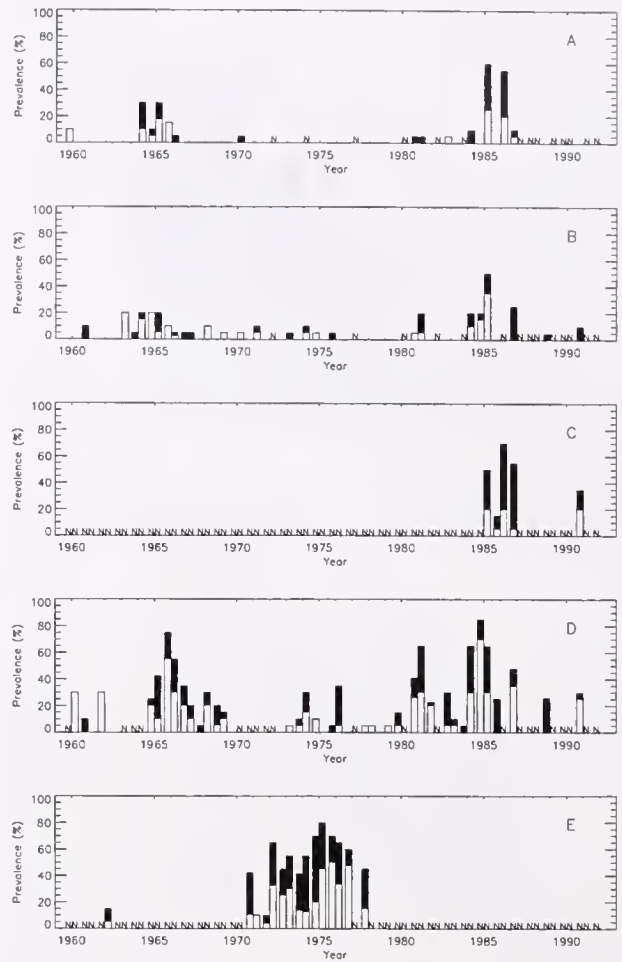


Figure 6. Prevalence and intensity of *Haplasporidium nelsoni* infections for A) Arnolds Bed, B) Cohansey Bed, C) Shell Rock Bed, D) Bennies Bed, and E) Miah Maul Grounds constructed from long-term data of Haskin Shellfish Research Laboratory (Fegley et al. 1994). Solid portions of bars represent localized infections (BFU categories 1 and 2); clear portions represent systemic infections (BFU categories 3 and 4). Annual cycles are represented by 2 samples. Bars on the left of the tick mark represent the spring (May/June) measurements; bars on the right represent the early winter (November/December) measurements. Periods when no data were available are indicated by N. Note that after 1992, *Perkinsus marinus*, cause of Dermo disease in oysters, became prevalent on the seed beds and confounded *H. nelsoni* patterns.

were performed on the simulated output. The maximum difference, average difference, root mean square (RMS), and correlation coefficient (*r*) were calculated for each site to compare each simulation to the reference simulation. Comparisons were made for each infection intensity (BFU) category between time series for each hypothetical extreme-condition simulated and the corresponding reference time series.

The maximum difference is the greatest percentage point difference in prevalence for a particular infection category between the simulated extreme and reference annual infection cycles. A high value means that an extreme condition caused a relatively large change in that category of infection compared to the reference simulation; a low value means that the extreme condition caused relatively little change:

$$\text{maximum difference} = \max(|A_{l,i} - B_{l,i}|) \quad (10)$$

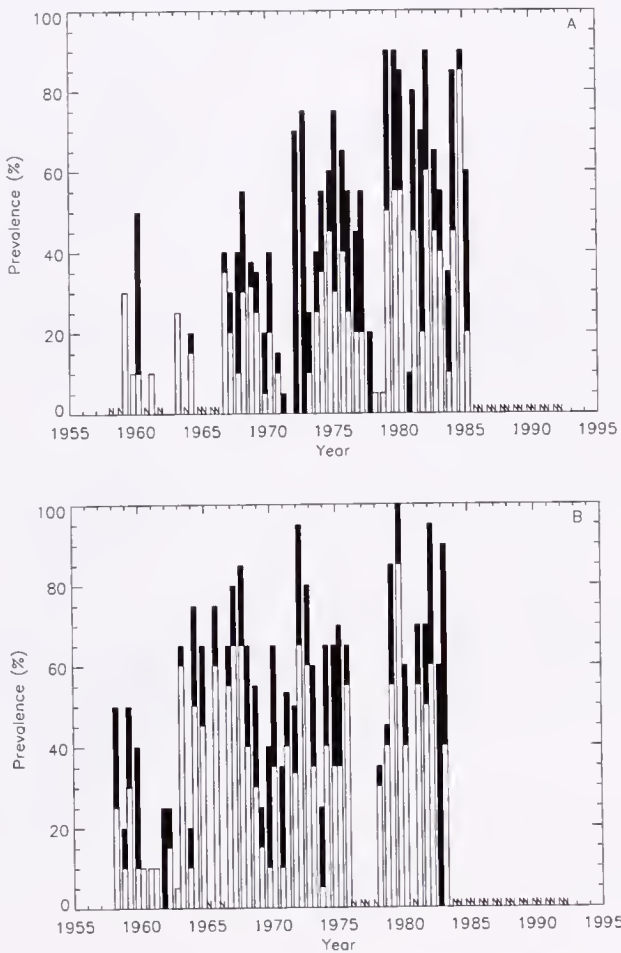


Figure 7. Prevalence and intensity of *Haplosporidium nelsoni* infections in oysters at A) Southwest Line and B) Deepwater planted grounds from records of the Haskin Shellfish Research Laboratory (Fegley et al. 1994). Solid portions of bars represent localized infections (BFU categories 1 and 2); clear portions represent systemic infections (BFU categories 3 and 4). Annual cycles are represented by 2 samples. Bars on the left of the tick mark represent the spring (May/June) measurements; bars on the right represent the early winter (November/December) measurements. Periods when no data were available are indicated by N. Note that after 1990, *Perkinsus marinus*, cause of Dermo disease in oysters, became prevalent on the New Jersey planted and confounded *H. nelsoni* patterns.

where  $A_{l,i} = A(t)$  or the MSX prevalence time series for a given location ( $l$ ) and MSX intensity category ( $i$ ) and  $B_{l,i} = B(t)$  for the reference simulation at the same site and intensity category.

The average difference is the average percentage point difference in prevalence of a particular infection category between simulated extreme and reference annual infection cycles. A high value means that an extreme condition caused a relatively large change in that category of infection; a low value means that the extreme condition caused relatively little change:

$$\text{average difference} = \frac{1}{T} \sum_{t=1}^T |(A_{l,i} - B_{l,i})| \quad (11)$$

where  $T$  is the length of the simulated time series produced by model simulations.

TABLE 3.

Environmental time series for model simulations. Salinity for Arnolds, Cohansey, Shell Rock, and Bennies Beds, and Miah Maul Grounds are indicated by A, C, S, B, and M, respectively. "Dry" and "wet" indicate low and high freshwater runoff, respectively. Timing of freshwater discharge is indicated by the month to which the spring discharge event was moved. R and E represent time series constructed from field measurements at Ridge and Section E sites, respectively.

Simulation	Site	Temperature (°C)	salinity (ppt)	Food Supply ( $\mu\text{g chl } a \text{ l}^{-1}$ )	Turbidity ( $\text{mg l}^{-1}$ )
Reference	A	E	A	E	E
	C	E	C	E	E
	S	E	S	E	E
	B	E	B	E	E
	M	R	M	R	R
Low fresh-water	A	E	A dry	E	E
	C	E	C dry	E	E
	S	E	S dry	E	E
	B	E	B dry	E	E
	M	R	M dry	R	R
High fresh-water	A	E	A wet	E	E
	C	E	C wet	E	E
	S	E	S wet	E	E
	B	E	B wet	E	E
	M	R	M wet	R	R
Early spring runoff	A	E	A Feb	E	E
	C	E	C Feb	E	E
	S	E	S Feb	E	E
	B	E	B Feb	E	E
	M	R	M Feb	R	R
Late spring runoff	A	E	A May	E	E
	C	E	C May	E	E
	S	E	S May	E	E
	B	E	B May	E	E
	M	R	M May	R	R

The RMS (root mean square) is a standard measure of the difference between 2 time series. Squaring the prevalence differences between the extreme  $A(t)$  and  $B(t)$  reference time series before taking the average enhances the larger differences and diminishes the smaller differences. Therefore, a relatively large difference will result in a larger value than a smaller difference. Again, a high value means that an extreme condition caused a relatively large change in that category of infection; a low value indicates that the extreme condition caused relatively little change:

$$\text{RMS} = \sqrt{\frac{1}{T} \sum_{t=1}^T (A_{l,i} - B_{l,i})^2} \quad (12)$$

Spearman's Rank Correlation Coefficient ( $r$ ) which is given by:

$$r = \frac{C_{AB}}{\sqrt{C_{AA}} \sqrt{C_{BB}}} \quad (13)$$

was used to evaluate correlations of ranked prevalences of each infection category between simulated extreme  $A(t)$  and reference  $B(t)$  annual infection cycles. The coefficient  $C_{AB}$  is defined as the  $\sum_{t=1}^T (A_{l,i} - \bar{A})(B_{l,i} - \bar{B})$ , where  $\bar{A}$  and  $\bar{B}$  are the mean for time series  $A_{l,i}$  and  $B_{l,i}$ , respectively. Values of  $r$  near 1 indicate similar temporal patterns (although absolute values can still vary). Values of  $r$  near zero indicate different temporal patterns.

RESULTS

Model Simulations

Reference Time Series Prevalence

The first set of simulations provided the basic time development of *H. nelsoni* infection prevalence and intensity at the seed bed and planted ground sites. These reference simulations were forced by average environmental conditions (Table 3) at each location and provide a basis for comparison with simulations produced by extreme environmental conditions.

At Arnolds Bed, the most upbay and least saline bed, mean environmental conditions produce no detectable infections (Fig. 8A). Infections are first detected just downbay at Cohansey Bed during August, increase in prevalence and intensity during the fall, and reach a winter prevalence maximum of 9% by late October (Fig. 8B). At Shell Rock Bed, a similar pattern is seen with new infections appearing a little earlier, in July, and winter prevalence rising to 20% by November (Fig. 8C). At both sites, little change

in total prevalence occurs over the winter. All detectable infections are lost in February and do not reappear in the spring.

Bennies Bed is the first site where the simulation results in an annual cycle more typical of higher salinity regions (with 2 peaks) and retention of some infections throughout the year (Fig. 8D). Prevalence and intensity increase through summer and fall, reaching a maximum by November, when the prevalence is about 45%. Loss of infections in all categories, but primarily systemic, occurs in March; infections re-appear in the spring, coincident with rising water temperatures and salinities. Prevalence reaches 25% in the late May/early June peak.

The reference simulation for Miah Maull, in the planted grounds, produces the highest infection levels (Fig. 8E). From a low of 5% to 10% in July, prevalence increases to 60% to 65%, with a little more than half the infections being systemic by November. A loss of primarily systemic infections causes a total prevalence decline in March and April, followed by a second prevalence peak of 55% in late May, in which most infections are advanced. A rapid loss of infections follows by early summer (see also Ford et al. this volume).

Reference Time Series Intensity

Under average conditions, approximately half the simulated infections in seed bed oysters are systemic at the November/December and late May/early June peaks (Fig. 8A–D). At the Miah Maull Grounds site, about half the winter infections are systemic. In contrast, the spring peak was predominantly systemic, with a large fraction in category 4, advanced systemic infections (Fig. 8E).

Extremes in Freshwater Runoff Simulations

Low Freshwater Runoff. During periods when freshwater runoff is lower than average, salinity in Delaware Bay increases (Fig. 5A) and elevated *H. nelsoni* infection prevalence results at all sites. Unlike average conditions, low freshwater runoff simulations produce detectable infections at Arnolds Bed, beginning in August (Figs. 8A, 9A). Infections develop slowly through the fall, reaching a winter prevalence peak of 8%. At Cohansey Bed, detectable infections appear in June and reach a maximum prevalence of about 35% by November. At Arnolds Bed, all detectable infections are lost in January; the decline occurs in February at Cohansey, where nearly all infections are lost. At neither site do infections re-appear in the spring.

Under the low runoff conditions, the simulated annual cycle for Shell Rock Bed is similar to that for Bennies Bed under average conditions: a 2-peak annual cycle with a substantial fraction of infections persisting throughout the year (Fig. 9C). The winter prevalence peak is approximately 45%. Loss of infections, primarily systemic, occurs in March. The spring peak prevalence is about 30%. Low-flow simulations produce almost identical cycle patterns and levels at Bennies Bed, representing the lower seed beds, as they do at Miah Maull, in the planting grounds (Fig. 9D, E). Both cycles show distinct winter and spring peaks with prevalence maxima of 60% to 65%, a moderate prevalence decrease in late winter, and a major loss of infections after the May peak.

As in the reference simulations, about half the infections are systemic by early winter (Fig. 9). The spring peak, however, produces relatively heavier infections, as well as higher prevalences at Shell Rock and Bennies Beds (Fig. 9C, D). The pattern at Bennies is nearly identical with that of the Miah Maull simulations, with a substantial number of category 4 infections (Fig. 9E).

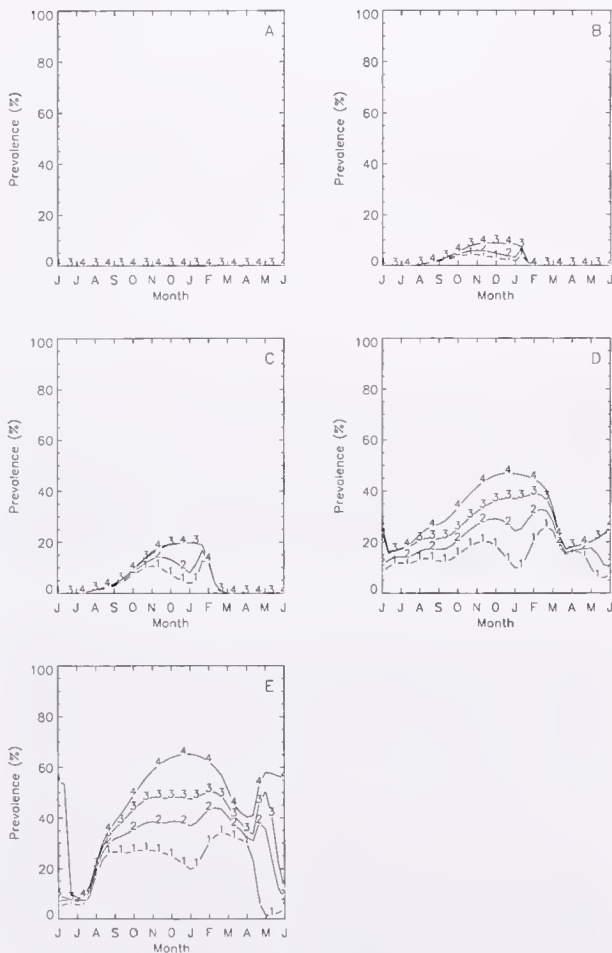
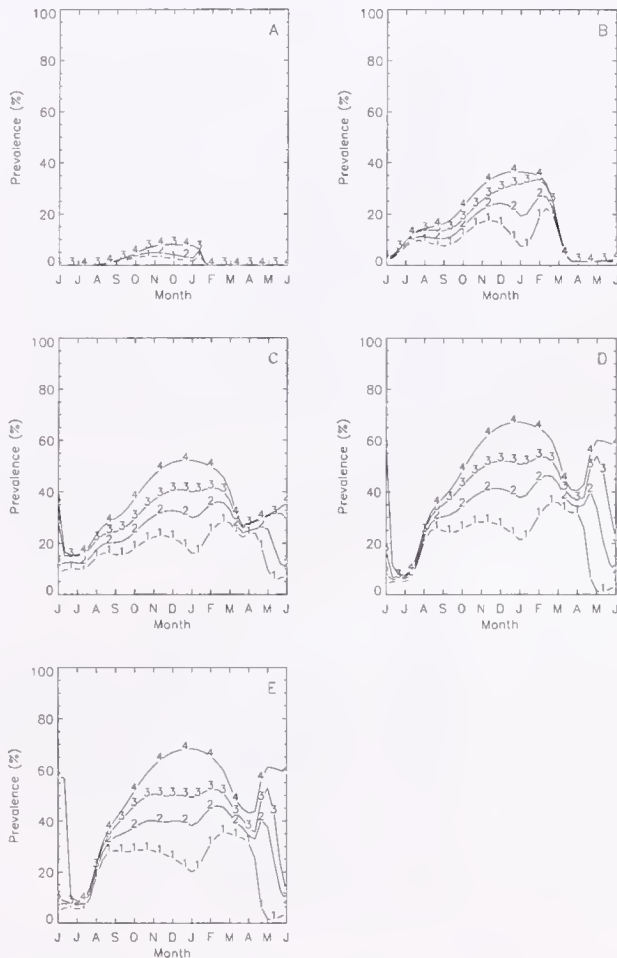


Figure 8. Simulated, time evolution of *Haplosporidium nelsoni* prevalence and intensity produced by average environmental conditions for A) Arnolds Bed, B) Cohansey Bed, C) Shell Rock Bed, D) Bennies Bed, and E) Miah Maull Grounds. Numbers 1 through 4 refer to the cumulative percentage of total oysters (prevalence) with epithelial (1), subepithelial local (2), light systemic (3), and advanced systemic (4) infections. The area above the category 4 line represents the portion of oysters in the undetectable infection category.

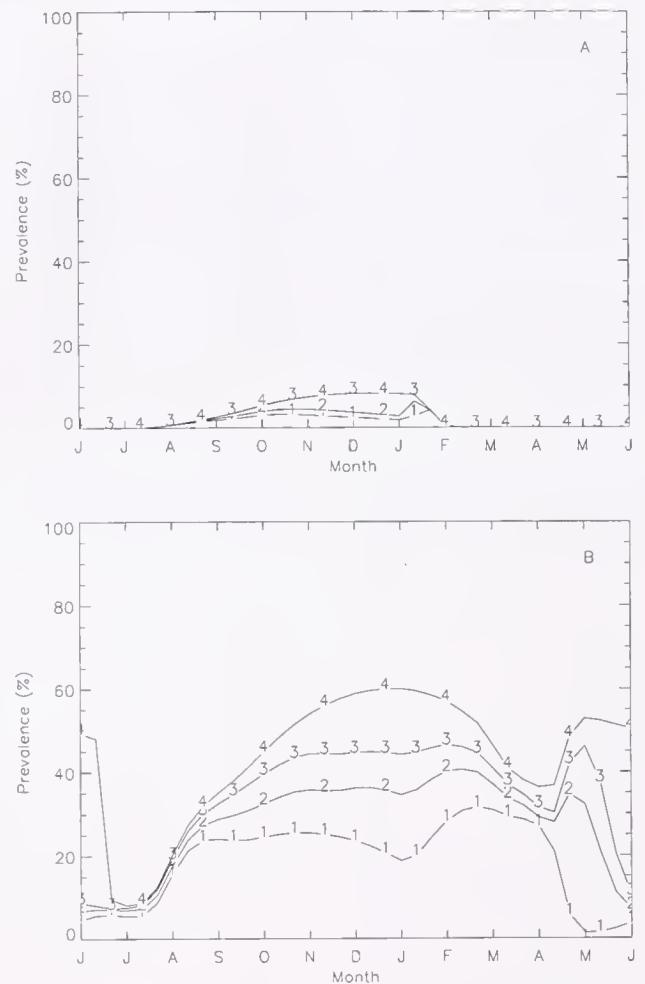


**Figure 9.** Simulated time evolution of *Haplosporidium nelsoni* prevalence and intensity produced by low freshwater conditions at A) Arnolds Bed, B) Cohansey Bed, C) Shell Rock Bed, D) Bennies Bed, and E) Miah Maull Grounds. Numbers 1 through 4 refer to the cumulative percentage of total oysters (prevalence) with epithelial (1), subepithelial local (2), light systemic (3), and advanced systemic (4) infections. The area above the category 4 line represents the portion of oysters in the undetectable infection category.

High Freshwater Runoff. Salinity conditions during periods of high freshwater runoff (Fig. 5B) result in no detectable *H. nelsoni* infections at Arnolds, Cohansey, or Shell Rock Beds. Infections appear at Bennies Bed in July, but reach a maximum prevalence of only 9% from November through January, considerably lower than the 40% to 45% prevalence found under average conditions (Figs. 8D, 10A). Infections disappear in February and do not reappear in spring. Compared to average conditions, there is little change in the Miah Maull Grounds cycle under high runoff.

#### Changes in Timing of Freshwater Runoff

Variations in the timing of maximum runoff from early (Fig. 5C) to late spring (Fig. 5D) affects *H. nelsoni* prevalence and intensity at Bennies Bed only. The major differences between the 2 extreme cycles (Fig. 11A, B) and that produced under average conditions (Fig. 8D) are in the degree of late winter infection loss and the extent of the late spring prevalence peak and subsequent decline. A February runoff produces a major loss of infections (from 45% to about 5%) in March followed by a relatively small



**Figure 10.** Simulated, time evolution of *Haplosporidium nelsoni* prevalence and intensity produced by high freshwater discharge conditions at A) Bennies Bed and B) Miah Maull Grounds. Numbers 1 through 4 refer to the cumulative percentage of total oysters (prevalence) with epithelial (1), subepithelial local (2), light systemic (3), and advanced systemic (4) infections. The area above the category 4 line represents the portion of oysters in the undetectable infection category.

resurgence, to 20%, in late May, and no subsequent decline (Fig. 11A). Understandably, a May runoff does not affect the late winter prevalence decline, which remains the same as under average conditions. It does, however, allow the late May prevalence peak to become higher than under average conditions (45% vs. 25%). More importantly, most of the infections become systemic (BFUs 3 and 4), resulting in a pronounced decline in prevalence in July (Fig. 11B) which was produced by a larger-than-normal number of heavily infected individuals in June that undergo attempted sporulation. After this decline, however, newly acquired infections quickly proliferate so that by late fall, and over the winter, there are no differences in prevalence or intensity among the early, average, and late discharge simulations.

#### Statistical Comparisons Between Extreme and Average Model Simulations

Because the first 2 years of the 3-year model runs were needed for the solution to reach a steady state, statistical analyses were done using third-year simulations only. The maximum difference,



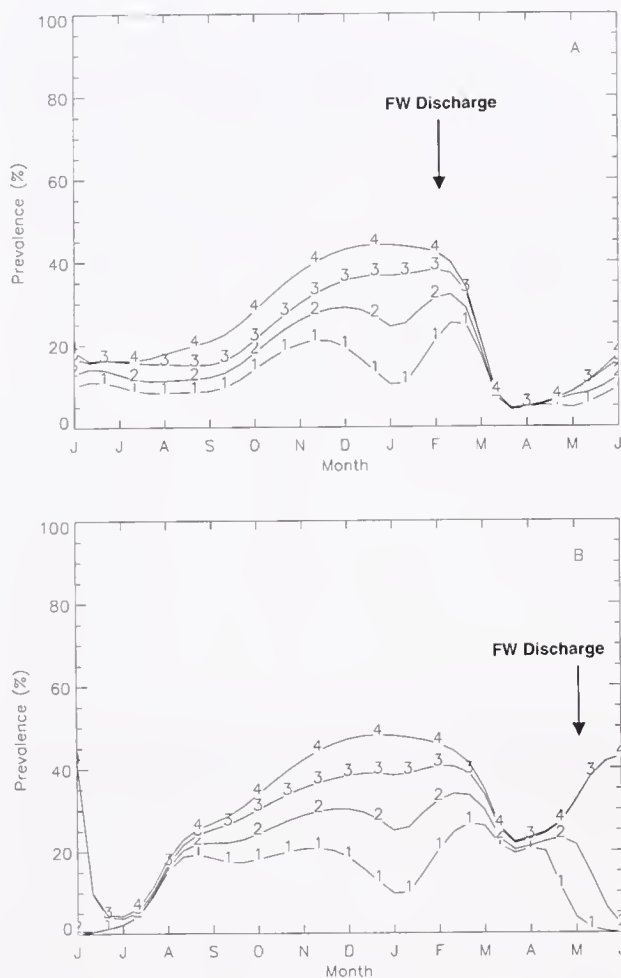


Figure 11. Simulated, time evolution of *Haplosporidium nelsoni* prevalence and intensity at Bennies Bed for A) early (February) and B) late (May) high freshwater discharge events. Numbers 1 through 4 refer to the cumulative percentage of total oysters (prevalence) with epithelial (1), subepithelial local (2), light systemic (3), and advanced systemic (4) infections. The area above the category 4 line represents the portion of oysters with undetectable infections. Arrows show the timing of the freshwater discharge.

average difference, root mean square (RMS), and correlation coefficients (*r*) between the reference simulation and each simulation with varied environmental conditions were calculated to quantify the effects of salinity variation of *H. nelsoni* on each infection category.

**Variations in the Magnitude of Runoff**

**Low Freshwater Discharge.** Conditions of low freshwater discharge have the largest effect on the undetectable infection category, as shown by high maximum difference, average difference, and RMS values (Table 4). Undetectable infections are, in effect, an inverse measure of percent infection. Thus, the decrease in this category in a high salinity regime simply means an increase in total prevalence. The largest differences associated with low discharge occurs at mid-bay sites, as suggested by the high maximum difference, average difference, and RMS values. Low correlation coefficients (<0.50) and relatively large maximum difference values were also calculated at Shell Rock and Bennies Beds for infection

TABLE 4.

Results of statistical analysis of the differences between low freshwater runoff and reference simulations at the seed bed and planted ground sites. *Haplosporidium nelsoni* infection intensity categories are UD = undetectable infections, 1 = epithelial, 2 = subepithelial local, 3 = light systemic, and 4 = systemic infections. "Maximum" and "Average" represent the maximum and average prevalence differences over an annual infection cycle. RMS represents the root mean square error, and *r* is the correlation coefficient. Bold is used to emphasize extreme differences.

Site	Intensity category	Maximum (%)	Average (%)	RMS (%)	<i>r</i>
Arnolds Bed	UD	8.03	2.42	4.02	<b>0.44</b>
	1	3.62	1.07	1.70	0.93
	2	2.03	0.44	0.78	0.87
	3	4.86	0.90	1.76	0.92
	4	0.10	0.01	0.03	—
Cohansey Bed	UD	<b>34.93</b>	<b>15.26</b>	<b>18.62</b>	0.84
	1	<b>22.29</b>	7.86	9.60	0.71
	2	<b>11.20</b>	3.04	4.47	0.89
	3	<b>11.46</b>	2.24	3.41	0.87
	4	5.95	2.10	3.11	0.91
Shell Rock Bed	UD	<b>44.68</b>	<b>29.32</b>	<b>30.12</b>	0.72
	1	<b>27.85</b>	<b>13.92</b>	<b>15.08</b>	<b>0.44</b>
	2	<b>15.99</b>	5.39	6.22	0.60
	3	<b>19.67</b>	4.85	6.61	<b>0.48</b>
	4	<b>12.15</b>	5.29	6.63	0.71
Bennies Bed	UD	<b>40.03</b>	<b>19.00</b>	<b>21.19</b>	0.81
	1	<b>16.82</b>	9.21	9.83	0.92
	2	<b>30.84</b>	4.30	7.37	0.81
	3	<b>20.89</b>	3.52	5.50	0.73
	4	<b>37.87</b>	5.73	9.89	<b>0.40</b>
Miah Maull Grounds	UD	3.32	2.58	2.74	<b>1.00</b>
	1	1.86	0.85	1.07	<b>1.00</b>
	2	2.10	0.61	0.78	<b>1.00</b>
	3	0.65	0.34	0.41	<b>1.00</b>
	4	3.15	0.78	1.11	<b>1.00</b>

categories 1, 3, and 4, reflecting increased intensity as infections move from category 1 to categories 3 and 4 under high salinity conditions. Correlation coefficients of 1.0 for every infection category at Miah Maull Grounds, as well as low values for the other 3 measures, indicate that there was almost no difference in prevalence or intensity associated with low runoff at this site.

**High Freshwater Discharge.** The greatest variation in simulated prevalences at all sites during high freshwater runoff occurs in the undetectable infection category (Table 5). This is consistent with the results of low freshwater discharge conditions and reflects the loss of parasites under low salinity conditions and a decrease in total prevalence. The greatest variability in maximum and total difference, and in RMS, is at Bennies Bed. Overall, correlation coefficients are positive and high (>0.50 in all but one comparison, at Bennies Bed), and are 0.99 in all infection categories at Miah Maull Grounds. Values for the other three measures are lowest at the Miah Maull site, indicating little change in infection intensity and prevalence associated with high freshwater discharge at this location.

*Variation in Timing of Freshwater Discharge*

The simulated prevalence and intensity obtained for early and late freshwater runoff conditions at Arnolds, Cohansey, and Shell

TABLE 5.

Results of statistical analysis of the differences between high freshwater runoff and reference simulations at the seed bed and planted ground sites. *Haplosporidium nelsoni* infection intensity categories are UD = undetectable infections, 1 = epithelial, 2 = subepithelial local, 3 = light systemic, and 4 = systemic infections. "Maximum" and "Average" represent the maximum and average prevalence differences over an annual infection cycle. RMS represents the root mean square error, and *r* is the correlation coefficient. Bold is used to emphasize extreme differences

Station	Intensity category	Maximum (%)	Average (%)	RMS (%)	<i>r</i>
Cohansey Bed	US	8.82	2.73	4.49	—
	1	4.31	1.27	2.01	0.86
	2	2.58	0.53	0.93	0.74
	3	5.15	0.92	1.81	0.84
	4	0.22	0.02	0.06	—
Shell Rock Bed	UD	<b>19.72</b>	6.57	<b>10.18</b>	0.74
	1	<b>12.67</b>	3.45	5.24	0.88
	2	6.43	1.36	2.47	0.90
	3	<b>11.77</b>	1.65	3.42	0.96
	4	0.70	0.11	0.23	—
Bennies Bed	UD	<b>44.43</b>	<b>28.04</b>	<b>29.35</b>	0.81
	1	<b>26.06</b>	<b>13.43</b>	<b>14.28</b>	<b>0.40</b>
	2	13.50	5.60	6.70	0.64
	3	<b>12.93</b>	4.56	5.55	0.55
	4	<b>10.04</b>	4.46	5.92	0.79
Miah Maull Grounds	UD	5.36	4.14	4.40	<b>1.00</b>
	1	2.91	1.47	1.75	<b>0.99</b>
	2	3.25	0.93	1.19	<b>1.00</b>
	3	1.27	0.55	0.64	<b>1.00</b>
	4	4.89	1.20	1.71	<b>1.00</b>

Rock Beds and at Miah Maull Grounds do not differ significantly from those obtained with the reference simulations (RMS < 0.37% and *r* > 0.98). The only significant modifications of the annual infection cycle associated with early or late discharge occur at Bennies Bed. Under early (February) runoff conditions, the greatest deviation from average was produced in undetectable and category 1 infections, as indicated by the maximum difference value at these categories (Table 6). A comparatively low correlation (0.59) in category 1 reflects the light infections present during the late spring and early summer, which never progress to higher levels (Fig. 11A) as they do under average conditions (Fig. 8D). The undetectable and category 1 infections were also affected by late runoff, as indicated by the maximum difference values. The greatest maximum difference value was in category 3 infections (Table 6), reflecting the high percentage of light systemic infections in the late spring peak (Fig. 11B) compared to the average cycle (Fig. 8D).

## DISCUSSION

### Comparison of Model Simulations with Field Observations

A test of the accuracy of the oyster population-*H. nelsoni* model is to compare simulated with observed infection prevalence and intensity patterns and levels under different environmental conditions. This was done in two ways. For the high salinity region of lower Delaware Bay, where considerable data are available on *H. nelsoni* infections throughout the year (Fig. 2), model simula-

TABLE 6.

Results of statistical analysis of the differences between early spring freshwater runoff, late spring freshwater runoff and reference simulations at Bennies Bed. *Haplosporidium nelsoni* infection intensity categories are UD = undetectable infections, 1 = epithelial, 2 = subepithelial local, 3 = light systemic, and 4 = systemic infections. "Maximum" and "Average" represent the maximum and average prevalence differences over an annual infection cycle. RMS represents the root mean square error, and *r* is the correlation coefficient. Bold is used to emphasize extreme differences.

Simulation	Intensity category	Maximum (%)	Average (%)	RMS (%)	<i>r</i>
Early freshwater runoff	UD	<b>14.46</b>	6.01	7.21	0.94
	1	<b>11.26</b>	3.07	4.63	<b>0.59</b>
	2	6.61	1.28	1.79	0.84
	3	9.30	1.58	2.78	0.78
	4	2.45	1.01	1.37	0.96
Late freshwater runoff	UD	<b>18.40</b>	5.01	7.60	0.90
	1	<b>10.45</b>	3.53	4.66	0.92
	2	9.73	1.66	2.64	0.89
	3	<b>27.94</b>	4.01	8.20	0.79
	4	4.74	1.37	2.04	0.93

tions for Miah Maull in the planted grounds were compared with the annual infection cycle for that region, as described by Ford and Haskin (1982). On the other hand, data from the low salinity seed beds consist primarily of samples taken at winter and spring infection peaks (Haskin and Ford 1982, Fegley et al. 1994). Consequently, model output for this region of the bay was tested by comparing simulated prevalences and intensities at the peaks with observed values under similar ambient conditions.

### The Annual *H. nelsoni* Infection Cycle in a High-Salinity Location

The onset of the simulated infection cycle at the Miah Maull Grounds begins in June. Prevalences and intensities increase, reaching a maximum prevalence of 65% in December (Fig. 8E). This value is consistent with prevalences reported by Ford and Haskin (1982) for oysters in the planted ground area, which averaged 40% to 60% in most years, with minimum and maximum prevalences of 15% and 100%, respectively. As discussed by Ford et al. (this volume) the simulated proliferation of parasites during warm weather is primarily determined by temperature, but the reduction in proliferation rates apparent by late fall is caused by a combination of falling temperatures and high parasite density in oyster tissue. The latter inhibits proliferation because parasites are competing for a limited and declining nutrient resource within the host.

In late winter, the simulated infection cycle shows a marked loss of infected oysters, as does the observed cycle (Figs. 2, 8E). Observed mortality of severely infected oysters occurs at this time, reducing both prevalence and intensity. In addition, intensity lessens because parasites do not tolerate overwinter conditions well. Perhaps this is due to a direct low temperature intolerance or other unfavorable influences facing the parasite under the prolonged anaerobic conditions that occur inside the oyster, over the winter (Ford and Haskin 1982). For instance, *H. nelsoni* plasmodia contain numerous mitochondria (Muller 1967, Perkins 1968), which suggests that the parasite is well equipped for aerobic metabolism and may not be able to tolerate low oxygen well. The model

simulates the degeneration and disappearance of parasites at this time by including a susceptibility factor, which is applied to parasites and which increases as a function of degree days below 5 °C (Ford et al. this volume). As susceptibility increases, the parasites become more vulnerable to attack by host hemocytes. Hemocytes themselves are becoming more active with increasing spring temperatures and, as a consequence, can readily eliminate the damaged parasites from the host.

Both simulated and observed annual cycles contain a distinct second peak, reached in late May or early June. This peak contains many very heavily infected oysters due to increasing temperatures that cause a swift resurgence of those infections that were suppressed over the winter (Andrews 1966, Ford 1985b, Ford and Haskin 1982). The model, however, was unable to reproduce the very rapid infection development by temperature alone. A second factor was added, which eased the density-dependent limits on parasite growth as a consequence of improving conditions within the oyster (Ford et al. this volume). The increasing quality of the parasite's environment, hypothesized to occur at this time, is due to the spring bloom, increased feeding rates of the host, and the cleansing of waste products that have accumulated in the host over the winter.

Finally, both simulated and observed cycles show a marked decline in prevalence following the spring peak (Figs. 2, 8E). Heavily infected oysters die at this time, accounting for some of the decline. Additionally, parasites are lost from living oysters for reasons that are not clear, but may have to do with attempted, but unsuccessful, sporulation and consequently a failure on the part of the parasite to complete its life cycle (Ford and Haskin 1982, Ford et al. this volume).

**Peak Infection Levels at Low Salinity Seed Bed Locations**

To compare simulations for the seed bed sites (Fig. 1) with field measurements, the latter were grouped, by site, according to years

of high, average, and low freshwater runoff. The grouping criteria were the same as those used to produce salinity time series for these conditions. That is, the average years were those in the middle 60% of the range; the high and low runoff years were the 20% at either extreme. For each flow regime and site, the mean, median, and range of observed prevalences at both late fall and spring peaks were obtained (Table 7). Because of the limited number of observations (Fig. 6) Shell Rock Bed was not included in this comparison.

Comparison of initial simulations with observations showed that the timing of infections was correct, but that prevalences at the seed bed sites were too high. The relationship used to model the host-parasite relationship under varying salinity was derived from a study in which parasites were subjected to acute *in vitro* salinity change (Ford and Haskin 1988), modified to simulate more gradual *in vivo* changes in salt content of host fluids (equation 3). The inability of the model to reproduce observed prevalences using this relationship alone suggested that prevalence was influenced by an additional factor or factors.

Although temperatures from only a single site, Section E (Fig. 4A), were used in reference simulations for all seed bed sites, monthly-averaged temperatures varied by a maximum of only 1.4 °C from the planted grounds to the seed bed areas (Fig. 4A). Further, temperature variations within the seed bed area are relatively small (Fegley et al. 1994). Thus, temperature differences are unlikely to be responsible for the disparity.

Another possible factor could be salinity-associated differences in the rate of infection. The infective stage of *H. nelsoni* remains unknown. Spores, typically an important element in parasite transmission, are rare in adult oysters and their absence has led to the hypothesis that another host may be involved in the life cycle (Burreson 1988, Haskin and Andrews 1988). On the other hand, spores appear to be produced regularly in juvenile oysters with advanced infections (Barber and Ford 1992, Burreson 1994), so

TABLE 7.

Comparison of simulated total prevalence with observed mean, median, and range of prevalences on Delaware Bay sites (Fig. 1) under High, Average, and Low Delaware River runoff conditions. PG indicates various planted grounds (Fig. 7).

	High runoff		Average runoff		Low runoff	
	%MSX Fall	%MSX Spring	%MSX Fall	%MSX Spring	%MSX Fall	%MSX Spring
Arnolds Model	0	0	0	0	8	0
observed mean	1	<1	13	2	11	4
observed median	0	0	0	0	8	0
observed range	0-5	0-5	0-60	0-10	0-30	0-15
Cohansey Model	0	0	9	0	37	3
observed mean	5	<1	16	5	19	8
observed median	5	0	10	0	20	5
observed range	0-10	0-5	0-52	0-25	0-40	0-20
Shell Rock Model	0	0	20	0	52	35
Bennies Model	9	0	47	25	67	60
observed mean	10	9	30	21	44	28
observed median	5	8	22	15	51	15
observed range	0-30	0-30	0-65	0-85	0-65	0-75
Miah Maull Model	60	55	65	55	67	62
PG observed mean	43-65	40-53	40-58	40-54	43-60	42-55
PG observed median	55-65	43-53	35-65	35-50	33-63	0
PG observed range	5-95	0-80	10-95	5-90	0-85	0-100

direct transmission between oysters remains a possibility. Further, Barber and Ford (1992) found that haplosporidian spores, morphologically similar to those of *H. nelsoni*, are present in the water column in Delaware Bay in approximately equal abundance in upper and lower Bay sites. These authors speculated that if these spores are, in fact, *H. nelsoni* and are the infective element, the observed disparity between their concentration and infection prevalence in the upper and lower Bay could be explained by reduced infectivity under low salinity conditions. Alternatively, if another host is involved, the disparity could be explained by a salinity effect on another host or on its relationship with *H. nelsoni*.

The discrepancy was resolved in the model by adding an infection efficiency factor (equation 6), which reduces infection rates with decreasing salinity. This relationship implies nothing about the source or form of the infective particle; however, it does suggest that some element in the life cycle other than the plasmodial stage in the oyster, is sensitive to low salinity. This includes, but is not limited to, an intermediate or reservoir (alternate) host, the abundance of infective stages in the water, or the ability of infective stages to invade and establish themselves in oysters.

Addition of infection efficiency to the model resulted in simulated prevalences that largely matched those observed (Table 7). The simulated levels were most like the median prevalences, which they reproduced almost exactly in many cases. Notably, the model produced no detectable infections on Arnolds Bed except under low flow conditions, as observed, and reproduced the elimination of the spring peak in cases where it was observed in the field. The model also accurately simulated the relative insensitivity to river flow changes of the lower bay infection cycle that is observed in nature.

Only on Bennies Bed for average conditions and at Bennies and Cohansey Beds for low flow conditions, did the simulations not closely reproduce the mean or median observed prevalences (Table 7). The simulated prevalences were higher, especially under the low flow conditions, although they were clearly within the range of observations. Similarly, the modeled prevalence for Miah Maull Grounds tended to be at the high end of the observed mean or median range for the lower Bay planted grounds. It is clear that observed prevalence in the moderate to high salinity region of the bay varies widely and in some years no infected oysters are found, regardless of salinity (Figs. 6, 7; Table 7). Multiyear cycles in *H. nelsoni* prevalence, totally independent of river flow and salinity, were described by Ford and Haskin (1982) for lower Delaware Bay. The salinity in lower Delaware Bay, in fact, is always high enough to support full *H. nelsoni* activity so that year-to-year variations must be due to some other condition. The fact that disparities between observed and simulated prevalences were maximum at mid-Bay sites under low flow, high salinity conditions, which produce infection levels and patterns similar to the lower Bay, suggests that they are influenced by factors other than the local salinity. The possible factors are included in a more complex disease transmission model as described in Powell et al. (this volume).

Another element of the basic model that was modified to simulate observed conditions involved infection intensity. Long-term observed averages show that despite decreasing prevalence, the ratio of systemic to localized infections remains essentially 1:1 along the salinity gradient in the seed bed regions of Delaware Bay (Ford and Haskin 1982, Fegley et al. 1994). However, the simulated intensity decreased upbay. To improve the model, the trans-

fer rate of parasites from the epithelium to the systemic tissue was increased with decreasing salinity. The addition of this formulation to the model caused about half of the infections to remain systemic at peak prevalence on seed bed sites, while still allowing more intense infections to develop at the spring peak under the normally high salinity conditions of the lower bay and, at low river flow, on the lower seed beds. When a strong spring peak occurs, which it does only under high salinity situations, oysters often have the most advanced infections of the year, so that simulations reproduce observed intensities under high salinity conditions also.

The biological basis for the maintenance of high systemic infection levels under conditions of decreasing prevalence along the salinity gradient is unclear. Further, the mechanism used by parasites to penetrate the basal lamina between the epithelium and the underlying tissue is not known (Scro and Ford 1990). Thus, there is no direct evidence that low salinity enhances the transfer rate between the two tissues. On the other hand, model simulations were run for a population in which all oysters were the same size (1 g dry weight). However, adult size generally decreases along the salinity gradient in Delaware Bay, and along with it occurs an increase in the surface (epithelium) to volume (systemic) ratio. A given concentration of parasites per unit area of epithelium in a small oyster is actually a larger total parasite load in the surface layer than it is in a larger oyster. In some way, this large overall burden may speed the movement of parasites into the underlying tissue and thus help explain the observed relatively high intensity levels on the seed beds. The addition of multiple oyster size classes to the model is needed to test this hypothesis.

Simulations were also run with variations in food availability in the spring and with warm winter conditions (>5 °C) to determine the relative effects of salinity compared to food and temperature on oyster populations infected with *H. nelsoni*. Variations in salinity had a larger effect on the simulated infection levels than did variations in food availability and winter temperatures in a population of 1-g oysters. However, the simulated time development of infections did demonstrate the importance of cold winters in producing a reduction in infections the following spring, and therefore modulating the annual cycle.

When absolute differences between average and extreme conditions were compared statistically, the mid-Bay locations of Shell Rock and Bennies Beds showed the greatest variations. The explanation lies in the fact that mean mid-tide salinities at these locations range from 9.5–11.2 ppt to 19.3–21.1 ppt (Table 2), and it is in this range that a shift in salinity causes the greatest change in parasite survival (Fig. 3). In addition, Bennies Bed (salinity range 13.3 to 18.7 under average conditions) was the only site at which simulated variation in the timing of "spring" runoff caused a significant change from the reference annual cycle. This is because Bennies Bed, under average conditions, was the only seed bed to exhibit a late spring prevalence peak (Fig. 8D) and thus the only bed that had any infections to be altered by differences in timing. Salinity was always low enough to permit a spring MSX prevalence peak at the upper bay sites regardless of the timing of the spring runoff and salinity in the planted grounds was high enough (>20 ppt) nearly all the time to support infections so that changes in timing, like changes in volume of discharge, would have little effect on the spring peak at Miah Maull Grounds.

#### Summary and Conclusions

The simulations obtained with the oyster population–*H. nelsoni* model were able to reproduce observed infection patterns and lev-

els along the salinity gradient in Delaware Bay. The base relationship used by the model was developed from *in vitro* parasite survival data. The inability of initial simulations, using this direct salinity-parasite function, to produce infection levels that adequately represented field observations, indicate a more complex host-parasite relationship at low salinity. Two additional functions, both based on assumptions, were added to the model to improve simulations. One allowed infection intensity to remain stable along the decreasing salinity gradient by increasing the transfer rate of parasites from epithelial to systemic tissues. The biological rationale may be linked to decreasing size of oysters and increasing surface-to-volume ratios along the salinity gradient. It is also possible that the transfer rate change may simply be a substitute for some other mechanism that allows intense infections to occur at low prevalence. The second modification was to diminish infection efficiency with decreasing salinity. Because the mode of transmission of *H. nelsoni* and the form of its infective stage remain unknown, this assumption cannot be verified. Reduced infection efficiency may also be a surrogate for another mechanism, such as abundance of a potential intermediate or alternate host, the abundance of infective stages, or both.

The importance of these modifications is not necessarily the assumptions that were made, but that they show that factors other than a simple, direct salinity effect on *H. nelsoni* proliferation and death rates in the oyster are required to explain field observations. The model results emphasize that in the *H. nelsoni* relationship, salinity is a permissive/restrictive element, modifying the degree and pattern of infections, during transmission as well as after oysters become infected.

Another, overriding factor, is the presence and abundance of infective particles, which varies from year to year. Model simula-

tions show that in low salinity environments, infection levels are clearly correlated with river flow and that salinity changes over the 10–20 ppt range have the greatest effect on infection patterns and level. This association, however, breaks down at high salinity. Infection levels in high salinity (>20 ppt) regions of Delaware Bay are uncorrelated with river flow, as was noted earlier (Haskin and Ford 1982), and is attributed to fluctuations in infective particle abundance. The model shows that under drought conditions, the same factor or factors override local salinity to control infections in mid-Bay locations.

The ability to investigate *H. nelsoni*-oyster-salinity relationships with relative ease using the coupled model demonstrates its usefulness as a practical tool. In many regions other than Delaware Bay, low salinity areas are critical as setting and storage areas for seed oysters. The capacity of the model to simulate, with only minor modifications, observed *H. nelsoni* levels in mid-Chesapeake Bay demonstrates that it is robust and can be employed in other areas (Powell et al. this volume).

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## MODELING THE MSX PARASITE IN EASTERN OYSTER (*CRASSOSTREA VIRGINICA*) POPULATIONS. III. REGIONAL APPLICATION AND THE PROBLEM OF TRANSMISSION

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**ABSTRACT** A model of transmission for *Haplosporidium nelsoni*, the disease agent for MSX disease, is developed and applied to sites in Delaware Bay and Chesapeake Bay. The environmental factors that force the oyster population-*H. nelsoni* model are salinity, temperature, food, and total suspended solids. The simulated development of MSX disease was verified using 3 time series of disease prevalence and intensity: 1960 to 1970 and 1980 to 1990 for Delaware Bay, and 1980 to 1994 for Chesapeake Bay, and for a series of sites covering the salinity gradient in each bay. Additional simulations consider the implications of assumptions made in development of the model for constraining the mode of transmission of *H. nelsoni* disease in oyster populations.

Transmission of *H. nelsoni* includes non-local factors that exert a paramount influence on the transmission process. Key environmental forcing factors of season, salinity, and winter temperature exert a direct control on the transmission process, either by controlling the availability of infective particles in the water column or by controlling the population dynamics of an alternate host. Salinity's role is a dual one. Salinity acts on the local host population by varying the infectivity of infective particles as they impinge the oyster gill during the filtration process. In addition, salinity exerts a regional influence on the transmission process by controlling, in part and on a bay-wide scale, the concentration of infective particles in the water column (or perhaps the abundance of an alternate host). In addition to the effect of salinity, infective particle concentration also decreases for 1 to 2 years after a cold winter and returns to high levels faster after a warm winter. It is the presence in the *H. nelsoni* transmission model of these bay-wide influences of environmental change that make this model different from most other transmission models. Simulations suggest that epizootic cycles are principally the product of enhanced transmission rather than enhanced intensification. These influences of transmission on the course of infection, in many cases, have multiyear implications for prevalence and infection intensity, and the root of much of this multiyear behavior is in the processes that control the concentration of infective particles in the water column.

**KEY WORDS:** oyster, disease, parasite, population modeling, transmission

### INTRODUCTION

Perhaps the most significant task facing any parasite or disease model is to adequately simulate the geographic distribution of prevalence and infection intensity. Models attempting to do so always rely heavily on the dynamic modeling of transmission. (Dietz 1982, Ackerman et al. 1984, Kermack and McKendrick 1991, Dwyer and Elkinton 1993), because the geographic variation in infection intensity cannot be adequately simulated if the geographic and temporal dynamics of prevalence are inadequately defined. Transmission may have local and regional dynamics. Models of disease principally influenced by local processes usually rely on estimates of contact rate, host density, and the frequency of infected individuals (Godfrey and Briggs 1995, Hethcote and van den Driessche 1995, Frank 1996). The spread of disease beyond local populations, however, often involves the integration of variations in landscape and environmental condition that significantly influence the infection process (Gill 1928, Mollison 1987, Myers 1993, Ostfeld 1997). Dermo disease in oysters, produced by the protistan *Perkinsus marinus*, is an example of a disease modeled adequately using local processes of population prevalence, infection intensity, and host density, a surrogate for contact rate in an immobile species (Hofmann et al. 1995, Powell et al. 1996), and this seems to agree with the neighborhood model of disease transmission that has evolved from experimental and

observational studies (Mackin 1952, Andrews 1965, Andrews 1988, Andrews and Ray 1988, Ford 1992). MSX disease in oysters, produced by the haplosporidian protozoan *Haplosporidium nelsoni*, is a good example of a disease that shows significant temporal and geographic variation in prevalence and infection intensity that cannot be modeled assuming a local process-driven mode of transmission. Regional-scale processes are inferred to be important from observational studies (Andrews 1964, Haskin and Andrews 1988) and will be shown herein to be important in modeling the disease.

The full life cycle of *H. nelsoni* is unknown (Ford and Tripp 1996). Transmission between oysters has never been confirmed experimentally. Disease epizootics occur spontaneously over wide regional areas, more or less simultaneously across broad expanses of the salinity gradient, and independent of the proximity of infected oysters (Andrews 1968, Ford and Haskin 1982, Haskin and Ford 1982). These observations suggest bay-wide response patterns in transmission that are considerably decoupled from the local processes occurring on any one oyster bed. One well-considered hypothesis is that an intermediate host is involved in the life cycle, however no direct confirmation of this hypothesis exists (Haskin and Andrews 1988, Ford and Tripp 1996). The purpose of this paper is first to develop a model of transmission in MSX disease. We will then apply this model to 3 case histories using long-term time series, 2 from Delaware Bay (1960 to 1970, 1980 to 1990) and 1 from Chesapeake Bay (1989 to 1994), that

come from a series of sites covering much of the salinity gradient in each bay. Finally, we will consider the implications of the assumptions made in formulating the transmission model for constraining the mode of transmission of *H. nelsoni* in oyster populations.

### THE *CRASSOSTREA VIRGINICA*-*HAPLOSPORIDIUM NELSONI* MODEL

The *Crassostrea virginica*-*Haplosporidium nelsoni* model can be separated into a transmission component and an intensification component.

#### The Intensification Component

The intensification component is described in detail by Ford et al. (this volume). Only a brief review is given here.

#### Disease Quantification

The majority of the observations on MSX disease use a semi-quantitative scale that categorizes *H. nelsoni* infection level according to parasite distribution (local or diffuse) and visual rankings of cell density. The infection rating system used for the model is based on a semi-quantitative scale developed for use in Delaware Bay (Ford and Haskin 1982). In contrast, the oyster-*H. nelsoni* model is based on the number of parasites per oyster. It was therefore necessary to establish, at the outset, a relationship between *H. nelsoni* cells per oyster and the 0-to-6-point semi-quantitative scale (referred to as Little Ford Units [LFU]). This conversion, developed by Ford et al. (this volume), is based on a logarithmic relationship of the form:

$$LFU = a_{e/s} \ln \left( \frac{C_{e/s}}{bc_{e/s}W_0 \text{frac}_{e/s}} \right) \quad (1)$$

where  $a_{e/s}$  is a constant that differs for the epithelial ( $e$ ) and systemic ( $s$ ) tissue (the notation  $e/s$  will be used to denote constants that have different values for epithelial and systemic tissue).  $C_{e/s}$  is the number of *H. nelsoni* cells in the epithelial or systemic tissue,  $b$  is a scaling factor, and  $c_{e/s}$  is a constant.  $W_0$  is the ash-free dry weight of the oyster and  $\text{frac}_{e/s}$  is the fraction of epithelial or systemic tissue in the animal. The values of the coefficients in equation (1) and all other equations are given in Ford et al. (this volume).

Rare or very light epithelial infections (LFU = 1) may be identified by as few as 1 or 2 *H. nelsoni* cells in the gill epithelium in a standard tissue cross-section analysis. Thus, some oysters diagnosed as having no infections (LFU = 0) are probably infected (Stokes et al. 1995). The model is constructed to reflect this circumstance. The distinction between an uninfected oyster (LFU = 0) and one in the very lightest infection category (LFU = 1) is based on a detection limit of  $1.3 \times 10^4$  and  $6.5 \times 10^3$  parasites per gram wet weight for epithelial and systemic tissue, respectively and not on the absolute absence of infection.

For simplicity, a 0-to-4-point scale was chosen to depict the final output from the model. These units are referred to as Big Ford Units (BFU). Conversion between the scales simply involves combining the 4 highest LFUs into 2 BFUs (Table 1 and Fig. 4 in Ford et al. this volume). Most of the information and parameterizations used in the model originated from data provided in terms of LFU. Thus, in effect, the model converts from LFU to parasite number for calculation and from cell number back to LFU (and then to BFU) for data presentation. As a result, the values for most of the

constants used in the model equations depend upon the conversion given by equation (1), but, unlike the case for *Perkinsus marinus* where a relatively accurate conversion exists between cell density and Mackin units derived from whole body counts (Choi et al. 1989, Bushek et al. 1994), the relationship between LFUs and *H. nelsoni* number is more indirectly estimated and therefore subject to greater error.

#### Model Equations

The model is structured as a two-dimensional array (Fig. 3 in Ford et al. this volume) with 55 epithelial and 55 systemic infection categories. The infection level in each category is defined by the average number of *H. nelsoni* in it, with the maximum difference between adjacent classes being 1 population doubling. The difference between infection classes at the higher parasite densities is less than 1 population doubling because of the nonlinear distribution of LFUs with respect to parasite number. The nonlinear arrangement was required to provide multiple infection classes within each LFU infection category and, consequently, necessitated scaling the transfers between infection categories by the ratio of the parasite cell number ( $C$ ) between adjacent classes as:

- for transfers up in epithelial tissue:  $C_e/(C_{e+1} - C_e)$
- for transfers down in epithelial tissue:  $C_e/(C_e - C_{e-1})$
- for transfers up in systemic tissue:  $C_s/(C_{s+1} - C_s)$
- for transfers down in systemic tissue:  $C_s/(C_s - C_{s-1})$

The governing equation for determining the prevalence and intensity of *H. nelsoni* infections in the epithelial ( $e$ ) and systemic ( $s$ ) tissue of post-settlement infected oyster ( $O$ ) populations is given by:

$$\begin{aligned} \frac{dO_{e,s}}{dt} = & -\alpha_{e,s}O_{e,s} - \beta_{e,s}O_{e,s} + \alpha_{e,s-1}O_{e,s-1} + \beta_{e-1,s}O_{e-1,s} \\ & + \alpha_{e,s+1}O_{e,s+1} + \beta_{e+1,s}O_{e+1,s} - M_{e,s}O_{e,s} - \gamma_{e,s_4}O_{e,s_4} \\ & + \delta_{e_1}\delta_{s_0}\frac{1}{2}\sum_{s=s_4}^N\sum_{e=0}^N\gamma_{e,s}O_{e,s} \end{aligned} \quad (2)$$

where the first 6 terms represent the movement of oysters between infection intensity classes through gains or losses of *H. nelsoni* cells in the epithelial and systemic tissue. The coefficients,  $\alpha$  and  $\beta$ , determine the rate at which *H. nelsoni* cells are gained or lost. The parameterizations used to determine these coefficients are given in Ford et al. (this volume). The seventh term in equation (2) represents the loss of parasites through oyster mortality from lethal infections as determined by the rate of mortality,  $M$ . The final 2 terms in equation (2) represent the transfer of oysters from heavy infection classes to lower infection classes due to the formation or attempted formation of spores by *H. nelsoni* in the oysters with advanced infections, which results in a loss of parasites from infection classes with BFU = 4 ( $s_4$  in equation 2), and a gain of infected oysters into infection class [1,0]. The  $\delta$  functions represent a step-function process in which the oysters are introduced into the [1,0] infection class only. The coefficient  $\gamma$  determines the rate of this transfer process.

The establishment of infection in uninfected oysters ([0,0] class) is determined by the equation:

$$\frac{dO_{0,0}}{dt} = -\beta_{0,0}O_{0,0} + \frac{1}{2}\delta_{e,0}\delta_{0,s}\sum_{s=s_4}^N\sum_{e=0}^N\gamma_{e,s}O_{e,s} \quad (3)$$

where the first term represents the acquisition of *H. nelsoni* infective particles at a rate determined by  $\beta_{0,0}$ . The second term rep-



resents addition of oysters to the uninfected class after hypothesized abortive *H. nelsoni* sporulation events, as given by the eighth term in equation (2). These oysters are divided evenly between the [0.0] and [1.0] infection classes as given by the last terms in equations (2) and (3).

Certain rules apply to the movement of oysters among infection categories, all based on histological observations of the disease process (Farley 1968, Ford and Haskin 1982). To reflect the fact that infections are initiated in the gill epithelium, uninfected oysters [0.0 class] must move first into an epithelial class (x,0) before entering a systemic class (Fig. 3 in Ford et al. this volume). Oysters never reach high epithelial infections,  $LFU_c > 6.5$ , without developing systemic infections. Oysters in systemic classes  $\geq 7.0$  are automatically placed in the dead oyster category because parasite densities represented by these classes are higher than those found in live oysters. Additional mortality processes are discussed in Ford et al. (this volume).

**The Transmission Model**

The transmission model deals with the initiation of true new infections. MSX, like Dermo, is assayed using semi-quantitative methods that have a limit of resolution defined as the *H. nelsoni* cell density below which false negatives commonly occur. Epidemiological data cannot distinguish false negatives from truly uninfected animals and, as such, particularly during certain times of the year, many infected oysters will appear to be uninfected (e.g., Bushek et al. 1994, Hofmann et al. 1995). Accordingly, a transmission model cannot be fit to field data alone; coupling to the intensification model is required to generate the false negatives that can be prevalent in field observational data.

The development of this component of the *Crassostrea virginica*-*Haplosporidium nelsoni* model faced a particularly stringent hurdle due to the limited information beyond epizootiological observations of the infection process. Accordingly, the strategy taken was to develop relationships that produced the broad regional and temporal trends in prevalence observed in observational data. An attempt was made to ground these relationships in observed or anticipated physiological and ecological processes. Nevertheless, the few available data do not permit verification of all of these processes and so the approach taken in the model may well be incorrect even though the answer would appear to be correct, and the relationships are admittedly crude in many cases. Nevertheless, our approach has been to utilize a large number of sites covering a wide region with a significant salinity gradient and a significant temporal time series based on the assumption that any relationship fitting a multitude of sites is likely, to some significant extent, to express a real process controlling *H. nelsoni* transmission.

Finally, for convenience, we will refer to the *H. nelsoni* life stage responsible for infection as the infective particle. The *H. nelsoni* life cycle includes a spore phase (Couch et al. 1966, Ford and Tripp 1996) that is free in the water column, judging from the observation of spores in oyster guts (Barber and Ford 1992). However, the evidence supporting spores as the primary infective stage for oysters is meager. An, as yet unknown, stage may be responsible for infection.

**Governing Equations**

The transmission model covers a series of basic processes. (1) The model is initialized with a base infective particle concentration

(infective particles  $L^{-1}$ ). (2) The infective particle concentration is modified by season such that more new infections are acquired in the warmer months. (3) The infective particle concentration is affected by local changes in salinity such that lower salinity is assumed to increase infective particle mortality or decrease particle infectivity, producing a salinity-dependent gradient in prevalence. (4) The infective particle concentration is affected by 2 regional processes. Winters with unusually cold temperatures reduce infective particle concentrations for up to 2 years. Oscillations in average bay salinity above and below 15.5‰ introduce a source of variation in infective particle concentration that occurs uniformly throughout the bay. (5) Changes in oyster filtration rate affect the rate of infective particle uptake and, hence, the rate of infection. Only the last process involves the host population.

The processes by which *H. nelsoni* is transmitted to uninfected oysters, and the form of the infective particle, are not known. However, observations that the earliest infections are in the gill epithelium indicate that infective particles are acquired through filtration (Farley 1968, Ford and Haskin 1982). Hence the rate at which new *H. nelsoni* infections occur in uninfected oysters ( $O_{00}$ ) is dependent upon the number of infective particles filtered out of solution. This rate,  $\beta_{00}$ , is given by:

$$\beta_{00} = \frac{I_1}{1 + \frac{I_1 - I_2}{I_2} e^{I_3 IPfilter}} \tag{4}$$

where *IPfilter* is the number of infective particles filtered by the oyster. The relationship assumes a threshold level of infective particles needed to generate a new infection, the infective dose, of 8,700 particles filtered  $d^{-1}$ . This infective dose was estimated from information provided by Barber and Ford (1992) on the number of haplosporidian spores in oyster guts, an estimate of the filtration rate of these oysters, and a series of simulations of varying infective dose compared to field observations of the infection process. The remainder of the transmission submodel is designed to estimate *IPfilter*.

**Oyster Filtration Rate**

The number of infective particles filtered by the oyster was modeled as:

$$IPfilter = IPconc \text{ filt}(size) IPseason IPsal IPtemp \tag{5}$$

where *IPconc* is the ambient infective particle concentration in the water column, *filt(size)* is oyster filtration rate, *IPseason* is the seasonal variation in infective particle availability, and *IPsal* and *IPtemp* are the salinity and temperature effects on infective particles, respectively. Oyster filtration rate is calculated using the relationships given in Hofmann et al. (1992, 1994) and is parameterized as a function of temperature, salinity, and total seston as described in these references. The relationships used to specify the seasonal, salinity, and temperature dependencies of the infective particles are described in the next sections.

**Base Infective Particle Number**

The base concentration of infective particles,  $IPconc_o$ , was chosen by comparing results of simulations using a range of values to field observations of prevalence. This value was used to initialize the model and to establish, as twice its value, a maximum concentration of infective particles ( $IPconc_{max}$ ) that could not be exceeded during a simulation.

### Seasonal Cycle in Infective Particle Number

Early studies with timed imports of oysters into enzootic regions of Delaware and Chesapeake Bays showed that oysters became infected only during a period from late May through early October (Andrews 1968, Ford and Haskin 1982), suggesting a seasonal dependence in the ambient concentration of infective particles. The concentration of infective particles in the water is a critical element in modeling transmission; however no measurements are available to parameterize this process. Recently, however, Barber and Ford (1992) reported finding haplosporidian spores in the digestive tract lumina of oysters in Delaware Bay and other regions enzootic for *H. nelsoni*. These spores, morphologically similar to spores produced by *H. nelsoni*, had obviously been ingested during feeding. Further, numbers were higher from May through October, the known infective period for *H. nelsoni*. Barber and Ford (1992) extrapolated their findings to estimate that mean spore abundance in the water the oysters were filtering was several hundred spores per liter. These may not be *H. nelsoni* spores, and if they are, they may not be the stage that infects oysters. Nevertheless, they represent the only available data on which to base a rough estimate of the seasonal variation in concentration of infective particles.

The base concentration of infective particles was modified seasonally based on the data obtained from examination of oyster gut contents. This time series (Fig. 1) was taken to reflect the relative abundance of infective particles. The seasonal change in relative abundance was included in equation (5) as  $IP_{season}$ .

### Salinity Effect on Infectivity

Initial simulations of *H. nelsoni* prevalence in oysters from low salinity environments suggested that the rate of infection decreases with decreasing salinity. An effect of salinity on *H. nelsoni* mortality within the oyster (Paraso et al. this volume) did not adequately simulate changes in prevalence, even when false negatives were taken into account. A function that decreased infection efficiency at low salinity resulted in simulated prevalence levels that better matched those recorded on the low-salinity Delaware Bay seed beds (Paraso et al. this volume). The function was obtained by using the model to simulate infections over a broad range of salinities in Delaware Bay and comparing these to long-term

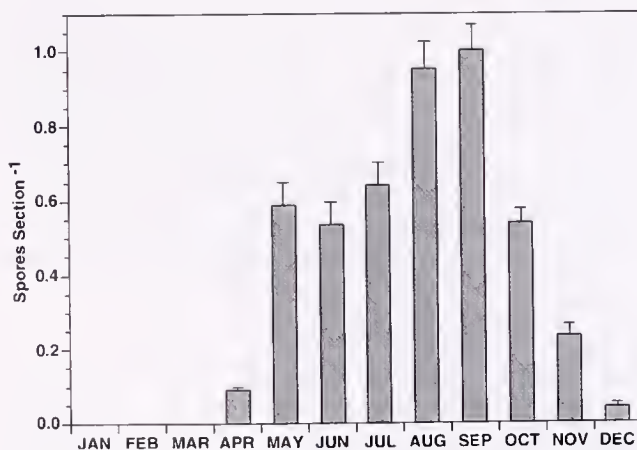


Figure 1. Time series of putative *Haplosporidium nelsoni* spores observed in sectioned *Crassostrea virginica* gut lumina (from information in Barber and Ford 1992).

time series (Ford and Haskin 1982, Haskin and Ford 1982). Based on these comparisons, the effect of local salinity on transmission rate was modeled as:

$$IP_{sal} = \frac{1 + \tanh\left(SM_1 \frac{(S - SM_0)}{SM_2}\right)}{2} \quad (6)$$

The relationship is biologically realistic because the salinity range affecting transmission is similar to the range affecting mortality in the host animal (Paraso et al. this volume). Whether equation (6) simulates the mortality of infective particles or their decreased ability to infect oysters at low salinity is unknown.

### Bay-wide Oscillations Induced by Salinity

Simulations with long-term time series that were designed to test the adequacy of the transmission model, using the basic process of oyster filtration, infective dose, the seasonal cycle of infective particle availability, and a local effect of salinity on infectivity, showed adequate simulations for oyster populations over some portion of the salinity range in Delaware Bay (e.g., Paraso et al. this volume) during most years. However, the same parameterizations failed in Chesapeake Bay and over the entire salinity range in Delaware Bay. Although the seasonal cycle of infective particle availability may be somewhat different, certainly most factors influencing transmission should be equivalent in both bays. This suggested an additional process was needed to model transmission rate.

Review of long-term time series of *H. nelsoni* prevalence taken simultaneously at multiple sites across the salinity gradient in both bays revealed relatively simultaneous oscillations in disease prevalence over a wide portion of the salinity gradient, even at locations where salinity remained high enough to have only a limited effect on *H. nelsoni* growth and mortality. Addition of bay-wide salinity-dependent multiyear oscillations in infective particle availability allowed both bays to be modeled with very minor differences in the values of only 3 variables,  $IP_{conc_0}$ ,  $IP_{conc_{max}}$ , and  $DD_o$ . (Variations in  $DD_o$  are discussed in the following section.) These oscillations are parameterized as follows.

The rate of salinity change was calculated as:

$$IP_{salrate} = IP_{salrate_0} \left( \frac{S_{IP} - IP_{sal_0}}{IP_{sal_1}} \right) \quad (7)$$

where  $IP_{salrate_0}$  specifies the response time of the infective particles to changes in salinity, which was taken to be 180 days. The salinity value used to specify  $S_{IP}$  is assumed to be representative of the salinity at which an *H. nelsoni* secondary host lives or where some other reservoir of infective particles is found. For the simulations given in the following sections, the value of  $S_{IP}$  was taken from the most downestuary (highest salinity) site showing strong salinity excursions across the 15‰ isohaline in both Delaware and Chesapeake bays. Lower salinity sites failed to provide adequate simulations in either bay and higher salinity sites were not present in the suite of available Chesapeake Bay time series. The concentration of infective particles was updated each time step, based on this rate ( $IP_{salrate}$ ) forced by the direction and magnitude of salinity change. So, for increasing salinities ( $IP_{salrate} \geq 0$ ),

$$\frac{dIP_{conc}}{dt} = IP_{salrate} (IP_{conc_{max}} - IP_{conc}). \quad (8)$$

For decreasing salinities ( $IPsalrate < 0$ ).

$$\frac{dIPconc}{dt} = IPsalrate (IPconc - IPconc_{min}) \quad (9)$$

and, a model initialization,  $IPconc = IPconc_0$ . The new value of  $IPconc$  was then inserted into equation (5). Not having any evidence to the contrary, the value of  $IPconc_{max}$  was routinely set at twice the initial base infective particle concentration ( $IPconc_0$ ) and  $IPconc_{min}$  was set near zero. Values for all constants are given in Table 3 of Ford et al. (this volume).

**Effect of Cold Winters**

Long-term observations from Delaware Bay show a cyclic pattern of *H. nelsoni* activity in which years of low infection prevalence follow, typically with a lag of 1 to 2 years, very cold winters (Ford and Haskin 1982). Examination of the long-term data sets for Chesapeake Bay showed the same phenomenon. Thus, in some years, very few oysters become infected, even when appropriate salinity conditions are present (Haskin and Ford 1982, Paraso et al. this volume). This pattern suggests that, in some way, the abundance of infective particles is diminished after cold winters.

In the model, direct temperature effects on infective particle abundance were included through a calculation of degree days that is based on 10 °C. The number of degree-days in which the temperature ( $T$ ) is below 10 °C ( $DD10$ ) from January (Julian Day 1) to May (Julian Day 150) is accumulated as:

$$DD10 = \sum_{t=1JD}^{t=150JD} 10 - T \quad (10)$$

and the value of  $DD10$  is then used to estimate the extent to which cold temperature affects the survival of infective particles ( $IPtemp_{est}$ ) as:

$$IPtemp_{est} = \frac{1}{2} \left\{ 1 - \tanh \left[ DD_2 \left( \frac{DD10 - DD_0}{DD_1} \right) \right] \right\} \quad (11)$$

where  $DD_0$  is a threshold value at which the temperature effect becomes active. Values for the constants are given in Table 3 of Ford et al. (this volume).

Equation (11) provides a value for the temperature effect that is based upon the current degree-day calculation. To simulate the observed delay in the manifestation of winter temperature effects on *H. nelsoni* infective particles, the value of  $IPtemp$  in equation (5), determined from the current  $DD10$  value, was modified based on the value calculated for the previous year. If the current value of  $DD10$  is less than one-half of the threshold value ( $DD_0$ ), then this indicates that the current year's winter is considerably warmer (an extreme difference) than that in the previous year, and the current value of  $IPtemp_{est}$  is used as  $IPtemp$ . If the current value of  $DD10$  is greater than one-half  $DD_0$  and less than the value for the previous year, such that the current year's winter is only somewhat warmer than the previous year's winter, the current and previous year's values are averaged to obtain the value for  $IPtemp$ . This allows the conditions in the previous winter to affect the level of infectivity by *H. nelsoni* and thereby allows for persistence of the effects of harsh winters over a period of more than 1 year, as observed. If  $DD10$  is greater than one-half  $DD_0$  and greater than the value calculated for the previous year, then the current conditions are colder than the previous year's conditions and also characteristic of an average to cold winter. In this case,  $IPtemp$  is specified using the current value of  $IPtemp_{est}$ .

**FIELD OBSERVATIONS**

The environmental factors that force the oyster population-*H. nelsoni* model are salinity, temperature, food, and total seston (total suspended solids). We compared simulations of 3 time series to field data of *H. nelsoni* prevalence for verifying the model. In each case, the simulations and field observations covered a substantial fraction of the salinity gradient in which oysters live. These time series were: 1960 to 1970 for Delaware Bay, 1980 to 1990 for Delaware Bay, and 1989 to 1994 for Chesapeake Bay.

*Delaware Bay*

Prevalence and infection intensity of *H. nelsoni* were measured over the period 1959 to 1992 by personnel from the Haskin Shellfish Research Laboratory at Rutgers University (Ford and Haskin 1982, Haskin and Ford 1982, Fegley et al. 1994). Oysters from the downbay planted grounds were obtained by dredge at regular intervals during the year. Oysters from the upbay seed beds were typically collected during late autumn/early winter and the following late spring. Five locations, identified in Fig. 1 of Paraso et al. (this volume), distributed along the salinity gradient, were chosen for simulation. From lowest to highest salinity, these were Arnolds Bed, Cohansey Bed, Shell Rock Bed, Bennies Bed, and Miah Maull Grounds. These sites are described in more detail in Paraso et al. (this volume). The time series of *H. nelsoni* prevalence for these sites show that the decades of the 1960s and 1980s contain epizootics of 3 to 5 years duration, preceded and followed by periods of relatively low *H. nelsoni* prevalence (Fig. 2). These decades, therefore, test the full range of the model's capabilities.

Temperature time series for these 2 decades were obtained from long-term temperature recordings at the Haskin Shellfish Research Laboratory pier in the lower Maurice River, supplemented by once-to-twice monthly recordings at each of the 5 sites for the 1960s and 1980s, except for 1989 to 1990 when very few measurements were available (Fig. 3). Simulations of the 1980s decade are credible only through 1988. The same temperature time series was used for all 5 locations along the salinity gradient. The  $DD10$  value, given for each winter (Fig. 3), is a measure of the severity of the winter, with higher values indicating colder winters. By this measure, the decade of the 1960s was characterized by particularly cold winters in 1962 and the period 1968 to 1970 (Fig. 3). A relatively warm period from 1963 to 1967 coincided with a prolonged drought and relatively high-salinity conditions. The 1980s were a period of consistently warmer temperatures. Only 1982 would be considered cold by 1960s standards and 7 winters were warmer than any winter recorded in the 1960s decade.

Salinity time series were derived from monthly-averaged Delaware River flow measurements taken at Trenton, New Jersey, by the U.S. Geological Survey using the relationship between Delaware River flow and salinity derived by Haskin (1972) as described in Paraso et al. (this volume). The Haskin relationship was clearly appropriate for the 1960s time series. However, the 1980s time series followed the establishment of the relationship by about a decade and, so, salinities may not be predicted as accurately for this decade. In particular, the century-long gradual increase in Bay salinity observed by Haskin (1972) presents the possibility that the salinities used for the 1980s time series were somewhat underestimated.

The Delaware Bay salinity time series (Figs. 4, 5) is characterized by spring lows and summer/fall highs coincident with the typical variation in Delaware River flows (Fig. 2). The decade of

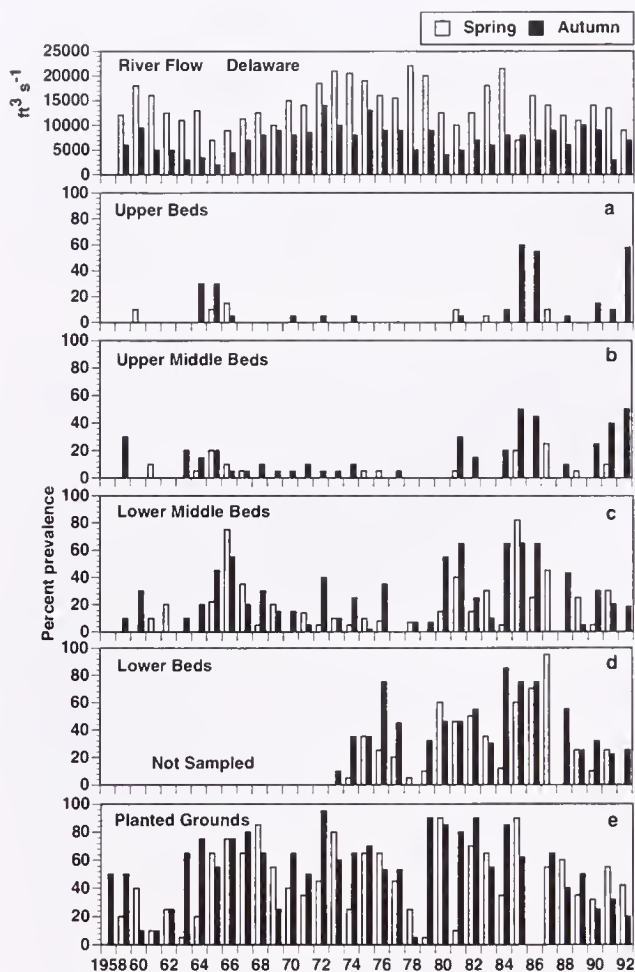


Figure 2. Delaware River flow and prevalence of *Haplosporidium nelsoni* for (a) Arnolds Bed, (b) Cohansey Bed, (c) Shell Rock Bed, (d) Bennies Bed, and (e) Miah Maull Grounds constructed from data in Fegley et al. (1994). Annual cycles are represented by two measurements. Open bars on the left are May/June conditions; shaded bars on the right are November/December conditions. Location map is Figure 1 in Paraso et al. (this volume).

the 1960s was characterized by increasingly saline conditions, on the average, in the first 6 years of the decade, followed by a strong freshening trend that began in 1967 (Fig. 4). The 1963 to 1967 drought coincided with a period of relatively mild winters compared to other years in the decade. The decade of the 1980s (Fig. 5) had fewer anomalously wet or dry years, although 1985 was characterized by an unusually dry spring and salinity, on average, decreased in the latter years of the decade at all sites.

Neither food nor total seston data were available for most of the 1980s or for any of the 1960s. Accordingly the food and total seston data sets developed by Powell et al. (1997) were employed. These time series are based on total seston and chlorophyll measurements made at several locations in Delaware Bay by Haskin Shellfish Research Laboratory (HSRL) scientists at about monthly intervals from 1981 to 1986, with the sampling frequency increased to bi-weekly between 1982 and 1984. For this study, the time series from a site just east of the Delaware Bay Ship Channel opposite Kelly Island, Delaware ( $39^{\circ}14.44'N$   $75^{\circ}16.47'W$ ), was taken to be representative of conditions on the 4 seed beds. Measurements at a site just south of Egg Island, New Jersey

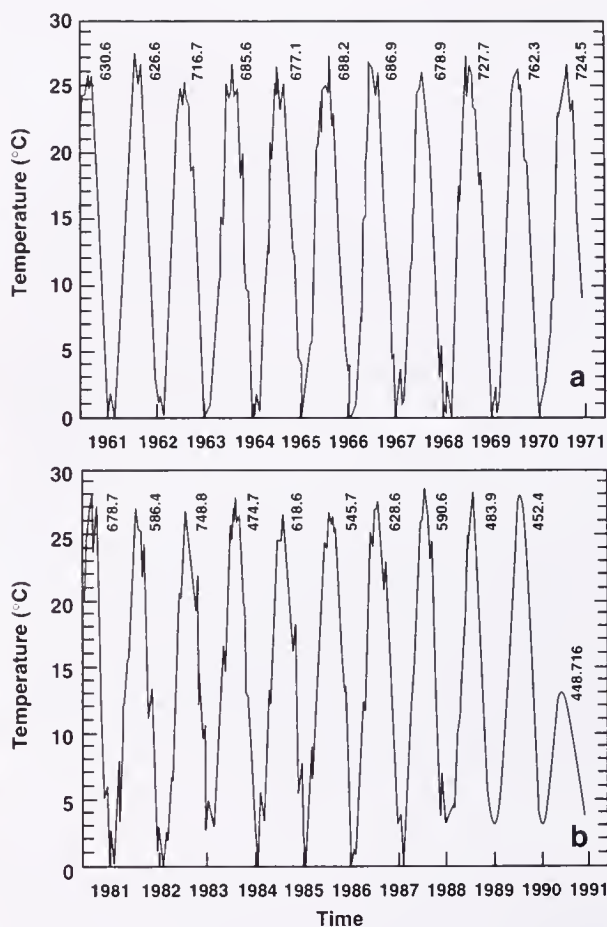


Figure 3. Temperature time series used for the decadal runs of the (a) 1960s and (b) 1980s in Delaware Bay. Values for 1989 and 1990 are based on only a few measurements and should be discounted. Numbers above each winter are values of the model variable *DD10*, a measure of the coldness of the winter. Higher values indicate colder conditions. Location map is Figure 1 in Paraso et al. (this volume).

$39^{\circ}10.46'N$   $75^{\circ}5.05'W$ ), were assumed to be representative of the planted grounds. The 6 years of data were averaged to obtain a 1-year average data set at both locations that was used each year during the decade-long simulations. The same data sets were used for the 1960s and 1980s simulations.

Total suspended solids at both sites show variability throughout the year, with maximum values tending to occur in late spring to early autumn (Fig. 6). Total suspended solids average about twice as high on the planted grounds than over the 4 seed bed sites throughout the year. The chlorophyll time series shows a distinctive spring bloom that occurs in March to May, with the maximum in March downbay and about one-half month later over the seed beds (Fig. 6). A consistent fall bloom does not occur at either site, although transient increases in chlorophyll content do occur from time to time. Rather, at both sites, chlorophyll values drop to seasonally low levels in July and remain, for the most part, at or near these levels until the next spring. Chlorophyll values average higher over the planted grounds than any of the seed bed sites throughout the year, including the spring bloom and the much lower summer and fall mean. As a consequence, food supply drops to very low levels in the summer and fall over the seed beds.

Chlorophyll *a* in  $\mu g L^{-1}$  was converted to oyster food in  $mg DW L^{-1}$  using the relationship derived by Powell et al. (1997):

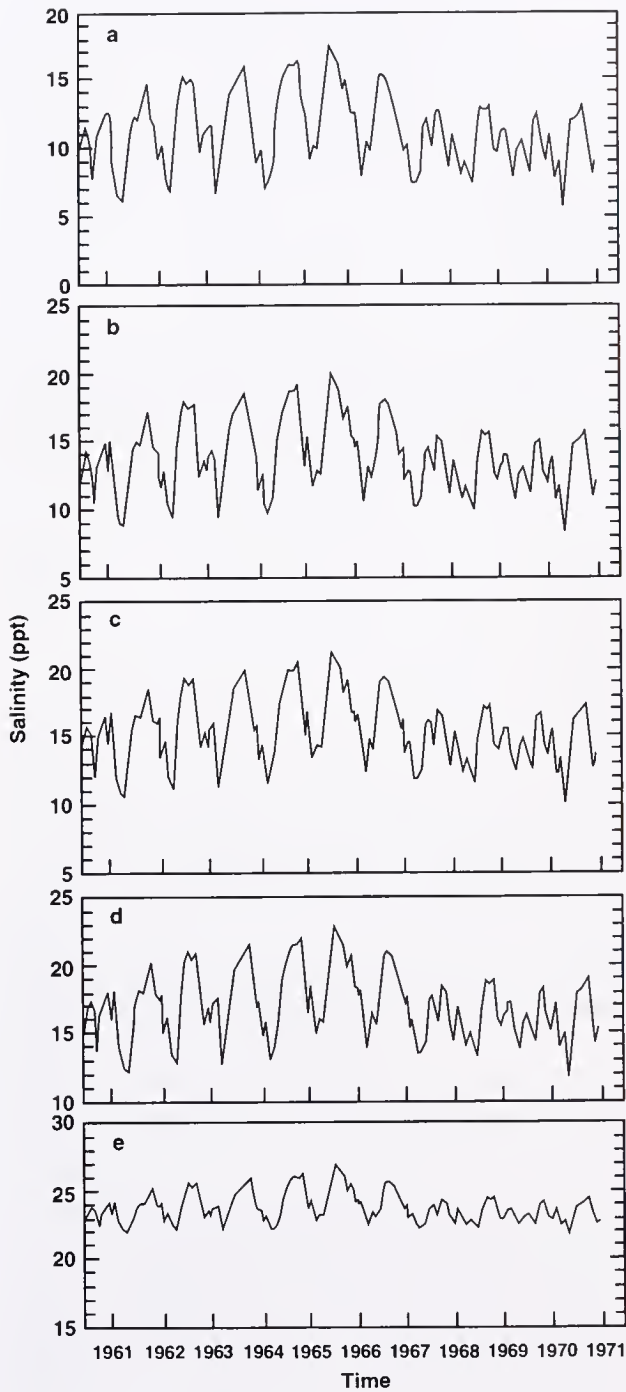


Figure 4. Salinity time series used for the decadal runs of the 1960s in Delaware Bay for (a) Arnolds Bed, (b) Cohanseey Bed, (c) Shell Rock Bed, (d) Bennies Bed, and (e) Miah Maull Grounds. Location map is Figure 1 in Paraso et al. (this volume).

$$Food = \alpha \times chlorophyll\ a + \beta \quad (12)$$

where  $\alpha = 0.088\ mg\ \mu g^{-1}$  and  $\beta = 0.26\ mg\ L^{-1}$ .

*Chesapeake Bay*

*H. nelsoni* prevalence was derived from hemolymph assays measured by personnel at the Oxford Cooperative Laboratory, Maryland Department of Natural Resources (DNR), for the periods

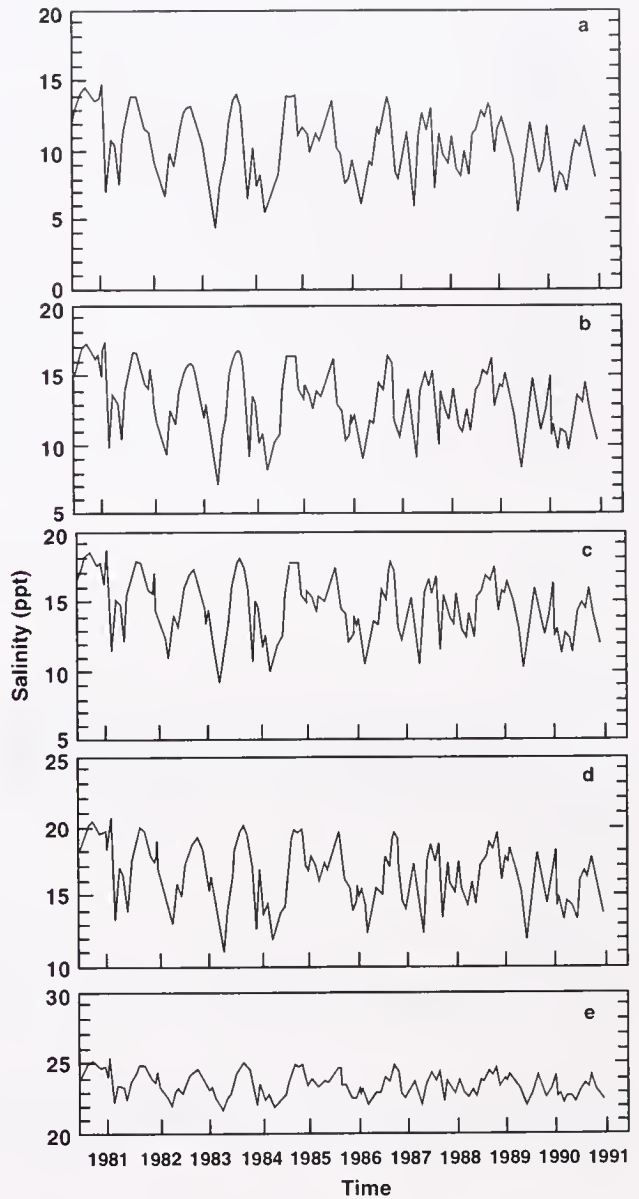


Figure 5. Salinity time series used for the decadal runs of the 1980s in Delaware Bay for (a) Arnolds Bed, (b) Cohanseey Bed, (c) Shell Rock Bed, (d) Bennies Bed, and (e) Miah Maull Grounds. Location map in Figure 1 in Paraso et al. (this volume).

1990 to 1995 for a series of sites in the Maryland portion of Chesapeake Bay covering the full range of the salinity gradient in Maryland waters (averaging near 0‰ to around 15‰). Sites TSOW (Tangier Sound-Old Woman’s Leg), TSSS (Tangier Sound-Sharkfin Shoal), FBGC (Fishing Bay-Goose Creed), LCRP (Little Choptank River-Ragged Point), MESR (Middle Eastern Shore-Stone Rock), and PRCH (Potamic River-Cornfield Harbor) were selected for simulations (Fig. 7). Hemolymph prevalence data for each of these stations were used for verification (Fig. 8). Oyster shells were drilled. After purging the drill site in seawater for 12–24 h, 0.5 mL hemolymph was withdrawn from the adductor muscle with a sterile syringe. Four drops of hemolymph were placed in a well slide with 0.5 mL sterile seawater and allowed to settle for 30 min. After settling, seawater was decanted from the well and replaced with Davidson’s fixative. The preparation was

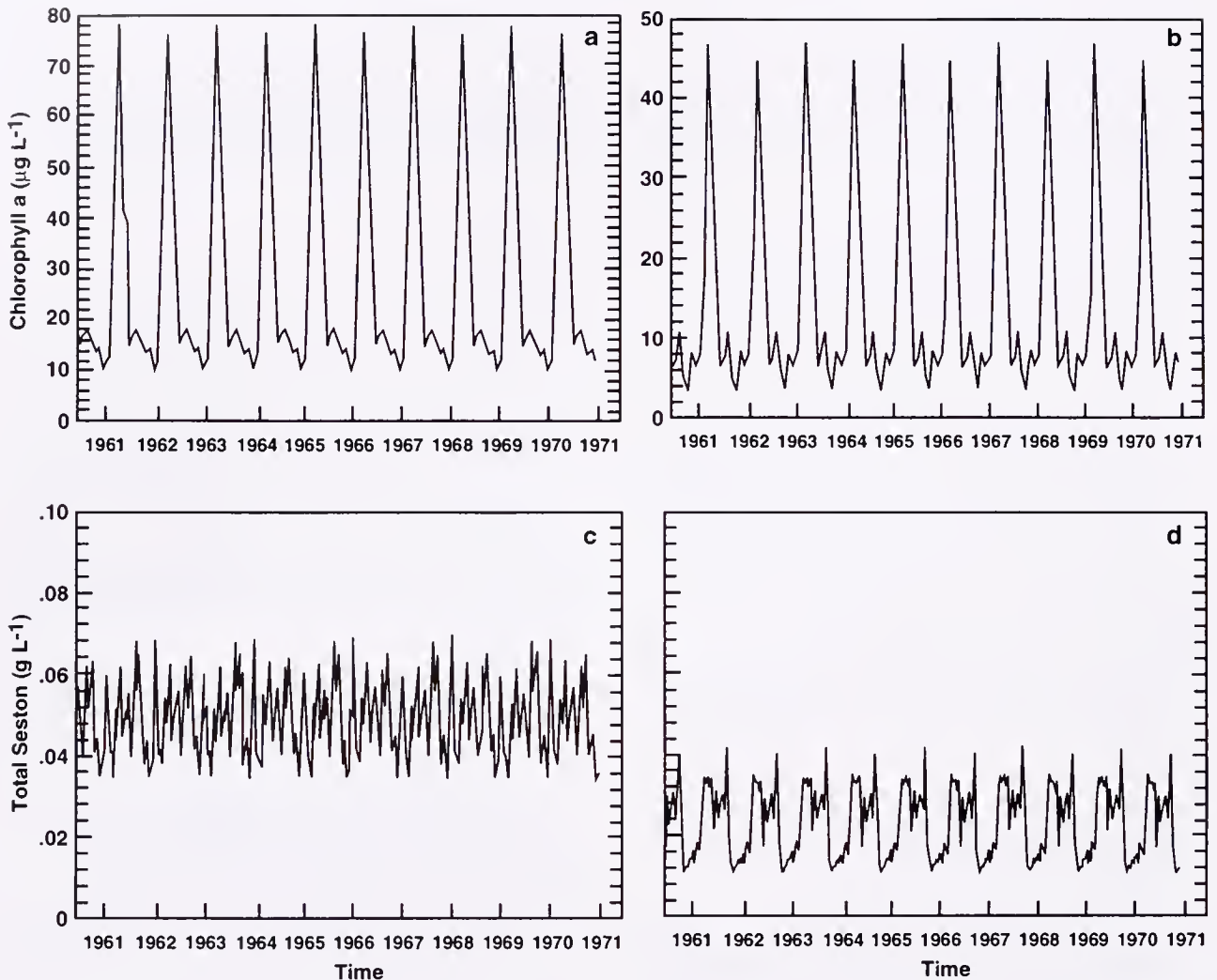


Figure 6. Chlorophyll *a* and total seston time series used for the decadal runs of the 1960s and 1980s in Delaware Bay. Time series are composed of 10 repeats of an average year derived by averaging data obtained during the time period 1981 to 1986. (a,c) Data for Miah Maull Grounds; (b,d) data for the oyster seed beds, including Arnolds Bed, Cohanseay Bed, Shell Rock Bed, and Bennies Bed. The same time series were used for the 1960s and the 1980s simulations.

then stained with Mayer's hematoxylin and eosin, and cover-slipped. The resulting slides were read, with counts of MSX plasmodia in standard microscope fields recorded on a semi-quantitative scale. Occasional values of tissue prevalence, done histologically, were always higher, so that the hemolymph values plotted in Figure 8 should be viewed as conservative estimates of prevalence.

Time series of salinity, temperature, food, and total seston were obtained from stations near the oyster bed sampling sites that are part of the Environmental Protection Agency (EPA) Chesapeake Bay Monitoring Network (Fig. 7). Data were obtained from the EPA public access web site: [www.chesapeakebay.net/bayprogram/data/wqual/wqgate.html](http://www.chesapeakebay.net/bayprogram/data/wqual/wqgate.html). Maryland DNR (EPA) station pairs are given in Figure 7. Data for the deepest depth sampled were used except in cases where the EPA station was in a channel. In this case, a mid-water depth was used to be more representative of the conditions on the nearby oyster bed.

Salinity was highest at Tangier Sound-Old Woman's Leg (EE3.2) and lowest at Middle Eastern Shore-Stone Rock (CB4.2) (Fig. 9). Each of the 6 data sets was characterized by a relatively

wet period in 1990/1991 and a relatively dry period in 1992/1993 followed by a period of extreme oscillations in 1994/1995.

The temperature time series were nearly identical among the 6 sites (Fig. 10). Years 1990 and 1994 were markedly colder than the remaining years. Year 1992 was markedly warmer.

Chlorophyll values typically showed a strong spring bloom and a much smaller, but distinct, fall bloom (Fig. 11). The spring bloom was more intense and predictable at Tangier Sound and Little Choptank River stations. With the exception of EE2.2 in the Little Choptank River, 1992 was an anomalous year with unusually low chlorophyll values and the near absence of a spring bloom. Chlorophyll *a* data were converted to oyster food using equation (12).

Total seston was chaotic in its time series, as it was in Delaware Bay (Fig. 12). Stations in the main bay stem, Middle Eastern Shore-Stone Rock (CB4.2), and Potomac River-Cornfield Harbor (LE2.3), had lower suspended loads in contrast to stations on the eastern side in Tangier Sound (Old Woman's Leg and Sharkfin Shoal) and Little Choptank River-Ragged Point (EE2.2), where total seston typically varied between 20 and 50 mg L<sup>-1</sup>.

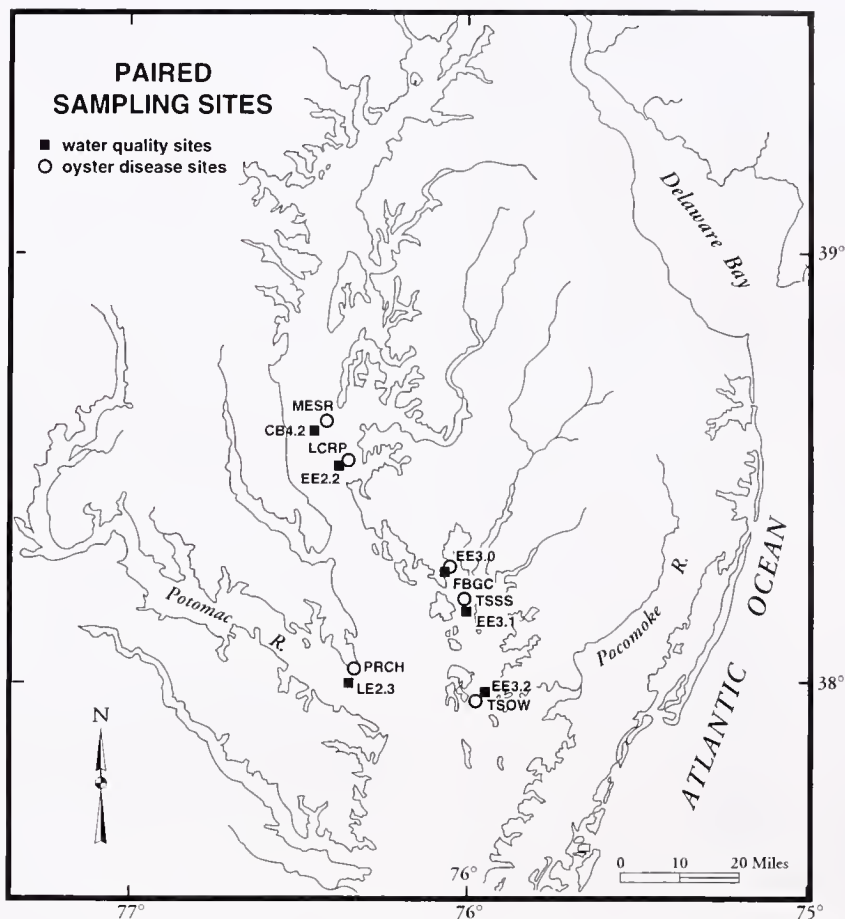


Figure 7. Distribution of Oxford Cooperative Laboratory sampling sites for *Haplosporidium nelsoni* prevalence and EPA sampling sites for environmental variables. The sites used for simulations are (Oxford/EPA) TSOV/EE3.2, TSSS/EE3.1, FBGC/EE3.0, LCRP/EE2.2, MESR/CB4.2, PRCH/LE2.3. TSSS, Tangier Sound-Sharkfin Shoal; TSOV, Tangier Sound-Old Woman's Leg; LCRP, Little Choptank River-Ragged Point; FBGC, Fishing Bay-Goose Creek; MESR, Middle Eastern Shore-Stone Rock; PRCH, Potomac River-Cornfield Harbor. Additional information is available in Smith (1997).

#### MODEL IMPLEMENTATION

Details of model implementation are presented in Ford et al. (this volume). The limited understanding of the true process of transmission required that the model be verified using observations of the time series of prevalence in oyster populations. That is, the model was verified based on knowledge of the result of the process rather than the process itself. In approaching this task, our *modus operandi* included two principal constraints: (1) that processes induced in the model had some ecological and biological relevance, that is, that the processes existed and could have been responsible for the observed results; and (2) that the two bays, Chesapeake and Delaware, and the two time periods in Delaware Bay should be simulated adequately with very few differences in model construction and variable values, that is, that the transmission process was common to all places and times.

#### RESULTS

##### Base Cases for Verification

##### Delaware Bay, 1960s

A drought during the middle 1960s generated an *H. nelsoni* epizootic in Delaware Bay. Prevalences were observed to increase

down the salinity gradient from about 30‰ at Arnolds Bed to near 80‰ at Bennies Bed in 1965. Simulated *H. nelsoni* prevalence during the 1960s using an initial infective particle concentration of 900 infective particles  $L^{-1}$  shows a similar pattern. Prevalence increases downbay from about 20% at Arnolds Bed to 70% at Bennies Bed in 1965 (Fig. 13). About one-half of the simulated *H. nelsoni* infections are strong systemic (BFU = 3 or 4) infections, which corresponds to observation. The epizootic in the simulated population diminishes over time and *H. nelsoni* nearly disappears from the bay by 1970, as was observed. Observations at Bennies Bed showed that the epizootic was strongest in 1965 through 1967. This is also true in simulation. Simulated cumulative mortalities reach about 80% by 1968 at Bennies Bed and decline upbay to less than 10% during the same period as Arnolds Bed. Accordingly, the simulations of the time-dependence of *H. nelsoni* infection agree, to the level represented in the observed disease prevalence and intensity time series, with what occurred in Delaware Bay during the decade of the 1960s.

##### Delaware Bay, 1980s

The 1980s period was characterized by an *H. nelsoni* epizootic during 1982 and 1985. *H. nelsoni* prevalence declined late in the decade and during the 1982 to 1983 period (Fig. 2). In the simu-

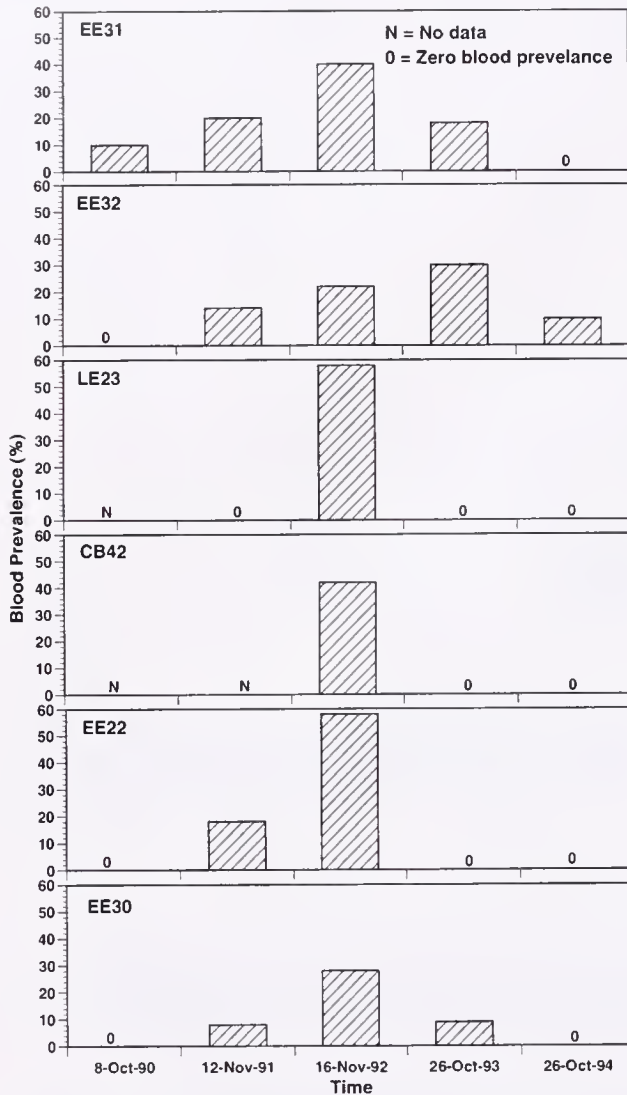


Figure 8. *Haplosporidium nelsoni* prevalence from blood assays taken by the Oxford Cooperative Laboratory at the 6 sites identified in Figure 7. TSSS/EE3.1, Tangier Sound-Sharkin Shoal; TSOW-EE3.2, Tangier Sound-Old Woman's Leg; LCRP/EE2.2, Little Choptank River-Ragged Point; FBGC/EE3.0, Fishing Bay-Goose Creek; MESR/CB4.2, Middle Eastern Shore-Stone Rock; PRCH/LE2.3, Potomac River-Cornfield Harbor.

lations, the 1985 epizootic and the decline in prevalence of *H. nelsoni* in 1983/1984 and in the last few years of the decade are faithfully reproduced at most sites (Fig. 14). Prevalences in the 1982 epizootic are somewhat exaggerated because of adjustments that occur early in the simulation as *H. nelsoni* prevalence and infection intensity come into equilibrium with environmental conditions. These adjustments normally take about 24 months of simulation, but the time required is exaggerated during epizootic conditions.

Overall, the simulated prevalences for the 1980s underestimate observations throughout the salinity gradient, and particularly so at upbay sites, despite the use of a significantly higher maximum infective particle concentration (1,500 infective particles L<sup>-1</sup> versus 900 particles L<sup>-1</sup>) than used in the 1960s simulations. The necessity of using varying concentrations of infective particles as initial conditions will be discussed in a later section.

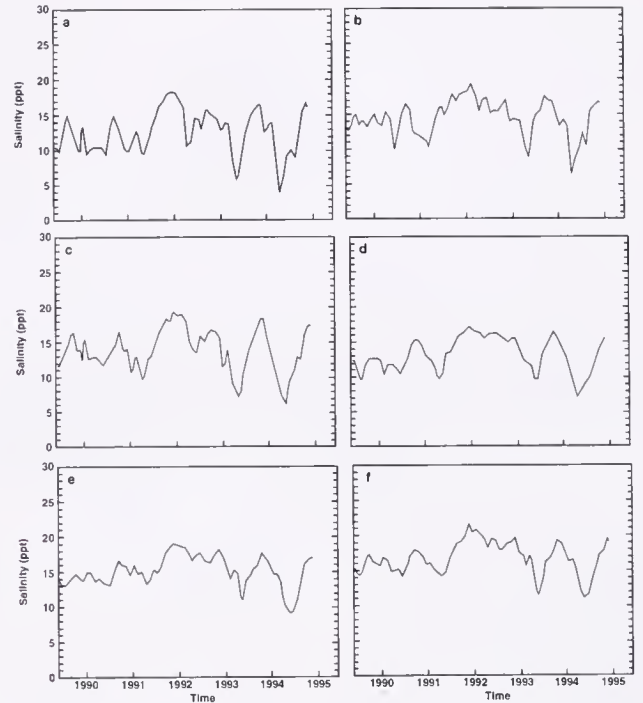


Figure 9. Salinity time series for Chesapeake Bay obtained from the EPA stations identified in Figure 7. (a) CB4.2; (b) EE2.2; (c) LE2.3; (d) EE3.0; (e) EE3.1; and (f) EE3.2.

Chesapeake Bay, Early 1990s

Observations show that an *H. nelsoni* epizootic occurred in 1992 at all sites (Fig. 8) and *H. nelsoni* prevalence increased again in 1995. The 1992 to 1993 epizootic was restricted temporally at most low-salinity sites in 1992, whereas higher salinity sites

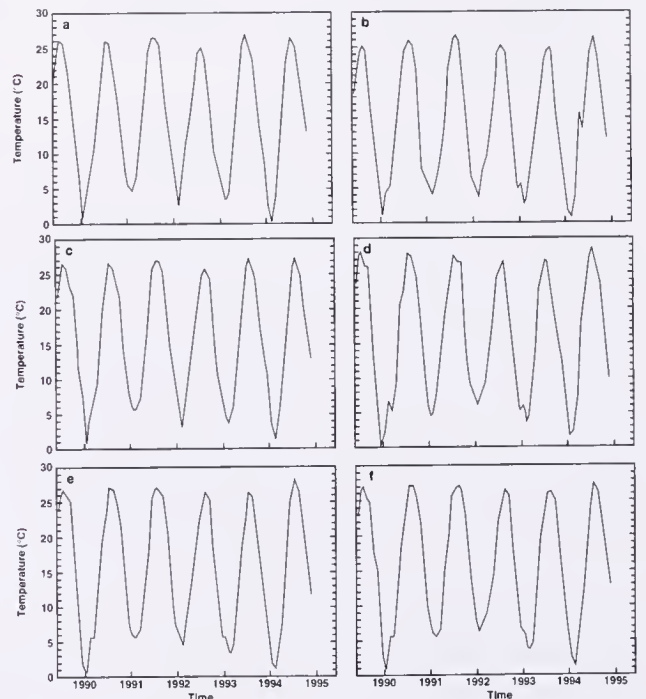


Figure 10. Temperature time series for Chesapeake Bay obtained from the EPA stations identified in Figure 7. (a) CB4.2; (b) EE2.2; (c) LE2.3; (d) EE3.0; (e) EE3.1; and (f) EE3.2.



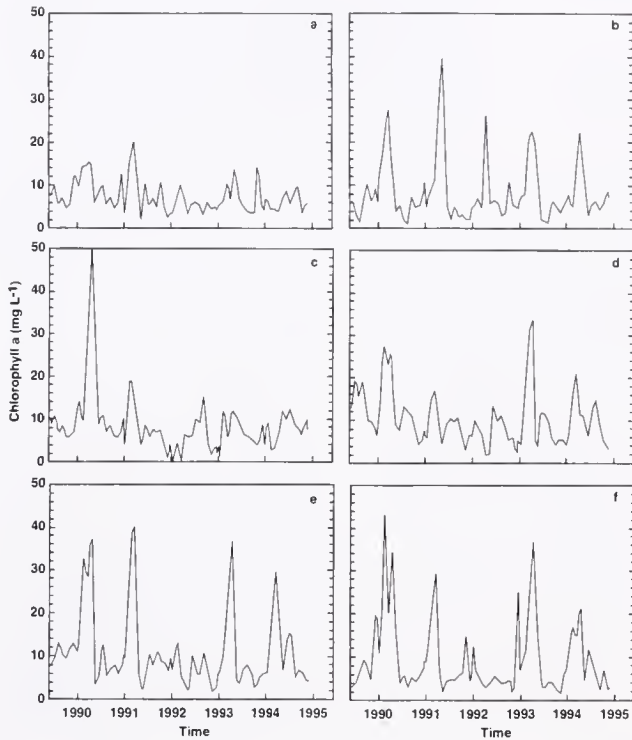


Figure 11. Chlorophyll *a* time series for Chesapeake Bay obtained from the EPA stations identified in Figure 7. (a) CB4.2; (b) EE2.2; (c) LE2.3; (d) EE3.0; (e) EE3.1; and (f) EE3.2.

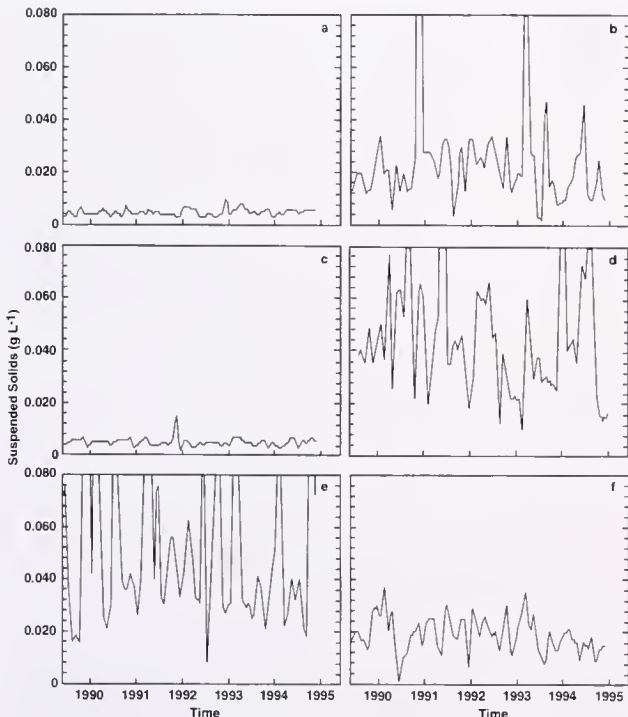


Figure 12. Total seston time series for Chesapeake Bay obtained from the EPA stations identified in Figure 7. (a) CB4.2; (b) EE2.2; (c) LE2.3; (d) EE3.0; (e) EE3.1; and (f) EE3.2.

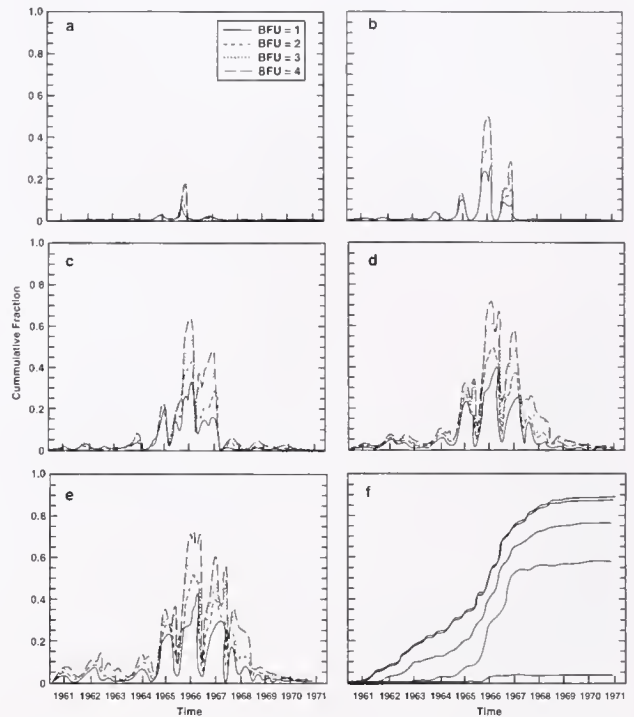


Figure 13. Time-development of *Haplosporidium nelsoni* infection in Delaware Bay during the 1960s using a maximum infective particle concentration of 900 infective particles  $L^{-1}$  and a standard 1 g AFDW oyster. (a) Arnolds Bed, (b) Cohansey Bed, (c) Shell Rock Bed, (d) Bennies Bed, and (e) Miah Maul Grounds. (f) Cumulative mortality during the simulation for each of the 5 simulated locations.

in Tangier Sound (TSOW, TSSS) were characterized by higher prevalences in 1993 as well and prevalences above 10% in most years. In contrast to the longer time span of the epizootic downbay, the epizootic reached highest prevalences upbay at the lower salinity sites, where prevalences in 1992, measured by hemolymph assay, exceeded 40%.

The observations of *H. nelsoni* prevalence (Fig. 8) were taken in late October/early November of each year. It is during this time that the model simulations typically show rapid changes in prevalence, making comparison to field data difficult. Nevertheless, the 1992 epizootic is present in each simulation, as are the low prevalences before and after (Fig. 15). The epizootic, as simulated, is temporally more extensive at Tangier Bay sites, as observed, and tends to be restricted to 1992 at upbay sites, also as observed. Simulated upbay prevalences are not necessarily higher than downbay prevalences, however. Imposition of a spatially variable concentration of infective particles at the two most-upbay sites (Little Choptank River and Middle Eastern Shore) would correct this error. Nevertheless, except for Little Choptank River (underestimated by about half) and Tangier Sound-Old Woman's Leg (overestimated by about a factor of 3), simulated prevalences are very close to observation. In both of the latter cases, similar prevalences exist in the simulations within 1 month of observation, so that small changes in timing are critical. In particular, the fall rise in prevalence is keyed to a fall increase in salinity at most sites and the exact timing of this salinity excursion is crucial because *H. nelsoni* cannot increase in intensity as long as salinities stay much below 15‰. Finally, the model simulates tissue, not hemolymph prevalence, and these can be significantly different (Ford and

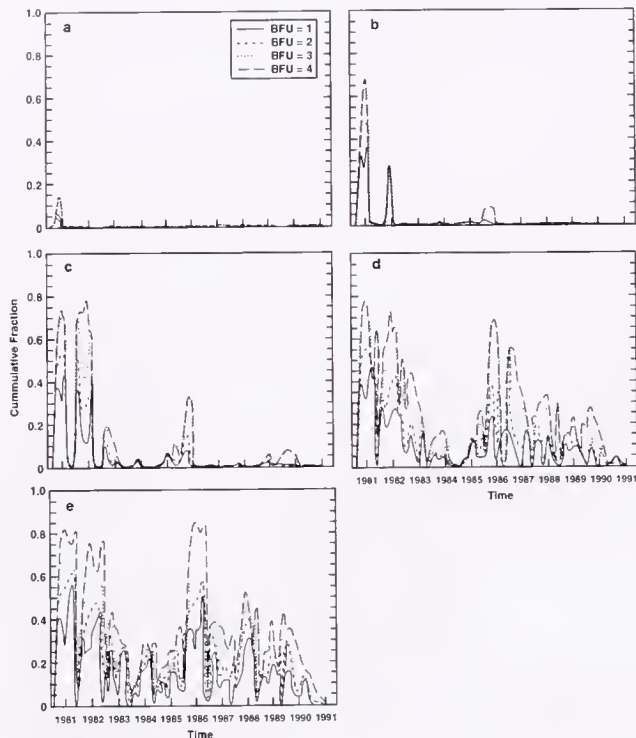


Figure 14. Time-development of *Haplosporidium nelsoni* infection in Delaware Bay during the 1980s using a maximum infective particle concentration of 1,500 infective particles  $L^{-1}$  and a standard 1-g AFDW oyster. (a) Arnolds Bed, (b) Cohansey Bed, (c) Shell Rock Bed, (d) Bennies Bed, and (e) Miah Maul Grounds.

Kanaley 1988). Accordingly, within the limitations of the model and observations, the model accurately simulates the time-dependent growth and decay of the 1992 to 1993 Chesapeake Bay epizootic.

#### Components of the Transmission Model

##### Oyster Filtration Rate

Initiation of *H. nelsoni* infection requires filtration of an infective dose. Total seston, among other environmental factors, can affect oyster filtration rate (Loosanoff 1962, Powell et al. 1992), as shown by a simulation in which the effect of a decrease in filtration rate was produced by an increase in total seston concentration (Fig. 16). Prevalence decreases as oysters filter infective particles less rapidly. Small changes in temperature, salinity, and total seston, as they affect filtration rate, will generate significant changes in prevalence. In this study, the rarity of environmental data and population data obtained from precisely the same location introduces a significant potential error in accurately simulating observed prevalence, because cross-bay and upbay-downbay variations in environmental variables are significant (e.g., Garvine et al. 1992, Wong 1994).

##### Initial Concentration of Infective Particles

The model requires initialization with a beginning infective particle concentration ( $IPconc_0$ ) and establishment of a maximum particle concentration that cannot be exceeded during the simulation ( $IPconc_{max}$ ) set at twice  $IPconc_0$ . No information exists on

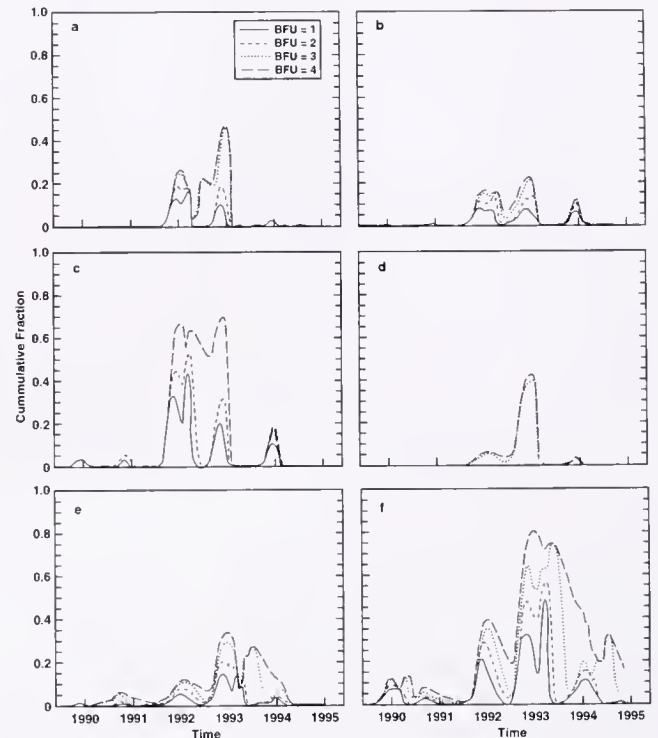


Figure 15. Time-development of *Haplosporidium nelsoni* infection in Chesapeake Bay during the early 1990s using a maximum infective particle concentration of 900 infective particles  $L^{-1}$  and a standard 1-g AFDW oyster. (a) Middle Eastern Shore-Stone Rock; (b) Little Choptank River-Ragged Point; (c) Potomac River-Cornfield Harbor; (d) Fishing Bay-Goose Creek; (e) Tangier Sound-Sharkfin Shoal; (f) Tangier Sound-Old Woman's Leg.

infective particle concentration except for estimates by Barber and Ford (1992), obtained from spore concentrations in gut lumina, that infective particle concentrations should be in the hundreds per liter.

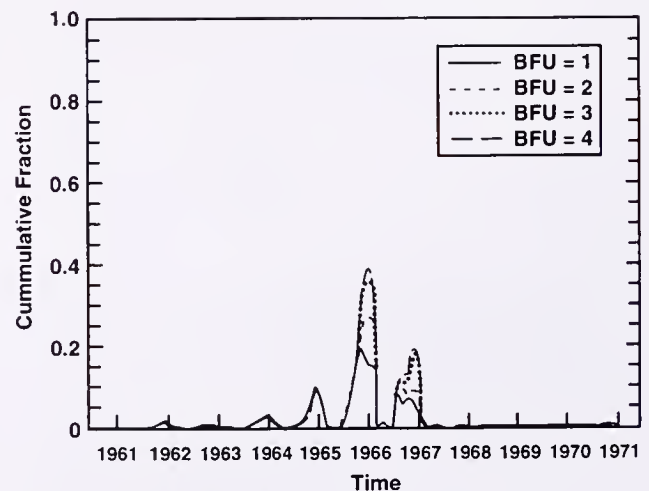


Figure 16. Time-development of *Haplosporidium nelsoni* infection on Cohansey Bed in Delaware Bay during the decade of the 1960s using a maximum infective particle concentration of 900 infective particles  $L^{-1}$  and a standard 1-g AFDW oyster, but using the higher total seston time series of Miah Maul Grounds (Fig. 6). The comparable reference case is given in Figure 13.

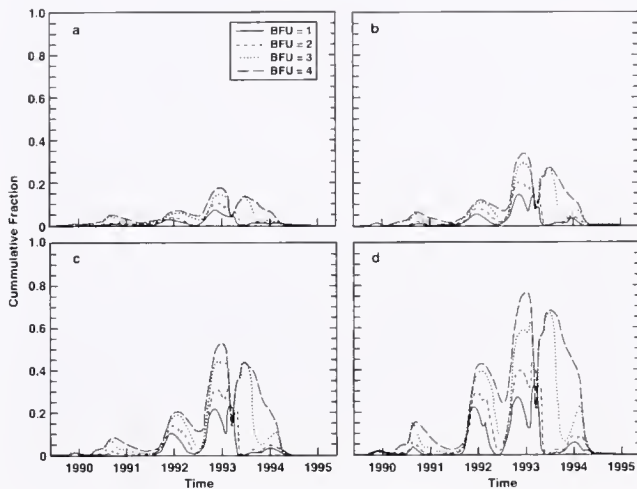


Figure 17. Time-development of *Haplosporidium nelsoni* infection in Chesapeake Bay during the early 1990s using a range of maximum infective particle concentrations and a standard 1-g AFDW oyster. The simulations are run using the environmental conditions for Tangier Sound-Sharkfin Shoal. Maximum allowable infective particle concentrations ( $IPconc_{max}$ ) for these simulations are: (a) 700 particles  $L^{-1}$ ; (b) 900 particles  $L^{-1}$  (reference case in Fig. 15); (c) 1,100 particles  $L^{-1}$ ; (d) 1,500 particles  $L^{-1}$ . Initial particle concentration ( $IPconc_o$ ) was set at half this value.

Variation in these two variables determining infective particle concentration significantly alters the simulated pattern of *H. nelsoni* prevalence. In Figure 17, simulations are presented in which the maximum allowed concentration of infective particles was varied from 700 to 1,500 particles  $L^{-1}$ . Simulated prevalence increases in proportion with the concentration of infective particles. In Chesapeake Bay in the early 1990s and in Delaware Bay in the 1960s, the simulations agree with observations for a maximum infective particle concentration of 900 particles  $L^{-1}$ ; the value of which also agrees with estimates made by Barber and Ford (1992).

In contrast, fitting simulated disease prevalences with observations for the decade of the 1980s in Delaware Bay required using a maximum infective particle concentration of 1,500 particles  $L^{-1}$ . Simulations using lower concentrations failed to provide the observed prevalences (Fig. 18). This case represents one of two (the other to be discussed later) cases where changing model parameter values proved necessary to provide adequate agreement between simulation and observation between sites or times. Several possible explanations exist for the higher infective particle concentration required in the 1980s in Delaware Bay. (1) The infective dose has decreased over time in Delaware Bay. That is, oysters have become more susceptible to infection. A decrease in infective dose would allow infection to occur at a lower particle concentration. However, no evidence supports an increase in disease susceptibility (e.g., Andrews 1968, Farley 1975, Haskin and Ford 1979, Ford and Haskin 1982, Chintala and Fisher 1991). (2) *H. nelsoni* has become more resistant to low salinity in Delaware Bay. However, an increase in infective particle concentration was required even at the highest salinity sites (Bennies Bed and Miah Maull), where the influence of low salinity is minor or non-existent. Thus, a change in salinity tolerance fails to meet the requirements of all simulations. (3) Salinities have been underestimated in the 1980s using Haskin's (1972) regression equations between salinity and Delaware River flow. If salinities were higher, particularly at upbay

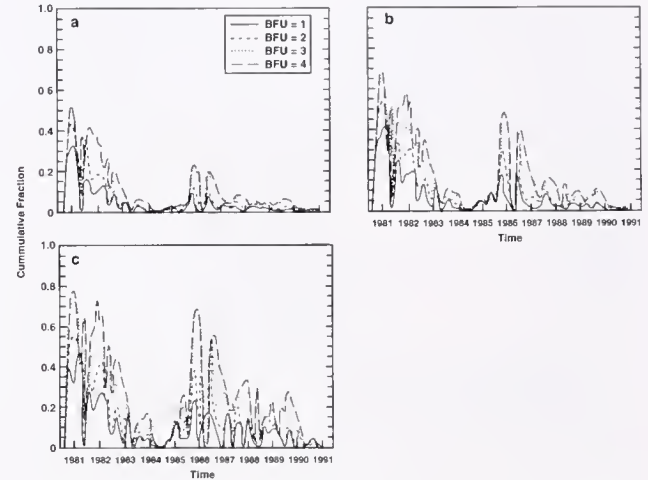


Figure 18. Time-development of *Haplosporidium nelsoni* infection in Delaware Bay during the 1980s using a range of maximum infective particle concentrations and a standard 1-g AFDW oyster. The simulations are run using the environmental conditions for Bennies Bed. Maximum infective particle concentrations ( $IPconc_{max}$ ) for these simulations are: (a) 700 particles  $L^{-1}$ ; (b) 1,000 particles  $L^{-1}$ ; (c) 1,500 particles  $L^{-1}$  (reference case in Fig. 14). Initial particle concentration ( $IPconc_o$ ) was set at half this value.

stations, than predicted from these equations, prevalence would increase for a given infective element concentration because the influence of low salinity on transmission is to decrease the rate of infection. Because the model simulations fit field observations increasingly poorly in the 1980s with decreasing salinity upbay, the evidence supports this option. However, an increase in infective particle concentration was required even at high-salinity sites (Bennies Bed and Miah Maull), where the influence of low salinity is minor or non-existent. (4) The concentration of infective particles has increased in Delaware Bay since the 1960s. Because modifications in the relationship of *H. nelsoni* with salinity do not adequately explain the discrepancy between simulation and observation and because *H. nelsoni* prevalence seems to have increased over time at high-salinity sites, this option remains the most viable of the four.

#### Seasonal Cycle in Infective Particle Number

Observations of *H. nelsoni* spores in oyster gut lumina suggest that infective particles are not present in the water column all year. Replacing the seasonal cycle with a constant value for infective particle availability gives an annual cycle of *H. nelsoni* prevalence (Fig. 19) that is relatively similar to the reference case (Fig. 13) because the temperature dependency of filtration rate reduces the filtration rate of infective particles in the winter. Nevertheless, infection intensities and prevalences average higher after removal of the seasonal cycle and this is due to higher infection rates during the spring warming than observed.

#### Salinity Effect on Infectivity

The effect of changes in salinity at the local population level has been investigated by Paraso et al. (this volume). Observations show that *H. nelsoni* prevalence declines with decreasing salinity. Although some part of this decline is induced by a reduction in infection intensity due to *H. nelsoni* cell mortality, which should increase the number of false negatives and thus decrease estimated prevalence, a direct effect of low salinity on cell survival in the host oyster could not fully explain the decline in prevalence with declining salinity.

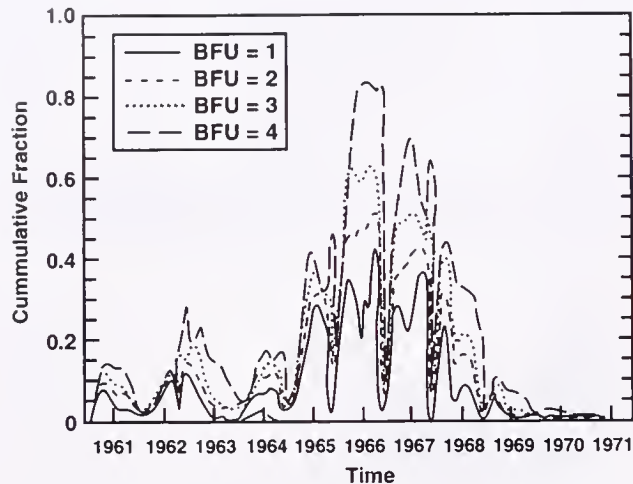


Figure 19. Time-development of *Haplosporidium nelsoni* infection in Delaware Bay during the 1960s without the inclusion of a seasonal cycle in infective particle availability (Fig. 1) using a standard 1-g AFDW oyster. The simulation is run using the environmental conditions for Miah Maull Grounds. Maximum infective particle concentration ( $IPconc_{max}$ ) was set at 900 particles  $L^{-1}$ . A reference case comparison plot is presented in Figure 13.

Paraso et al. (this volume), accordingly, included a relationship that altered infective particle concentration according to local conditions of salinity, thereby reducing infectivity at low salinity.

#### Bay-wide Oscillations Induced by Salinity

*H. nelsoni* epizootics tend to occur simultaneously over large areas, including over significant fractions of the salinity gradient (Andrews 1968, Haskin and Ford 1982) (e.g., Figs. 2 and 8). This generation of epizootics over large spatial scales requires that the infective particle populations be responsive to environmental change in two ways: the concentration of infective particles must increase dramatically prior to and during an epizootic and the concentration of infective particles must respond synchronously over large distances, even when environmental variables that might sometimes induce synchrony, such as simultaneous increases in salinity above 15‰, are not effective.

Both of these responses must be non-local functions of salinity. Thus, the model includes a series of relationships that varies the concentration of infective particles according to the general rise and fall of salinity throughout the bay. These relationships do not use the specific values of the local salinity regime for each particular site, because the absolute value and timing of salinity change vary considerably between sites. Rather, these relationships impose on all sites a simultaneous rise and fall in infective particle concentration based on the general, bay-wide variation in salinity caused by changes in freshwater inflow. The result is a simultaneous modification in infective particle concentration at all locations across the salinity gradient. When bay salinity increases, the concentration of infective particles increases. When bay salinity decreases, the concentration of infective particles decreases. The response time for this effect, determined by a series of simulations varying response time from 90 to 360 days, was determined to be 180 days. If this time-dependent and salinity-dependent variation in infective particle concentration is removed from the model, the simulated patterns of *H. nelsoni* prevalence no longer agree with field observations (Fig. 20). In particular, the magnitude

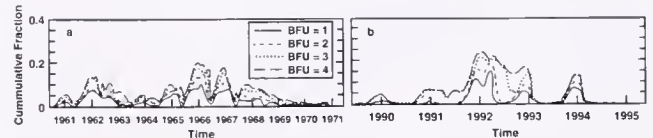


Figure 20. Time-development of *Haplosporidium nelsoni* infections in Delaware and Chesapeake Bays without the imposition of bay-wide synchrony in the time-dependent variation of infective particle concentration in a population of 1-g AFDW oysters. In this case, the relationship varying the concentration of infective particles according to the rise and fall in average bay salinity was deleted from the model. The simulation is run using the environmental conditions for (a) Bennies Bed in Delaware Bay during the 1960s and (b) Potomac River-Cornfield Harbor in Chesapeake Bay during the early 1990s. Reference case comparison plots are presented in Figures 13 and 15. Maximum infective particle concentration ( $IPconc_{max}$ ) was set at 900 particles  $L^{-1}$ .

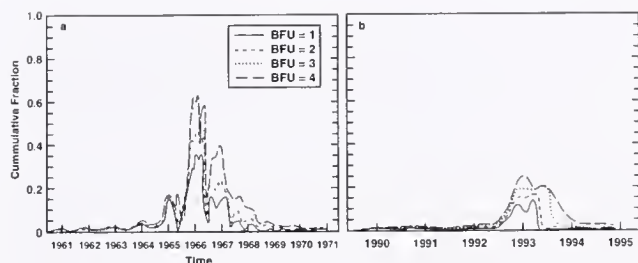
of change in prevalence between non-epizootic and epizootic conditions is significantly reduced. Clearly, generation of an epizootic requires an increase in the ambient concentration of infective particles as well as environmental conditions conducive to the proliferation of *H. nelsoni* in the oyster and this increase in particle concentration must occur independent of the local salinity excursions experienced by any individual population.

Possibly, a measure of residence time (e.g., Marshall and Alden 1997), which should decrease with increasing freshwater inflow, or a measure describing the change in average bay salinity would more effectively determine temporal variation in infective particle concentration. Unfortunately, data were not adequate in either bay to calculate either variable at all sites. So, in developing the model, the need arose to choose a salinity time series as a surrogate to impose synchrony among all sites. A series of trials using the 6 Chesapeake Bay time series showed that the highest salinity time series available, from Tangier Sound-Old Woman's Leg, produced simulations closest to observations at all Chesapeake sites. This time series was characterized by significant salinity excursions across the 15‰ line (Fig. 9f). A time series of nearly equivalent salinity range in Delaware Bay comes from Shell Rock Bed (Figs. 4c, 5c). This time series provided equally adequate simulations in Delaware Bay. Use of lower-salinity time series consistently reduced prevalences below observations at all sites in the salinity gradient (e.g., Fig. 21) and often modified the temporal pattern of an epizootic. For example, the 1960s epizootic on Bennies Bed is not only muted, but also shortened temporally by the utilization of a lower-salinity time series from Cohansey Bed (Fig. 21a).

The results of these simulations clearly target higher-salinity conditions as the conditions that provide the best time series for varying infective element concentration in the water column. Because the highest salinity time series available in Chesapeake Bay, which had concomitant information on *H. nelsoni* prevalence, came from Tangier Sound, an area characterized by average salinities around 15‰, this salinity range was used. The effectiveness of even higher salinity conditions remains to be tested.

#### Effect of Cold Winters

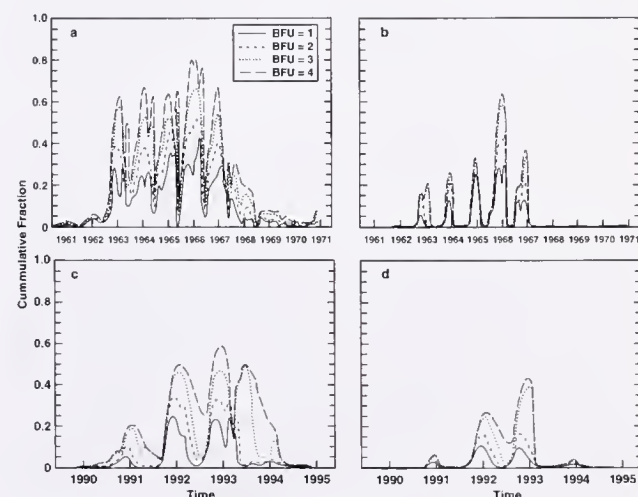
The year 1962 was particularly cold in Delaware Bay and 1990 was particularly cold in Chesapeake Bay. Simulations without an effect of cold winters on transmission rate showed epizootic conditions beginning early in both the decade of the 1960s in Delaware Bay and the early 1990s in Chesapeake Bay (Fig. 22). The



**Figure 21.** Time-development of *Haplosporidium nelsoni* infections in Delaware and Chesapeake Bays with the imposition of bay-wide synchrony in the time-dependent variation of infective particle concentration in a population of 1-g AFDW oysters, however with a lower salinity time series used to generate synchrony. The simulations were run using the environmental conditions for (a) Bennies Bed in Delaware Bay during the 1960s but using the lower-salinity time series from Cohansey Bed rather than Shell Rock Bed as the time series controlling synchrony, and (b) Tangier Sound-Old Woman's Leg in Chesapeake Bay during the early 1990s, but using the lower-salinity time series for the Potomac River-Cornfield Harbor to control synchrony rather than the higher salinity times series from Tangier Sound. Reference case comparison plots are presented in Figures 13 and 15. Maximum infective particle concentration ( $IP_{conc,max}$ ) was set at 900 particles  $L^{-1}$ .

suggestion of Ford and Haskin (1982) that the effect of cold winters extends beyond the year in which the winter occurred is supported by these simulations. Cold winters generate a multiyear response pattern in *H. nelsoni* transmission and, hence, in *H. nelsoni* prevalence.

Fitting simulation to observation required that the degree of cold exposure necessary to trigger the effect of a cold winter be varied between Chesapeake Bay and Delaware Bay (see Ford et al. this volume, Table 3, variable  $DD_o$ ). In effect, a cold winter was defined to be colder in Delaware Bay than in Chesapeake Bay.



**Figure 22.** Time-development of *Haplosporidium nelsoni* infections in Delaware and Chesapeake Bays without the influence of cold winters on transmission rate in a population of 1-g AFDW oysters. The simulations were run using the environmental conditions for (a) Bennies Bed, and (b) Cohansey Bed in Delaware Bay during the 1960s, (c) Tangier Sound-Sharklin Shoal, and (d) Fishing Bay-Gaosee Creek for Chesapeake Bay during the early 1990s. Reference case comparison plots are presented in Figures 13 and 15. Maximum infective particle concentration ( $IP_{conc,max}$ ) was set at 900 particles  $L^{-1}$ .

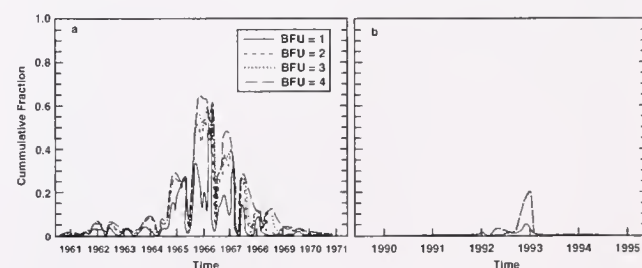
Temperature data came principally from moorings in Chesapeake Bay and from the Maurice River pier in Delaware Bay. Possibly, pier temperatures average lower than Delaware Bay temperatures during the winter. A small discrepancy would be sufficient to account for the variation required between the two bays. No alternative explanation is available and the absence of field information on the location and type of reservoir of infective particles prohibits the formulation of additional hypotheses. The difference in value for this variable ( $DD_o$ ) that controls the definition of a cold winter represents the sole significant difference in model setup between the Delaware Bay 1960s case and the case for Chesapeake Bay in the early 1990s.

All simulations reproduced observed trends in MSX disease except for the terminal years of the 1960s for Miah Maull Grounds in Delaware Bay where prevalences were considerably underestimated. The simulations suggest that high salinity, typical of Miah Maull grounds, might mitigate the effects of cold winters. Insufficient information from similar salinity sites was available in Chesapeake Bay to further test this possibility.

**Disease Resistance**

*H. nelsoni* resistance can clearly be bred into oysters and the development of disease resistance has occurred in wild populations (Andrews 1968, Haskin and Ford 1979, Bureson 1991). An increase in disease resistance might introduce another mechanism controlling prevalence, rather than transmission, by limiting proliferation and thereby increasing the number of false negatives. One mechanism of disease resistance is the restriction of *H. nelsoni* plasmodia to the epithelial tissue for longer periods of time. This effect was introduced into the model by increasing the doubling time ( $b_{0,es}$  in Table 3, Ford et al. this volume), thus decreasing the growth rate of *H. nelsoni* in the epithelial tissues. (Varying the diffusion rate,  $d_0$  in Table 3, Ford et al. [this volume], between epithelial and systemic tissue did not provide the desired result.)

A simulated increase in *H. nelsoni* growth rate results in little increase in prevalence, however, the duration of epizootics is lengthened because environmental effects limiting *H. nelsoni* proliferation, such as salinity, are lessened in importance (Fig. 23). An increase in resistance significantly reduces prevalence (Fig. 23),



**Figure 23.** Time-development of *Haplosporidium nelsoni* infections in Delaware and Chesapeake Bays with varying disease resistance in a population of 1-g AFDW oysters. The simulations were run using the environmental conditions for (a) Shell Rock Bed in Delaware Bay during the 1960s, and (b) Potomac River-Cornfield Harbor in Chesapeake Bay during the early 1990s. Reference case comparison plots are presented in Figures 13 and 15. (a) is a case of decreased resistance obtained by decreasing the doubling time of *H. nelsoni* plasmodia in the epithelium from 3 days to 1.5 days. (b) is a case of increased disease resistance obtained by increasing the doubling time of *H. nelsoni* plasmodia in the epithelium from 3 days to 12 days. Maximum infective particle concentration ( $IP_{conc,max}$ ) was set at 900 particles  $L^{-1}$ .

but not because infectivity changes. As anticipated, prevalence is reduced because false negatives are more frequently encountered.

Simulations for Delaware Bay in the 1960s and 1980s and Chesapeake Bay in the early 1990s agree with field observations using the same *H. nelsoni* doubling time. These simulations support the belief that resistance builds up slowly or not at all in natural populations following the first few years of exposure, possibly because reservoir populations of oysters at low salinity are protected from *H. nelsoni* exposure and continue to supply non-resistant larvae to downbay populations.

On the other hand, disease resistance might entail a decrease in susceptibility or an increase in infective dose. The need for a higher infective particle concentration in Delaware Bay during the 1980s might be explained by an increase in susceptibility or decrease in infective dose (e.g., Fig. 23a), however, no evidence exists in observations of natural populations for a decrease in resistance and better explanations for the difference in simulation conditions between the 1960s and 1980s decades are available (see earlier discussion). The successful simulation of the 1960s decade in Delaware Bay and the early 1990s in Chesapeake Bay using the same model conditions argues against a long-term decrease in susceptibility. Accordingly, variations in disease resistance and susceptibility were not included among the factors affecting *H. nelsoni* transmission and disease intensification.

## DISCUSSION

### Perspective

In the vast majority of disease models, transmission is modeled as a simple function of the number of infected individuals and the contact rate between infected and uninfected individuals (e.g., Kermack and McKendrick 1991, Hethcote and van den Driessche 1995, Frank 1996). Intermediate or alternate hosts and parasitoids can make the process more complex (e.g., Hassell 1982, Chesson and Murdoch 1986, Woolhouse and Chandiwana 1990). An example of the adequacy of the simple approach using prevalence and contact rate to model transmission in bivalve populations is the model of *Perkinsus marinus* in *Crassostrea virginica*, in which transmission was controlled by 3 variables, disease prevalence and infection intensity, both measures of the population of infected individuals, and host density, a surrogate of contact rate in an immobile host (Hofmann et al. 1995, Powell et al. 1996).

Contrast this simplicity with the present model of transmission in *H. nelsoni*. In *H. nelsoni*, the standard approach involving the fraction of the population infected and contact rate with uninfected individuals failed utterly to adequately simulate prevalence. It was clear from the earliest studies that transmission in the field did not depend on the presence of infected oysters and that transmission could not be achieved experimentally (Andrews and Wood, 1967, Ford and Haskin 1982, Haskin and Andrews 1988). These observations, along with the early failure to find spores, led to speculation that another host was involved in the life cycle, although a candidate species has yet to be identified. In the model, transmission in *H. nelsoni* includes non-local factors that exert a paramount influence on the transmission process. In fact, population-dependent processes are not sufficiently important to even be included in the model, save for filtration rate that, with infective particle concentration, determines whether an individual animal receives an infective dose. The resulting model is unusually complex in comparison to other disease models. It is the source of this complexity and the implication of another reservoir of infective

particles besides the oyster that is intriguing, because the full life cycle of *H. nelsoni* remains unknown and, in particular, present knowledge effectively provides no details of the transmission process beyond those that can be gleaned by observing temporal and spatial changes in prevalence.

Although the simulations provide good approximations to field observations of *H. nelsoni* prevalence, a few trends are not as faithfully rendered as one might wish. Examples include the base case for Tangier Sound-Old Woman's Leg (Fig. 15f) which overestimates prevalence and the upper bay cases for the decade of the 1980s in Delaware Bay where prevalence is underestimated (Fig. 14). Results presented in this contribution and in two companion papers (Ford et al. this volume, Paraso et al. this volume) show that the time-development of *H. nelsoni* proliferation is very sensitive to salinity, temperature, and food supply. In no case were we able to utilize a full suite of environmental data and observations of disease prevalence obtained from the same location at the same time. Soniat et al. (1998) provide a good example of the sensitivity of oyster population dynamics models to the frequency of collection of environmental data. It is likely that one important source of the discrepancies that remain between field observations and model simulations is the inability of the presently-available environmental time series to fully describe the temporal dynamics of the disease process.

### Forcing Factors in the Transmission Model

The transmission model has been constructed with little information on the transmission process itself beyond that gleaned from observations of the temporal progression of infection in host populations and the correlation of this progression with environmental variables such as temperature and salinity. Nevertheless, the modeling process permits identification of key environmental forcing factors that can be expected to exert a direct control on the transmission process, either by controlling the availability of infective particles in the water column or by controlling the population dynamics of an alternate host. These forcing factors include season, salinity, and winter temperature.

### Filtration Rate and Season

The form of the infective stage is unknown, but is assumed to be waterborne, at least for a short period before it infects, because the earliest infections are observed in the gill epithelium where they presumably lodged as a consequence of filter feeding. Further, timed transplants of uninfected oysters into zoootic areas showed that they became infected only during a period from late May through October (Ford and Tripp 1996). Lack of additional information required that assumptions be made about the quantity and availability of infective stages.

The model places infective stages in the water; they are not produced by infected oysters. In contrast, the density and infection level of oysters is a key element in the modeling of *P. marinus* transmission. The decoupling reflects the observed evidence that new infections are acquired independently of the infected oyster population. Based on a series of simulations compared to field observations of prevalence, an infective dose of 8,700 particles filtered  $d^{-1}$  was set as the threshold needed to initiate an infection. Compared to *P. marinus*, which can initiate an infection with as few as 10–100 parasites (Valiulis 1973, Chu and Volety 1997), this dose appears to be very high. The *P. marinus* experiments, however, were performed by injecting parasites directly into the gill

cavity, a method that is considerably more effective than allowing the oysters to filter *P. marinus* from the water as they do naturally (Chu 1996, Bushek et al. 1997). Recently, Li et al. (1993) reported levels of free *Perkinsus* spp. cells in estuarine water samples as high as 19,000 cells L<sup>-1</sup>. A 75–100-mm oyster filtering 133 L of water in a day (Powell et al. 1992), would encounter as many as 2,500,000 cells. Based on the results of Chu and Volety (1997) and Valiulis (1973) described above, it would seem impossible for any oysters in enzootic waters to be alive if all cells were infective. Ford et al. (1996) fed oysters a dose of 10<sup>6</sup> *P. marinus* g wet wt<sup>-1</sup> and did succeed in infecting all animals, but mortalities and infection intensities were very low through the 12-week experiment compared to groups injected with parasites, suggesting that a large fraction of parasites filtered from the water never cause infection. Thus, 8,700 infective stages filtered d<sup>-1</sup> for *H. nelsoni* is within the bounds set by observations.

Using the above infective dose, simulations were run with a variety of infective particle concentrations and a value of 900 particles L<sup>-1</sup> was found to provide the best results for the decade of the 1960s. Because no measurements of the abundance of infective stages in the water have been made, this figure cannot be verified, but it compares well with the 500–800 haplosporidian spores L<sup>-1</sup> estimated to be present in Delaware Bay based on evidence from spores that oysters had ingested through feeding (Barber and Ford 1992). Although these spores are identical, at the light microscope level, to those of *H. nelsoni*, their identity has not been confirmed. Nevertheless, because both these spores and *H. nelsoni* are abundant in Delaware Bay and because the temporal presence of the spores matched that of the known infective period for *H. nelsoni*, that they are, in fact, *H. nelsoni* must be seriously considered. Because of this strong circumstantial evidence linking the two, the seasonal abundance of ingested spores was used to vary the seasonal concentrations of *H. nelsoni* infective particles in the model. The fact that simulations subsequently reproduced the timing and degree of infection in oysters would seem to lend credence to the argument that these are *H. nelsoni* and that they are infective to the oyster. Nevertheless, some caution must be used in interpreting these results as correlation analysis failed to link interannual fluctuations in ingested spore abundance with subsequent *H. nelsoni* infection levels, a finding that lends support to the secondary host hypothesis (Barber and Ford 1992).

### Salinity

Salinity exerts the overriding influence on the transmission process and its role is a dual one. Initial simulations of prevalence and intensity at low salinity showed the appropriate timing, but produced prevalences that were higher than observed (Paraso et al. this volume). Decreasing the concentration of infective particles in low salinity water improved the model fit. Thus, salinity, in the model, acts on the local host population by varying the infectivity of infective particles as they impinge the oyster gill during the filtration process.

The model makes no biological distinction between whether this salinity effect produces fewer infective stages or lower infection efficiency of an undiminished number. In their report of ingested *H. nelsoni*-like spores in Delaware Bay, Barber and Ford (1992) noted that the spores were abundant in the low salinity regions of Delaware Bay where infection prevalence is typically low. They postulated that salinity might affect excystation of spores, and consequently infection success, if these are indeed a

life stage of *H. nelsoni* and responsible for infections in oysters. It is likely that a spore stage is involved in the transmission of *H. nelsoni* to oysters, even if it is not the one found by Barber and Ford (1992). This life stage should be more resistant to salinity change than the plasmodium and it could be quite long-lived and widely dispersed in the environment. However, the sporoplasm, no longer protected within the spore wall, would be immediately exposed to ambient salinity during excystation and, assuming that it has the same salinity tolerance as plasmodial stages, would quickly be killed at low salinity.

In addition, salinity exerts a regional influence on the transmission process by controlling, in part and on a bay-wide scale, the concentration of infective particles in the water column (or perhaps the abundance of an alternate host). This control on concentration must be distinguished from the local control on infectivity that may, in fact, be a local reduction in infective particle concentration due to cell mortality. The bay-wide control mechanism permits the concentration of infective particles to increase during high salinity times and decrease during low-salinity times, at all sites, regardless of the local salinity regime. Although the process has been modeled in a somewhat *ad hoc* fashion because of insufficient data, the process probably originates from the variation in residence time of bay waters. Very likely, as flushing rate declines with increased salinity, the concentration of infective particles increases.

### Temperature

An intriguing observation from long-term monitoring of *H. nelsoni* in lower Delaware Bay was that infection prevalence showed distinct interannual cycles (Ford and Haskin 1982, Haskin and Andrews 1988). Years of low prevalence followed, by 1 or 2 years, a very cold winter. Between the onset of the MSX epizootic in Delaware Bay in 1957 and 1990, this pattern was repeated half a dozen times. The model was fit to these observations by decreasing the abundance of infective stages according to the "coldness" (measured in degree days) of the preceding two winters. Infective particle concentration decreases for 1 to 2 years after a cold winter. It returns to high levels faster after a warm winter.

Simulations that included the cold-winter effect clearly replicated the cyclic prevalence pattern in Delaware Bay. The model also reproduced a post-cold-winter prevalence dip that appeared in a 1989 to 1994 data series from the Maryland portion of Chesapeake Bay, indicating that neither the model nor the cold-winter effect is restricted to a single location. How widespread the effect is remains uncertain, however, long-term (30-year) comparisons of prevalence cycles in lower Delaware Bay and the lower York River near its juncture with the Virginia portion of Chesapeake Bay, revealed dissimilar patterns (Haskin and Andrews 1988). The cold-winter effect that was obvious in the Delaware Bay location was not obvious at the Virginia location. Nevertheless, Haskin and Andrews (1988) pointed out hints of the same pattern in the York River, where it may have been masked by the more pronounced salinity effect. Again, the modeling device says nothing about the biological mechanism behind the observation, which has been speculated to be an effect on a secondary host (Ford and Haskin 1982, Haskin and Andrews 1988).

### Local versus Bay-wide Processes

Temperature, salinity (residence time effect), and season exert their influence over all sites regardless of the local salinity regime. It is the presence in the *H. nelsoni* transmission model of these

bay-wide influences of salinity, temperature and season that make this model different from most other transmission models and remarkably different from the other model most pertinent to oyster population dynamics, the model for *P. marinus*. In the latter case, temperature and salinity affect transmission indirectly by varying the infection intensity in the infected portion of the host population.

Despite the influence of bay-wide processes, which might be expected to carry with them some unique attributes of individual bays, particularly considering bays as different as Chesapeake Bay and Delaware Bay, bay-to-bay differences in the transmission model were minor. The only variable that varied between bays was  $DD_{10}$ , the variable defining a cold winter. Cold, as defined in the model, required more degree-days below 10 °C in Delaware Bay than in Chesapeake Bay. The possibility that this discrepancy originates in the acquisition of environmental time series and field observations of *H. nelsoni* prevalence from different locations cannot be discounted. Thus, this modeling exercise strongly supports the belief that an underlying basic process common throughout the disease range is responsible for transmission of *H. nelsoni* in oyster populations and that this process is dominantly one influenced by non-local, non-oyster-population-dependent processes.

#### *Multiyear-dependent Changes in the Infection Process*

Temporal changes in disease virulence and host resistance to disease are well-described and modeled (Lenski and May 1994, Frank 1996) and some evidence exists for geographic variation in virulence and resistance in another oyster disease-causing organism, *P. marinus* (Bushek and Allen 1996). Early in the development of the first epizootics of *H. nelsoni*, after its introduction in the late 1950s, it was observed that oyster populations seemed to build up resistance to the disease over the first few years of exposure (Haskin and Ford 1979). Controlled breeding has clearly produced oysters distinctly more resistant to *H. nelsoni* disease than native populations (Haskin and Ford 1979, Ford and Haskin 1987). Accordingly, some evidence of long-term changes in the infectivity of *H. nelsoni* might be sought through this modeling exercise.

In fact, such a search developed little supporting evidence. Simulations of disease-resistant oysters did not fit field observations in either bay. The same relative susceptibility to disease proved adequate to simulate the 1960s in Delaware Bay and the early 1990s in Chesapeake Bay. However, an increase in infective particle concentration was required in Delaware Bay to adequately simulate the decade of the 1980s. The suggestion that this necessity might be caused by a reduction in infective dose (increased virulence or decreased susceptibility) is not supported by any other information. The possibility that the necessity originates from inadequate field control on environmental time series cannot be discounted, but does not fit all nuances of the differences between field observation and simulation. A possibility that the concentration of infective particles has built up in the Bay over the decades remains a viable explanation, and this might be the result of increased abundance of an alternate host or increased capacity of some other reservoir. The modeling exercise clearly supports the requirement for the existence of a reservoir, alternate host or otherwise, independent of the oyster population (Ford and Tripp 1996).

#### *The Influence of Transmission in System Memory*

In the *P. marinus* model, the influence of the infection intensification cycle overwhelms the influence of transmission. Most epizootics are triggered by processes influencing the degree to which infected individuals retain significant parasite burdens over the winter (Powell et al. 1996) or processes permitting an increase in *P. marinus* growth relative to oyster growth. The *P. marinus* model is somewhat unusual in this regard. Transmission is normally an overwhelming component of the disease process in most animal diseases (e.g., Ackerman et al. 1984, Anderson 1991).

In this limited perspective, the *H. nelsoni* model is more typical, in that transmission has an overwhelming influence on the course of infection in oyster populations. Epizootic cycles seem to be principally the product of enhanced transmission rather than enhanced intensification. These influences of transmission on the course of infection, in many cases, have multiyear implications for prevalence and infection intensity, and the root of much of this multiyear behavior is in the processes that control the concentration of infective particles in the water column (or reservoir). It is typical of population dynamics models for the system modeled to demonstrate a multiyear system memory in which the present state is a product not just of the present or immediately past environmental conditions, but also of the environmental conditions integrated over a much longer period of time. Oyster population dynamics models are particularly good examples (e.g., Soniat et al. 1998, Powell et al. 1996). System memory is less pervasive in the *H. nelsoni* model than in the *P. marinus* model. The course of epizootics and the periodic near-disappearance of the disease originate through processes that have commenced within the year or, for cold winters, 1 or 2 years previously. In this extent, *H. nelsoni* is not typical of an epizootic-producing organism (e.g., Gill 1928) and it is likely that this atypical nature is due to the limited time-dependent description required of the alternate reservoir for infective particles. Either the reservoir is a large, persistent environmentally buffered one that does not involve an alternate host or involves a large long-lived animal or the alternate host has a 1-to-2-yr life cycle that allows it to rapidly respond to environmental change. The volatility of these latter species, however, would seem to preclude their involvement. In either case, it seems unlikely that any significant feedback between the oyster and alternate reservoir can exist.

Two interesting peculiarities can be considered with respect to the influence of large scale processes in *H. nelsoni* transmission. The first of these is the typical condition of *H. nelsoni* disease south of Cape Hatteras in which low *H. nelsoni* prevalence, but high infection intensity in infected animals, is characteristic of the disease process (e.g., Lewis et al. 1992). This event is easily recreated in the model by establishing a low concentration of infective particles, a condition very similar to the low-salinity cases from Delaware Bay where both simulation and observation show low prevalence, but high infection intensity in 50% or more of the infected population.

Second, most observational time series show periods when *H. nelsoni* more or less completely disappears from large areas of the bay. Examples include the terminal years of the 1960s and 1980s on the Delaware Bay seed beds (e.g., Fig. 2a-d). These events are faithfully reproduced in the model. They originate in processes set in motion 1 to 2 years earlier that gradually lead to the reduction in infective particle concentration and, thus, reduced transmission. They do not originate in processes resulting in the loss of patent



infections in the population produced by simultaneously occurring environmental conditions, although, of course, this must occur. It is particularly interesting, in Delaware Bay, that the decline of *H. nelsoni* in the latter part of the 1980s occurs even without the introduction of *P. marinus*. The coincidental change in the dominance of these two disease-causing organisms around 1990 in this bay has received some notoriety. It is significant, then, that the model indicates that processes set in motion in the late-1980s were responsible for the disappearance of *H. nelsoni* at the end of the decade. The failure of *H. nelsoni* to return in the 1990s, however, may still indicate a significant interaction between the two diseases. This possibility cannot be investigated with the present model.

The model suggests several processes that conspire to initiate and stifle epizootics, all related to changes in infective particle concentration. These include the presence of unusually warm or cold winters that would initiate and stifle, respectively, epizootics several years hence. Similarly, wet and dry years initiate the decrease and increase, respectively, in concentration of infective particles that then reduce and increase, respectively, the prevalence of disease. This process has a response time of about one-half year. The model strongly points to the need to evaluate the past several years of history of environmental change in understanding the process of epizootic initiation and cessation.

#### *The Intermediate Host Hypothesis*

The transmission model requires a reservoir of infective particles independent of the host oyster population that is modified according to multiyear variations in temperature and salinity. Several components of the model lend credence to the hypothesis that *H. nelsoni* has an alternate host in the life cycle, although no such host has yet been identified (Burrenson 1988, Haskin and Andrews 1988). These components include (1) the importance of bay-wide processes in transmission that impose a control on prevalence far beyond that exerted by the local population, including spore production in young oysters. This modeling exercise clearly indicates the importance of a time-varying concentration of infective particles that varies according to change in bay-wide salinity (wet versus dry conditions) and temperature (warm versus cold winters). (2) With the exception of local effects on infectivity as the expected by-product of the influence of low salinity on what is essentially a polyhaline disease organism, local effects do not occur in the transmission model. Oyster density, for example, plays no role in the transmission model. (3) The seasonal cycle of infection is an important component of the transmission model. However, the increase in infective particle concentration precedes the formation of any spores within the simulated oyster population (e.g., Ford et al. this volume). (4) A contact rate model fails because high prevalence and infection intensity can occur in host populations when infectivity is low and such a mismatch between the number of infected individuals and the rate of transmission cannot occur in a local-contact transmission model. (5) Cold winters significantly affect transmission rate, probably by reducing infective particle concentration, yet cold winters have a much less immediate effect on population infection intensity (Ford and Haskin 1982, Ford et al. this volume). All of these aspects of the transmission model support the alternate host hypothesis.

What are the characteristics of the alternate reservoir? (1) It must be capable of rapid, continuous release of large numbers of infective particles into the water column, at least during the

warmer months of the year. (2) It must be environmentally buffered to a significant extent, so that normal variations in temperature and salinity do not affect its capacity for infective element generation. (3) It must be independent of infection intensity of the oyster population, at least over significant time periods, which suggests that the reservoir either is capable of supporting the full life cycle of *H. nelsoni* or that the reservoir has a multiyear capacity under most conditions. (4) It must be sensitive to cold temperatures, but be capable of recovery over a 2-year period independent of the infection level in the oyster population. This sensitivity may be salinity-dependent. (5) It must exist at relatively high salinity to provide the observed response to salinity-driven changes in flushing rate.

#### *The Epidemiological Approach*

The transmission model points the way to epidemiological studies that might further elucidate the transmission cycle of *H. nelsoni* without requiring identification of the infective particle reservoir. That stumbling block has significantly impaired investigation of the transmission process. Investigation of the multiyear influence of temperature and salinity, for example, would be amenable to standard epidemiological approaches. This modeling exercise, however, emphasizes the need for such studies to extend over wide areas and significant time periods. Single-site studies and studies of 1 or 2 years duration are unlikely to significantly advance the understanding of transmission in this oyster disease. Formulation of the model, in fact, required the use of decadal time series. Clearly, large areas of bays are responding similarly and relatively synchronously and over time scales of several years or more. Understanding the generation of *H. nelsoni* epizootics will require epidemiological studies of similar scale.

The transmission model has been run in hindcasting mode for this contribution. Hindcasting was necessary to verify the model. Good agreement of model simulations with field observations over broad expanses of the salinity gradient and between bays suggests that the model should now be tested in nowcasting mode. Developing nowcasting, and then forecasting, capability will require a thorough understanding of environmental change, particularly near-real-time information on salinity, temperature, particulate load, and food supply. These data must be obtained in or very near the target population. Both salinity and temperature parameterization include highly non-linear equations that describe threshold responses in the oyster population. Accordingly, under certain circumstances, small changes in temperature and salinity, typical of cross-estuary and depth gradients, will be significant and this puts a premium on the collection of data very close to the target population.

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## REPRODUCTIVE CYCLES OF THE SURF BEACH CLAM *PAPHIES DONACINA* (SPENGLER, 1793) FROM NEW ZEALAND

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**ABSTRACT** The gametogenic cycle of the tuatua *Paphies donacina* is described from histological sections of clams collected monthly from November 1993 to April 1997. Trends in the dry weight condition index (CI) and gonad indices suggest an annual breeding cycle with continuous spawning over the summer and an inactive winter phase. There was considerable interannual variation with less than 50% of the population being reproductive during the 1994/5 season. There were significant correlations between seawater temperature, the population gonad index and gonosomatic indices of male and female tuatua. Food resources (chlorophyll *a* levels) fluctuated within and between seasons with values from 4 to 12  $\mu\text{g L}^{-1}$ . These values showed a positive correlation with seawater temperature, but not with any of the bivalve indices. Throughout the study, the population sex ratio was biased toward females, and often there were individuals of indeterminate sex within the population. Dry weight values for standard 40-mm and 90-mm length tuatua varied seasonally, with the smaller length group showing a higher coefficient of variation, consistent with seasonal patterns of somatic and gonad development. Both male and female tuatua show greater reproductive activity early rather than late in the breeding period and the proportion of reproductive tissue was similar for males and females of various lengths. The CI was not a good indicator of reproductive potential in male or female tuatua. The reproductive strategy of *P. donacina* is regarded as exploitive, closely determined by environmental seawater temperature. Somatic growth and gonad development occur simultaneously over the summer, but can extend into the winter if sufficient food resources are available.

**KEY WORDS:** bivalve, New Zealand, *Paphies donacina*, reproduction, gametogenic cycle, gonosomatic index, condition index

### INTRODUCTION

Surf clams form an important world-wide recreational and commercial shellfishery resource (McLachlan et al. 1996), and in New Zealand, several species have been identified as providing potential for aquaculture. These include two species of tuatua of the family Mesodesmatidae: *Paphies subtriangulata*, distributed mainly around the North Island, and *P. donacina*, the dominant tuatua around the South Island. It is optimally distributed from the low-tide down to about 3-m depth (Cranfield et al. 1994, Haddon et al. 1996).

The habitat of *P. donacina* within Pegasus Bay includes high wave action and well-sorted fine sand, resulting in a low beach profile and cyclic changes in beach morphology (Kirk 1980). The exposed coastline, with its highly dynamic meso-tidal surf beach, is classified within the range intermediate to dissipative on a world-wide scale (Short 1990). Bivalves within the surf zone typically are mobile and exposed to a shifting habitat with variable conditions of temperature, wave exposure, moisture levels, food supply, and salinity extremes (McLachlan and Erasmus 1983). Zonation patterns on exposed sand beaches are often indistinct, and environmental conditions are more extreme than those found in shallow estuaries or on rocky shores (Widdows 1978, Hummel 1985, McLachlan and Jaramillo 1995, Urrutia et al. 1997).

Along with the two species of tuatua in New Zealand, the superfamily Mactridae also includes the toheroa, *Paphies ventricosa*, the pipi, *P. australis*, *Mactra murchisoni*, and *Spisula aequilata* (Redfean 1974, Hooker and Creese 1995a, Hooker and Creese 1995b, Grant and Creese 1995, King 1997). The reproductive cycle has been described for all of the above species, but there are no comparable data for *P. donacina*. McLachlan et al. (1996) have reviewed the fisheries biology of surf clams, and report highly variable reproductive patterns both within and between species.

Although there have been numerous studies on the reproductive biology of a wide range of bivalves, including mussels, clams, and scallops, many of these have followed reproductive events over one or two annual cycles (Kanti et al. 1993, Garcia-Dominguez et al. 1994). Although some studies, including that on the sea scallop (Dibacco et al. 1995), include results from up to 7 years, it has not been possible to relate reproductive events to specific environmental conditions. There is also continued debate about the importance of temperature and food resources in influencing the reproductive cycle of bivalves (Manzi et al. 1985, Garcia-Dominguez et al. 1994, Urrutia et al. 1997).

The aims of the present study were to characterize the reproductive cycle of low-tide *P. donacina* and investigate the relationship between the gametogenic cycle and bivalve condition. This aspect has not previously been addressed in surf clams and is of interest in fisheries management. Another goal was to investigate the importance of temperature and potential food resources in the reproductive activity of the tuatua within the surf-zone habitat. For this reason the study was undertaken at approximately monthly intervals over a period of 3.5 years.

### MATERIALS AND METHODS

#### Sampling

Tuatua were opportunistically sampled along a 2-km section of South Brighton Beach within Pegasus Bay, New Zealand (43°33'S, 172°55'E) during extreme low-water spring tides between November 1993 and April 1997. They were hand collected by detecting a hydroid on the sand surface. This hydroid attaches to the posterior part of the bivalve shell. Each month, during extreme low-water spring tides, at least 40 tuatua within the size range of the population were collected. They were returned to the laboratory within an hour and processed after cooling to 5 °C. This allowed the shell to be opened easily and the tissues extracted with

minimal damage. There were two occasions (July 1994 and May 1996) when tidal and weather conditions prevented sampling due to onshore winds or exceptionally rough sea conditions. At each sampling time, the low-tide water temperature and air temperature were recorded and a sample of seawater was returned to the laboratory, where estimates were made for chlorophyll *a* using a Turner Fluorimeter calibrated using chlorophyll standards. The tidal range in the habitat was 2 to 2.5 m, and the average seasonal air temperature range 6 to 17.5 °C, measured at the nearby Avon-Heathcote Estuary (Estcourt 1962).

#### Weight Relationships

The shell length and width of 20 tuatua representing the population length range were measured using vernier callipers and the total wet body weight recorded after cutting the adductor muscle and draining the shell fluid. The tissue was removed from the shell and the wet weight recorded before drying at 60 °C for 3 days. This allowed calculation of a dry weight/wet weight ratio to estimate the dry weight of tissue from samples used in the histological study. The shell of each bivalve was dried at 60 °C and the physiological condition index (CI) (Lucas and Beninger 1985) was calculated for each individual used in the population survey and the histological study.

#### Histology

For each sample, the length and width of 20 tuatua greater than 45-mm shell length were processed for the histological study. Bivalves were measured and the whole-body wet weight recorded before fixing in Davidson's fluid (Morales-Alamo and Mann 1989). The sex of the tuatua could not be determined by eye, except for a few instances late in the breeding season when mature eggs were observed. The gonad of the tuatua is located along the posterior margin of the foot, and extends into the digestive diverticula. A standard cross section of approximately 5 mm was cut above the base of the foot, as in Hooker and Creese (1995a, 1995b) and Grant and Creese (1995). These cross sections were dehydrated in an ethanol series, blocked in paraffin wax, then sectioned at 7–9 µm. Two slides, each with 2 sections, were made from most blocks, and the slides were stained using Ehrlich's haematoxylin counterstained with eosin.

#### Gonad Indices

The slides were first examined under low power ( $\times 40$ ) to determine the presence of reproductive tissue. On one section of each slide, the maximum percentage of the cross section occupied by the gonad was measured (gonosomatic index). The sections were then examined using a high power binocular microscope ( $\times 200$ ) to determine the stages of development, as in Redfearn (1974) and Grant and Creese (1995). For female clams, these stages were: early active follicles, mixed follicles, mature follicles, and spent reproductive or reabsorbing gonad tissue. In males only early and mature follicles could be distinguished. The population gonad index was calculated as the percentage of reproductive individuals in each sample.

To investigate the effects of body size on reproductive tissue mass of male and female tuatua, the gonosomatic index was plotted against shell length, dry weight, and condition index for the samples combined into 3-month intervals representing early and later parts of the breeding season.

## RESULTS

#### Description of the Reproductive Tissues

During the winter (June to September) most *P. donacina* were reproductively inactive, characterized by the relatively few individuals with active gametogenic tissue. At this time it was not possible to distinguish between male and female tuatua and the sex ratio could not be accurately determined. Female gonad was found in bivalves of 52–101 mm shell length (Table 1). Early active developing female reproductive tissue was characterized by thick-

TABLE 1.

Sex ratio and shell length ranges of *P. donacina* from histological study. N = number of individuals used in sex ratio calculations; F length, female shell length range (mm); M length, male shell length (mm); I length, indeterminate shell length range (mm). N = 20 for each sample. NA indicates that no calculation was possible, \* indicates months with unequal numbers of females and males ( $\chi^2$  value,  $P < .05$ )

Date	Female:Male Ratio	N	F Length (mm)	M Length (mm)	I Length (mm)
Nov 93	1.83	17	62–95	62–90	74–87
Dec 93	0.86	13	52–95	81–98	88–98
Jan 94	1.43	17	68–92	78–100	78–95
Feb 94	3.00	12	65–92	85–92	85–102
Mar 94	0.22*	11	85–90	82–92	84–102
Apr 94	2.00	9	65–95	58–90	74–95
Apr 94	2.16	19	65–90	54–62	84
May 94	1.00	7	85–98	87–94	75–92
Jun 94	NA	0	–	–	81–98
Aug 94	5.0	6	77–93	90	81–98
Oct 94	1.50	10	85–98	84–93	74–100
Nov 94	0.22*	11	83–95	67–88	66–93
Dec 94	0.66	10	83–92	66–95	68–92
Jan 95	3.00	12	85–91	72–98	84–93
Feb 95	2.00	3	85	100	79–95
Mar 95	3.00	4	81–87	82	56–94
Mar 95	2.00	6	87–92	87–90	81–98
Apr 95	NA	2	78–85	–	67–94
May 95	2.00	3	83–87	90	66–92
Jun 95	1.00	2	83	88	63–94
Jul 95	2.00	3	77–80	82	55–92
Aug 95	NA	1	–	83	70–108
Sep 95	NA	1	–	85	47–98
Oct 95	5.50*	13	47–95	68–80	32–96
Oct 95	1.25	18	58–92	70–85	75–78
Nov 95	1.25	9	75–80	65–76	70–85
Dec 95	2.60	18	62–90	67–87	67–82
Jan 96	0.83	11	58–88	62–87	68–103
Feb 96	1.83	17	53–84	82–87	84–88
Mar 96	0.55	17	58–95	73–100	78–88
Apr 96	1.43	17	88–101	90–98	86–102
Jun 96	1.37	19	77–94	47–87	88
Jul 96	1.50	5	78–90	77–88	77–92
Aug 96	4.00	5	87–98	91	82–96
Aug 96	0.67	5	91–92	90–94	84–94
Sep 96	11.00*	12	67–92	78	80–90
Oct 96	7.00	8	71–100	80	73–95
Nov 96	3.30	13	68–86	72–80	50–93
Dec 96	1.00	16	70–84	72–90	84–88
Feb 97	1.70	19	69–90	64–82	77
Apr 97	1.00	16	68–88	65–85	73–95

ened follicle walls with many small oocytes. Later the follicle walls were thinner with both developing ova and mature oocytes (mixed stage). In the mature stage there was little ovogenic activity, the follicle walls could not be distinguished, and the whole of the lumen was filled with mature ova. At the end of the breeding season early active follicles were observed in some sections containing partially spawned ova and there was histological evidence of gonad tissue undergoing reabsorption. Male gonad was found in individuals of shell length 47 to 108 mm throughout the year. This was separated into an early active reproductive stage, which was characterized by uniformly thickened follicular walls, and a mature phase, which included dense bands of spermatozoa appearing to rotate towards the centre of the lumina. Individuals of *P. donacina* are gonochoristic and no hermaphrodites were found.

#### Sex Ratio

In most samples, the sex ratio of *P. donacina* deviated from a 1:1 female to male ratio (Table 1). In samples where more than 50% of individuals were able to be sexed, this ratio was 2.23 (SD = 2.08, n = 37). Unequal numbers of males and females occurred in 4 of the 23 samples, the combined ratio was biased in favor of females ( $\chi^2 = 9.95$ ,  $P < .05$ ) and there was significant heterogeneity between months (heterogeneity value = 37.88,  $P < .05$ ). When the data were separated into two length groups, there was an obvious effect of shell length on the sex ratio. There were fewer reproductive males less than 80 mm shell length where the sex ratio was 3.33: 1 in favor of females. More than 80 mm shell length, the ratio was 1:1.

#### Gametogenic Cycle

The gametogenic cycle of *P. donacina* was annual with significantly reduced reproductive activity over winter (July to September). In the early part of the cycle, the majority of females contained developing ova and there were many males with fully mature spermatogenic tissue (Fig. 1). The peak of breeding activity occurred in the summer (November to March) when the lumen of the female follicles contained large mature ova. The late part of the cycle, including both mature and partially spawned individuals, occurred in autumn (April), but extended some years into winter (June or July). Because few females were found with reabsorbing ova or gonads in an early development stage part way through the reproductive period, there is little evidence of multiple mass spawning events. The presence of mature ova throughout the breeding period suggests a prolonged breeding period with continuously released gametes reaching a maximum during summer. However, male tuatua with early developing gonad were present throughout the breeding season. This may indicate more than one gametogenic cycle during the breeding period, or considerable variability in the onset of gamete formation in some male tuatua. At the end of the breeding season, the remaining gonad tissue appeared to be reabsorbed before the onset of winter.

There was considerable variation in the timing of the reproductive cycle from year to year. This variation was particularly evident during the 1994/1995 breeding season when less than 50% of the individuals were reproductively active. This contrasted with the other 3 seasons when more than 90% of the population showed reproductive activity during the summer.

#### Environmental Variables

Seawater temperature varied seasonally, with minimum values close to 8 °C during winter and maximum values above 17 °C in

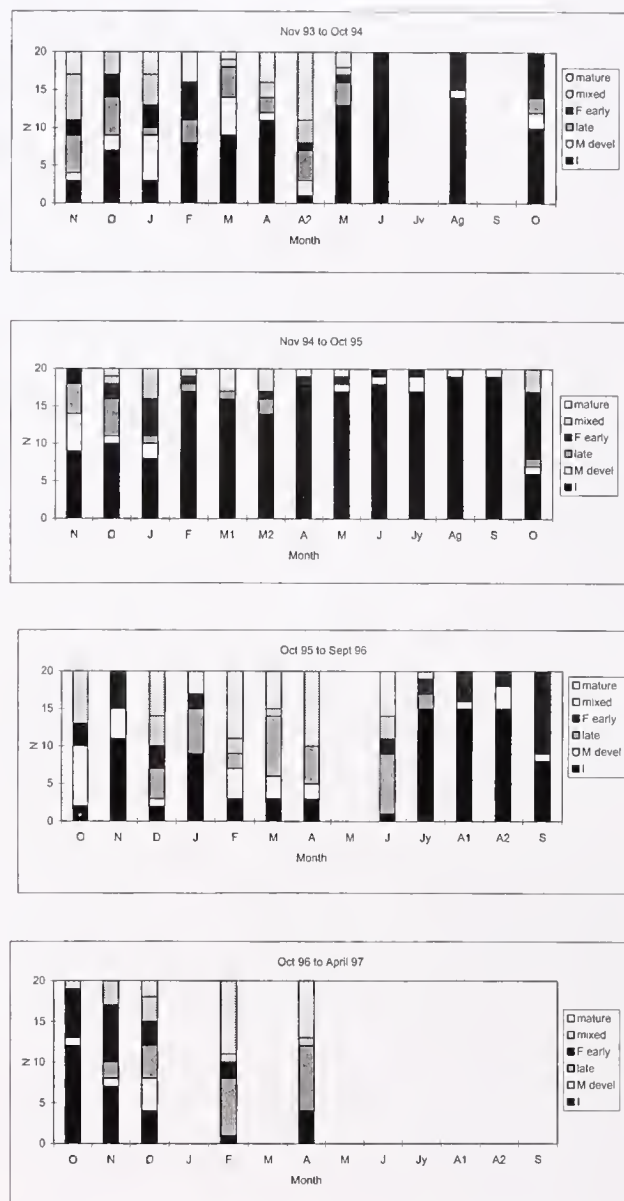


Figure 1. Developmental stages of tuatua at approximately 4-week intervals from November 1993 to April 1997. Symbols show female stages, F early development, mixed oocytes, and mature oocytes. Male development is shown as M devel, early development and late, mature, ripe, and spawning clams. I shows clams with no gametogenic activity.

summer, with an interannual variation of up to 3 °C (Fig. 2). Tuatua condition index followed the temperature cycle with similar maximum and minimum values between years, and the population gonad index varied between years, with consistently low levels in February and March 1995 (Fig. 2). The chlorophyll *a* levels fluctuated widely, both within and between seasons. Maximal values (> 12  $\mu\text{g chl } a \text{ L}^{-1}$ ) were recorded in May 1995 and June 1996, and minimum values, close to 4  $\mu\text{g chl } a \text{ L}^{-1}$ , in August 1994, 1995, 1996, and December 1996 (Fig. 2).

The relationship between environmental variables and tuatua reproductive condition was assessed using Spearman's rank correlation coefficient (Table 2). Of the factors examined, seawater temperature appeared to be the main variable affecting the num-





TABLE 3.

Seasonal variation in regression coefficients for the equation  $y = ax^b$ , where  $y$  = tissue dry wt (g) and  $x$  is the shell length (mm) in *P. donacina*, from November 1993 to April 1997.  $a$  is the intercept;  $b$ , slope of the regression line;  $r$ , correlation coefficient;  $P$ , probability level; n.s., not significant.  $n = 20$  in all cases.

	Date	$a$	$b$	$r$	$P$
1	27.11.93	$2.48 \times 10^{-5}$	2.77	0.95	0.01
2	15.2.93	$4.43 \times 10^{-5}$	2.63	0.92	0.01
3	8.1.94	$6.34 \times 10^{-6}$	3.04	0.96	0.01
4	11.2.94	$2.04 \times 10^{-4}$	2.26	0.85	0.01
5	12.3.94	$1.16 \times 10^{-4}$	2.37	0.84	0.01
6	1.4.94	$4.89 \times 10^{-5}$	2.56	0.95	0.01
7	28.4.94	$1.20 \times 10^{-5}$	2.91	0.97	0.01
8	24.5.94	$6.08 \times 10^{-5}$	3.01	0.95	0.01
9	24.6.94	$2.08 \times 10^{-4}$	2.19	0.79	0.01
11	12.8.94	$1.32 \times 10^{-2}$	1.26	0.30	n.s.
13	10.10.94	$1.75 \times 10^{-4}$	2.23	0.89	0.01
14	6.11.94	$2.23 \times 10^{-4}$	2.19	0.82	0.01
15	5.12.94	$5.08 \times 10^{-4}$	2.05	0.83	0.01
16	1.1.95	$9.96 \times 10^{-5}$	2.43	0.96	0.01
17	2.2.95	132.2	0.75	0.17	n.s.
18	1.3.95	$7.23 \times 10^{-5}$	2.47	0.92	0.01
19	29.3.95	$3.39 \times 10^{-3}$	1.65	0.78	0.01
20	19.4.95	$1.53 \times 10^{-5}$	2.81	0.94	0.01
21	15.5.95	$6.45 \times 10^{-6}$	2.99	0.92	0.01
22	16.6.95	$5.53 \times 10^{-6}$	3.00	0.93	0.01
23	14.7.95	$1.92 \times 10^{-5}$	2.72	0.96	0.01
24	11.8.95	$1.22 \times 10^{-4}$	2.30	0.89	0.01
25	10.9.95	$1.72 \times 10^{-4}$	2.23	0.97	0.01
26	6.10.95	$1.41 \times 10^{-5}$	2.78	0.94	0.01
27	27.10.95	$1.41 \times 10^{-5}$	2.78	0.94	0.01
28	23.11.95	$3.39 \times 10^{-5}$	2.60	0.98	0.01
29	27.12.95	$8.17 \times 10^{-6}$	3.02	0.99	0.01
30	25.1.96	$7.53 \times 10^{-6}$	3.05	0.99	0.01
31	21.2.96	$1.57 \times 10^{-5}$	2.86	0.97	0.01
32	20.3.96	5.75	0.25	0.37	n.s.
33	16.4.96	$1.45 \times 10^{-4}$	2.29	0.94	0.01
35	4.6.96	$5.74 \times 10^{-5}$	2.48	0.94	0.01
36	4.7.96	$2.87 \times 10^{-5}$	2.63	0.97	0.01
37	2.8.96	$5.28 \times 10^{-6}$	2.99	0.93	0.01
38	31.8.96	$1.21 \times 10^{-5}$	2.84	0.99	0.01
39	26.9.96	$6.72 \times 10^{-5}$	2.44	0.96	0.01
40	28.10.96	$1.37 \times 10^{-4}$	2.32	0.95	0.01
41	21.11.96	$2.93 \times 10^{-4}$	2.16	0.97	0.01
42	13.12.96	$2.55 \times 10^{-5}$	2.73	0.95	0.01
44	9.2.97	$3.67 \times 10^{-5}$	2.65	0.96	0.01
46	7.4.97	$1.53 \times 10^{-5}$	2.85	0.92	0.01

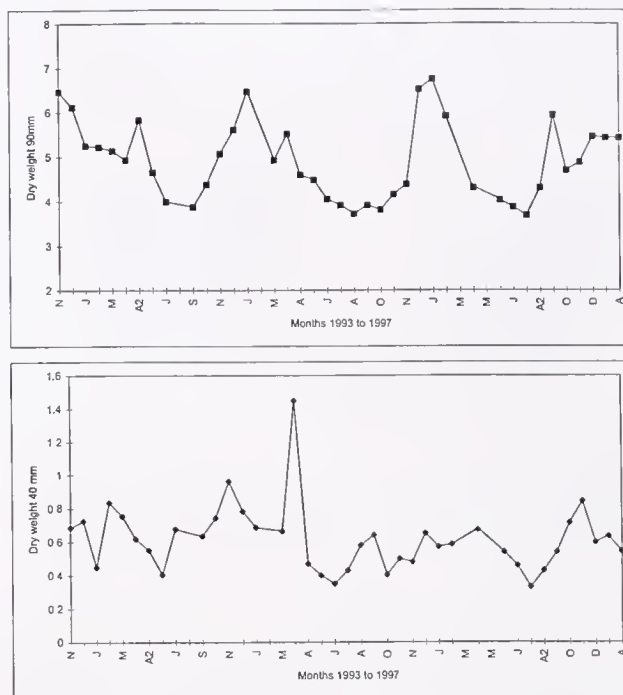


Figure 3. Seasonal variation in tissue dry weight (g) for *P. donacina* shell length 40 mm and 90 mm. Values calculated from the regression lines in Table 2.

*tricosca*, *P. australis*, and *P. subtriangulata* from warmer regions of northern New Zealand, where gametogenesis was continuous throughout the year, and there were one or two distinct spawning periods (Redfeam 1974, Grant and Creese 1995, Hooker and Creese 1995a). Several studies of bivalves have found differences in the reproductive cycle with geographic location (Keck et al. 1975, McLachlan et al. 1996). For example, *Donax trunculus*, which is distributed from Brittany to southern Morocco and the Mediterranean, shows increased duration of the breeding season in Southern Spain (Tirado and Salas 1998) compared with other areas. Also, within a species, populations in different areas may have two spawning periods (Ansell and Bodoy 1979) or spawnings may, as in *P. donacina*, be more evenly distributed throughout the summer (Ansell et al. 1980). This timing of gametogenic cycles suggests an exploitive reproductive strategy for surf clams, contrasting with more conservative gametogenesis patterns, where bivalves

male and female tuatua increased significantly with the population gonad index (linear regression analysis, Fig. 6). Therefore, as the number of reproductively active tuatua increased in the population, each individual was likely to have increased reproductive potential.

DISCUSSION

Gametogenesis in *Paphies donacina* is similar to that described for many other surf clams, including the New Zealand mactrids (Redfeam 1974, Grant and Creese 1995, Hooker and Creese 1995a, McLachlan et al. 1996, King 1997). In *P. donacina*, as in some other surf clams, there was a resting or inactive stage during winter (Kanti et al. 1993, King 1997). This contrasts with *P. ven-*

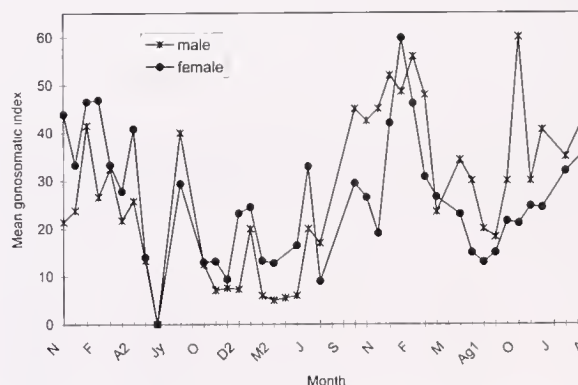


Figure 4. Seasonal variation in the gonosomatic index for male (x) and female (●) in *P. donacina* from November 1993 to April 1997.

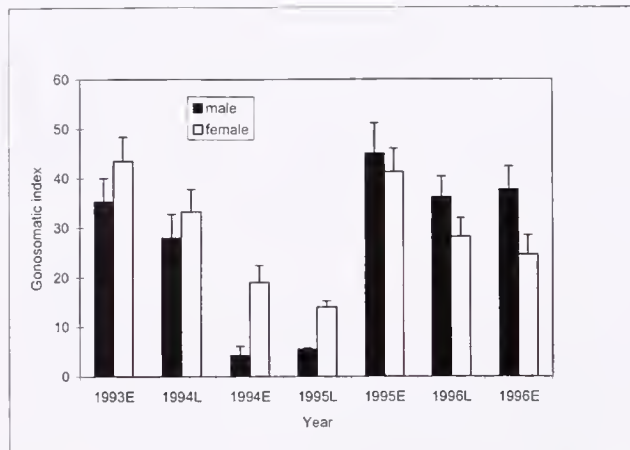


Figure 5. Comparison of gonosomatic index for male and female *P. donacina* early (E) (November to January) and (L) later (March to May) in the breeding season. Error bars show the standard error from the combined samples. N is shown in Table 4.

spawn over the winter using up resources that have built up over the summer (Bayne 1976).

In contrast with surf-clams such as *D. trunculus*, where males and females can be readily distinguished by their color during the breeding cycle, the sex of *P. donacina* could not be distinguished other than by histological sections. The sex ratio of 1.7 females to 1 male in this species differs from the more usual 1:1 ratio which is found in most other surf clams including *D. trunculus* (Tirado and Salas 1998), *D. serra*, *D. cuneatus*, *Tivela mactroides* (McLacelan et al. 1996), and other New Zealand surf clams (Redfearn 1974, Grant and Creese 1995, Hooker and Creese 1995a, King 1997). Several possible explanations have been forwarded to explain inequalities in the sex ratios of bivalves. These explanations include: a small sample size, differential rates of development, and differences in the maximal lengths for male and female bivalves. For *P. donacina*, the maximal shell length was similar for males and females, and the unequal sex ratio within the length range 45–108 mm was caused by females dominating the smaller length groups. The possibility of delayed sexual maturity in smaller males is supported by the presence of individuals of an indeterminate sex in the population throughout the year.

In studying the reproductive biology of bivalves, some authors have suggested that the condition index of bivalves can act as an indicator of reproductive activity (Dix and Ferguson 1984, Cheung 1991). For *P. donacina*, populations with a high proportion of reproductive individuals would be expected to have a high CI

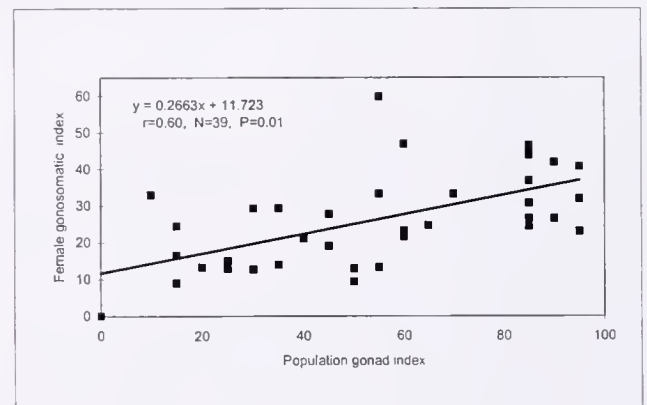
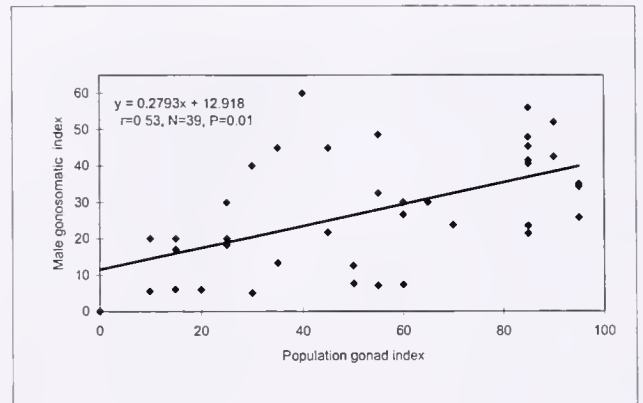


Figure 6. Relationship between gonosomatic index of male and female *P. donacina* plotted against the population gonad index. Both of the regression lines are significant at the 1% level and the slopes and elevations are similar.

index, but the index is not a good indicator of the reproductive potential. This is because both somatic tissue and gonad growth, as well as shell growth, may be occurring simultaneously in this species. This is supported by the seasonal weight relationships of smaller length tuatua that may not be reproductively active.

The effects of temperature on the gametogenic and spawning cycles of bivalves is complex, and depends on the overall reproductive strategy and the environmental temperature range. Some clams have an inactive period, which is the result of high environmental temperatures (Hesselman et al. 1989), and others, like *Spisula solidissima* (Kanti et al. 1993) and *P. donacina*, where low temperatures can delay gametogenesis and the periodicity of

TABLE 4.

Linear regression correlation coefficients relating shell length (L, mm), dry wt (W, g) and condition index (CI) to the gonosomatic index for male (M) and female (F) *P. donacina* early and late in the breeding season. N, number of individuals; \* values significant at  $P < .05$ ; \*\* values significant at  $P < .01$ ; -, negative correlations.

Season		ML	MD	MCI	MN	FL	FD	FCI	FN
1993/4	Early	0.13	0.02	0.14	22	0.11	0.14	0.35	28
	Late	0.33	0.31	0.25	18	-0.43*	-0.45*	0.04	22
1994/5	Early	-0.77**	-0.74**	0.10	17	0.36	0.28	0.02	15
	Late	0.38	0.89	0.59	4	0.03	0.25	0.48	9
1995/6	Early	0.01	0.16	0.23	15	0.36	0.29	0.58**	23
	Late	0.32	0.18	0.13	17	-0.55*	-0.60*	0.16	16
1996/7	Early	0.20	0.56	0.22	11	0.29	0.05	0.31	19

spawning. In *P. donacina* and *M. mercenaria* (Manzi et al. 1985), the gametogenic cycle depends on temperature, but in others, like the European cockle, *Cerastoderma edule* (Navarro et al. 1989) and the semi-tropical subtidal clam, *Megaitaria aurantica* (Garcia-Dominguez et al. 1994), the gametogenic cycle was temperature-independent. Although in *P. donacina*, both the number of reproductive individuals and the gonosomatic index correlated with seawater temperature, this factor was not a good predictor for reproductive success between years. A threshold temperature for spawning occurs in some oyster species (Burrell 1985) and in the northern tuatua *P. subtriangulata* (Grant and Creese 1995). However, no such threshold was detected in *P. donacina* or the cockle *Cerastoderma edule* (Navarro et al. 1989), where spawning can occur over a wide temperature range. For the cockle, annual variations are thought to be due to either the quantity of ingested food or to levels of previously stored nutrient reserves.

Relatively few studies have followed the reproductive cycles of bivalves over a number of years, and of these only a few have provided values for both the temperature and available food resources. Kautsky (1982), studying the mussel *Mytilus edulis* populations in the Baltic, concluded that annual differences in the dry weight of individuals was due to the food abundance not the temperature of the habitat. Although temperature has been identified as the major factor affecting the reproduction of *P. donacina*, it is interesting that there was no similar effect due to food levels as indicated by levels of chlorophyll *a*. Some studies have shown such a relationship may have a lag phase, which most likely results from the build up of reserves before gamete formation (Hawkins et al. 1985). There is some support for this observation in the present study, because chlorophyll *a* levels correlated with seawater temperature.

Although sand beaches have been described as low production habitats compared with estuaries or rocky shores, this generalization does not hold for tropical and subtropical exposed sand beaches where productivity levels are high. Species of *Donax* dominate these habitats (Ansell 1983), and opportunistic features of their life style include small size, rapid growth, early sexual maturity, and high fecundity. In southern temperate latitudes, there are fewer clam species including a few *Donax* species and macrtrids such as *Paphies* and *Mesodesma spp.* These much larger surf clams are most abundant within highly productive areas. For example, *D. serra* from South Africa occurs in warm water areas supplied with organic enrichment from upwellings (Brown et al. 1989). High production may also be due to surf zone algal blooms

or inputs from kelp strandings, land runoff, or river debris. These nutrients, including inorganic nitrogen from the beach itself, can be trapped inshore by semi-enclosing water patterns and on-shore winds. If these processes result in high productivity levels throughout the year, then they most likely explain the fast growth rates of species like *P. donacina*, their large maximal size (McLachlan et al. 1996, Cranfield et al. 1996), and high fecundity. Dawson (1954) estimated egg production by *P. donacina* to be 30–40 million per individual and values for the toheroa of 80 and 90 million eggs per individual. Similarly high values have been found in other surf-clams such as *Tivela stultorum* (11–20 million, Coe and Fitch 1950). This large reproductive output, together with an extended breeding period, allows larval release over an extended period without the risk of food limitation. A longer recruitment period may also assist dispersion and might increase larval survival within this physically dominated habitat where mortality is likely to be high.

For *P. donacina*, gonad development, somatic growth, and accumulation of energy resources overlap during the spring and summer and may extend into autumn because of continued high food levels. It is likely that *P. donacina* can maintain positive scope for growth at this time, because it shows seasonal adaptation in its oxygen consumption and feeding rate, maintaining a similar filtration rate at 10 °C in winter to 20 °C in summer (Marsden in press). The ecology and distribution patterns of *P. donacina* closely resemble *M. donacium* from southern Africa. Both species can occur—almost exclusively—intertidally (Dawson 1954) and also extend into the surf zone (Brown et al. 1989). The breeding cycle is exploitive, flexible, and largely determined by the temperature regime. As food is rarely limiting, these surf clam species would be expected to reproduce well during El Niño years, which are associated with increased seawater temperatures.

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## UPTAKE OF DISSOLVED FREE AMINO ACIDS BY NORTHERN QUAHOGS, *MERCENARIA MERCENARIA* AND ITS RELATIVE IMPORTANCE TO ORGANIC NITROGEN DEPOSITION IN NARRAGANSETT BAY, RHODE ISLAND

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**ABSTRACT** Studies were undertaken to determine the relationship between size of northern quahogs *Mercenaria mercenaria* and the rate at which they transport aspartic acid. Quahogs ranging from 25 to 103 mm valve length were collected in Narragansett Bay and placed in seawater aquaria (27 ppt, 20 °C) and allowed to pump water actively. Uptake experiments were carried out using 1  $\mu\text{mol/L}$   $\text{C}^{14}$  radiolabeled aspartic acid. Aspartate transport rates in  $\mu\text{mol/h}$  can be related to valve length by the allometric equation with  $a = 24.32$  and  $b = 0.905$  when valve length is in mm. In May 1990, near-bottom samples of seawater were taken from five locations in Narragansett Bay for analysis of dissolved free amino acids (DFAA) by high-pressure liquid chromatography (HPLC). Results showed that the mean total DFAA concentration was  $667.6 \text{ nmol/L} \pm 167.3 \text{ SD}$ , with the top five being serine, alanine, aspartic acid, glutamic acid, and glycine. A simple spreadsheet model was used to assess the relative importance of the uptake of DFAA *vis-à-vis* the filtration of particulate organic matter by *M. mercenaria*. In the model, environmental DFAA concentrations and uptake rates by quahogs determined in this study are compared with literature values for particulate organic nitrogen concentrations and filtration rates by quahogs. On an annual basis, uptake of DFAA can account for about 14% of the total organic nitrogen uptake by quahogs. Uptake of DFAA by these benthic filter feeders may be a pathway of benthic-pelagic nutrient coupling that is often overlooked in coastal ecosystem analyses.

**KEY WORDS:** DFAA, quahog, *Mercenaria*, Narragansett Bay, filter feeding, benthic-pelagic coupling

### INTRODUCTION

Since Stephens and Schinske (1961) demonstrated that several phyla of soft-bodied marine invertebrates are able to uptake dissolved free amino acids (DFAA) directly across epithelial surfaces from seawater, subsequent studies have shown that uptake of DFAA occurs at concentrations that are typical in estuarine and marine environments (Manahan et al. 1982). DFAA in estuarine and coastal waters are believed to be released from sediments via microbial decomposition of complex organic matter and as exudates from phytoplankton and macrophytes (Jorgensen et al. 1980, Jorgensen 1982, Bronk et al. 1994). These DFAA in estuarine and coastal waters are of potential nutritional significance to most soft-bodied marine invertebrates (reviewed by Stephens 1988, Wright and Manahan 1989).

Most work regarding the uptake of DFAA by invertebrates has focused on its significance at the organismal level. Several studies (e.g., Pequignat 1973, Manahan and Crisp 1983, Chien and Rice 1985) used histologic and autoradiographic methods to demonstrate the incorporation of transported DFAA into epithelial and subepithelial tissues. Other work has shown that DFAA can be transported by a number of invertebrate species at rates that are of nutritional significance, often supporting in excess of 50% of their measured oxygen consumption rates (e.g., Davis et al. 1985, O'Dell and Stephens 1986). In addition to nutritional significance, the uptake of DFAA has been demonstrated to be an important mechanism for the recovery of diffusively lost solutes from high-concentration DFAA pools in epithelial tissues (Gomme 1982, Wright and Secomb 1984).

In addition to work characterizing the organismal significance of DFAA uptake by marine invertebrates, some work has aimed at characterizing the nature and specificities of the DFAA carriers in epithelial tissues. Preston and Stevens (1982) showed that DFAA uptake by *Glycera dibranchiata*, polychaete, is a type of sodium

cotransport with a  $\text{Na}^+$ :amino acid coupling ratio of 3:1 necessary to energetically overcome a million-fold concentration gradient. Using competitive inhibition studies in bivalves, other workers have shown that the amino acid carriers, in general have broad specificities for  $\alpha$ -amino acids, but there is evidence of separate carriers for  $\beta$ -amino acids, such as taurine (Wright and Secomb 1984, Wright 1985, Rice and Stephens 1987). Eventually, Pajor and Wright (1987) isolated apical membrane vesicles from the ctenidium of the bivalve *Mytilus edulis*, and demonstrated active transport of alanine.

Despite the extensive work describing the physiological significance of the uptake of DFAA and the characterization of the amino acid carriers within epithelial membranes, there is a dearth of available information about the ecological significance of DFAA uptake by populations of invertebrates in estuarine or coastal marine waters. Stephens (1981, Stephens 1982) proposed that uptake of dissolved organic material (DOM), including DFAA, can provide supplemental nutrition to invertebrates residing in primary and secondary consumer trophic levels. He argued that this extra nutritional energy input directly to the higher trophic levels can potentially confound the accounting of trophic efficiency usually calculated based on the consumption of prey from lower trophic levels. Interestingly, Stephens's conclusions about trophic significance of DOM uptake mirrored that of Putter's (1909) postulation eight decades earlier.

Aside from the potential trophic significance of DFAA uptake by invertebrates, the amount of organic nitrogen being cycled within assemblages of invertebrates may be a significant contribution to nitrogen mass balance budgets in estuarine and coastal environments. *A priori*, we could expect that on a per hectare basis upward of 8 kg of nitrogen could be cycled daily via uptake of FAA alone (assuming 5-g mussels and 14-g N/mol DFAA). This estimation is based on published rates of DFAA uptake by some

marine mussels, *Mytilus edulis*, of  $1 \mu\text{mol/g} \cdot \text{h}$  from mixed amino acids in the environmentally realistic micromolar range (Manahan et al. 1983b) and numbers of mussels in some natural assemblages of  $500/\text{m}^2$  (Newell and Shumway 1993). The significance of the uptake of DFAA and other forms of dissolved organic nitrogen (DON) by benthic filter feeders deserves further attention in light of its potential significance to recent discussions of estuarine nitrogen budgets. Indeed, in their study of mussels in the Wadden Sea of Germany, Siebers and Winkler (1984) concluded that, "... mussel beds in shallow coastal waters exposed to tidal movements of large seawater masses play a significant role in recycling of dissolved organic material." But they did not estimate the relative importance of DFAA uptake and PON filtration at their site.

The first aim of this study is to determine the size-specific transport of a representative amino acid, aspartic acid, by an infaunal bivalve mollusk, *Mercenaria mercenaria*, one of the dominant benthic filter feeders in Narragansett Bay. The next aim is to determine the mean concentration of DFAA in Narragansett Bay waters and then to apply the size-specific transport rates by *M. mercenaria* to available information on *M. mercenaria* populations in Narragansett Bay, thereby estimating the bay-wide magnitude of DON cycling attributable to the DFAA uptake.

#### MATERIALS AND METHODS

Northern quahogs, *Mercenaria mercenaria*, ranging in size from 24–103 mm valve length ( $n = 20$ ) were collected from Narragansett Bay and placed in 40-L opaque-walled aquaria in the laboratory approximately 12 hours before commencement of aspartic acid uptake experiments. The experimental aquaria contained aerated Narragansett Bay seawater (27 ppt and  $20^\circ\text{C}$ ). Narragansett Bay seawater used during these experiments was passed through a  $0.45 \mu\text{m}$  nominal pore size filtration system and stored a minimum of 2 weeks in the laboratory to reduce the concentrations of naturally occurring DFAA. Labile dissolved organic material including DFAA are known to be greatly reduced in stored seawater (Waksman and Carey 1935, Stephens and Manahan 1984). Aspartic acid was chosen as a representative amino acid for study, because it is regularly among the top four most highly concentrated amino acids in seawater (Manahan et al. 1982, Siebers and Winkler 1984). Amino acid uptake experiments were begun only if the quahog siphons were visible as a sign of active water pumping. Water pumping by quahogs is easily disturbed in laboratory settings (e.g., Rice and Stephens 1988), but dim illumination and limiting outside disturbances can help improve the chances of inducing the quahogs to extend their siphons actively and to commence pumping water. Despite these precautions, some of the quahogs collected in Narragansett Bay did not extend their siphons during the 12-hour preliminary conditioning period, thus they were not used for the uptake experiments.

Uptake experiments were begun by the addition of stock aspartic acid to the experimental aquaria to give a final concentration of  $1.0 \mu\text{M}$ . In addition, uniformly labeled  $^{14}\text{C}$ -aspartic acid (New England Nuclear) was added to a final specific activity of approximately  $150 \text{ Bq/mL}$  ( $4 \text{ nCi/mL}$ ). Samples of the medium were taken initially and at the conclusion of the experiment at the end of a 2-hour period for liquid scintillation counting. Following the experiment, quahog valve lengths were measured, and they were shucked, blotted, and weighed, and the soft tissues were solubilized using formic acid. Samples of solubilized tissues and seawater (1 mL) were prepared for scintillation counting by adding a

scintillation cocktail suitable for aquatic samples (Aquasol, DuPont/New England Nuclear). The radioactivity in each of the samples was determined using a Packard Instruments Tri-Carb liquid scintillation counter corrected for background and quenching to count at approximately 96% efficiency as determined by an internal radiation standard and an external  $\text{C}^{14}$  standard. Aspartic acid uptake data were analyzed and plotted using Axum 4.0 (Seattle, WA) Statistical and Graphics software.

To determine the concentration of DFAA in *M. mercenaria* habitat, near-bottom seawater samples were taken in five locations in Narragansett Bay (Fig. 1) in mid-May 1990. Samples were taken from either a small boat or at the end of a pier with a 2-Liter alpha water sampler tripped with a lead "bullet" messenger. The five locations were Outer Wickford Harbor ( $41^\circ34.5' \text{N}$ ;  $71^\circ26.5' \text{W}$ ); South Ferry Pier ( $41^\circ29.5' \text{N}$ ;  $71^\circ25.1' \text{W}$ ), Jamestown Harbor Pier ( $41^\circ29.7' \text{N}$ ;  $71^\circ21.9' \text{W}$ ), Greenwich Bay ( $41^\circ40.1' \text{N}$ ,  $71^\circ21.9' \text{W}$ ), and Conimicut Point ( $41^\circ42.6' \text{N}$ ,  $71^\circ20.7' \text{W}$ ). Once brought to the surface, triplicate samples of the water were taken from the sampler and filtered through a  $0.1\text{-}\mu\text{m}$  pore size syringe filter into 1.5-mL polyethylene microcentrifuge tubes and placed on ice for transport to the laboratory. All water samples were stored in a  $-60^\circ\text{C}$  freezer awaiting DFAA analysis.

Amino acids in the seawater samples were analyzed by HPLC



Figure 1. Geographic segments of Narragansett Bay, Rhode Island, USA. Narragansett Bay has a total area of  $389.3 \text{ km}^2$  and is divided into 11 geographic regions. Closed circles on the map refer to five seawater collection stations for determination of dissolved free amino acids.

using a dual pump (Waters model 501) gradient mixing system (Waters model 481 controller). Separations were made on an Ultrasphere-ODS reverse-phase column (150 × 4.6 mm, 5 mm packing) and a Waters model 720 fluorescence detector was used with output to a 10 mV integrating chart recorder. Fluorescent derivatives of the DFAA were prepared using o-phthalaldehyde reagent (Lindroth and Mopper 1979), and eluent buffers were prepared and used as described by Jones et al. (1981), with modifications to improve separations. Duplicate samples of water from the five sites in Narragansett Bay were analyzed for DFAA, with the archived third sample maintained in reserve to be analyzed if there was a large discrepancy between the results of the duplicates. DFAAs in the natural waters were quantified by integrating peak area in comparison to 100 nanomolar amino acid standards. Further details on the procedure of DFAA analysis by HPLC are provided by Davis and Stephens (1984).

To make projections on the magnitude of nitrogen cycling in Narragansett Bay by uptake of DFAA by *M. mercenaria*, a simple model was developed using the Microsoft Excel spreadsheet microcomputer program. Using Geographical Information System (GIS) data from the University of Rhode Island Geographic Information Center, Narragansett Bay was broken up into 11 geographic segments (Fig. 1), and the area for each segment was determined. Standing crop densities and length-frequency profiles of *M. mercenaria* in each of the 11 segments was estimated by using best available data from fisheries surveys undertaken in Narragansett Bay since 1949 (most recently reviewed by Rice 1992). DFAA uptake rates are then compared to the best available data on particulate filtration rates by *M. mercenaria* (Doering and Oviatt 1986) along with estimates of phytoplankton, particulate detritus and total particulate organic nitrogen in Narragansett Bay (e.g., Oviatt and Nixon 1975, Durbin and Durbin 1981).

## RESULTS

The uptake of aspartic acid by actively pumping northern quahogs, *Mercenaria mercenaria*, was demonstrated by disappearance of radiolabel in the medium and appearance of the label in the molluscan soft tissues. The rates of aspartic acid transport by individual quahogs were determined by calculating a first-order rate constant based on radioactivity levels in the soft tissues at the end of 2 hours. The uptake rates of aspartic acid ranged from 0.70  $\mu\text{mol/h}$  in a 25 mm quahog and 2.55  $\mu\text{mol/h}$  in a 69 mm quahog. The rates of uptake of aspartic acid ( $J$ ) by actively pumping *M. mercenaria* at 20 °C is size dependent (Fig. 2) and can be described by the allometric equation:

$$J = a \cdot L^b \quad (1)$$

when,

$J$  = rate of aspartic acid uptake in  $\mu\text{mol/h}$

$L$  = valve length in mm

$a = 24.32$

and

$b = 0.905$

The concentrations of various amino acids in Narragansett Bay waters are presented in Table 1. Data represent the mean of replicate DFAA determinations from the five sites. Water was well mixed in the water sampler, so there was less than a 5% discrepancy between replicates. The top five amino acids in concentration at all five locations in Narragansett Bay were serine, alanine, aspartic acid, glutamic acid, and glycine, typically making up over

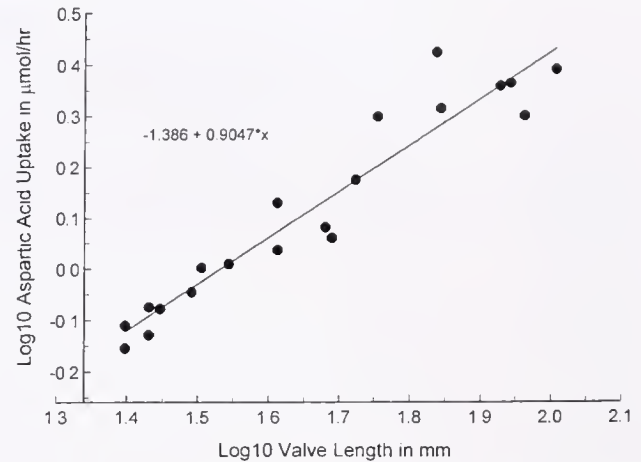


Figure 2. A double logarithmic plot relating aspartic acid uptake rate to quahog valve length. The uptake of aspartic acids by northern quahogs, *Mercenaria mercenaria*, is size dependent and can be related to valve length by the allometric equation with  $a = 24.32$  and  $b = 0.905$  when valve length is in mm. The correlation coefficient for the data is  $r^2 = 0.917$  ( $n = 20$ ). Quahog valve length can be related to whole-body mass by the allometric equation with  $a = 6.45 \times 10^{-4}$  and  $b = 2.81$  ( $r^2 = 0.985$ ,  $n = 20$ ), when valve length is in mm and mass is in g.

80% of the total DFAA. Total DFAA ranged from 478 nmol/L at Jamestown Harbor Pier to 852 nmol/L Outer Wickford Harbor, or nearly a two-fold difference in range. The sites closest to the mouth of Narragansett Bay (South Ferry and Jamestown Harbor) had the lowest concentrations of DFAAs, but it is uncertain if this difference is statistically significant, because only single water samples were taken at each of the five sites. From the data in Table 1, the mean DFAA concentration in Narragansett Bay is 667 nmol/L, so assuming there are 14 g of nitrogen per mole, the DON represented by DFAA is 9.35  $\mu\text{g/L}$ .

The spreadsheet model of DFAA uptake by the population of *M. mercenaria* in Narragansett Bay is presented in Table 2. Based upon literature estimates of the standing crop of *M. mercenaria* in Narragansett Bay, there are, on average, three animals/ $\text{m}^2$  (approx. 300  $\text{g}/\text{m}^2$  whole animal weight) with patchy distribution throughout 389  $\text{km}^2$  of the bay. In differing locations, average sizes range from 35 to 92 mm in valve length, based on the level of shellfishing. Higher levels of fishing effort for *M. mercenaria* are known to decrease the average valve lengths of a given subpopulation (e.g., Rice et al. 1989). Rates of DFAA uptake by quahogs in this analysis ranged from 1.02 to 2.55  $\mu\text{mol/h}$ , depending upon average size. The total uptake of DFAA by the Narragansett Bay population of quahogs is estimated to be 2.08 kmol/h or about 208 kg/h, and assuming 14 g of nitrogen per mole of DFAA, this represents an uptake of 2.91 kg of DON per hour.

To compare the relative level of dissolved nitrogen uptake by quahogs to the filtration of particulate organic nitrogen, it is useful to estimate the filtration rates of phytoplankton and detritus. Key to this calculation is the estimation of the population filtration rates by quahogs. On average, the quahogs in Narragansett Bay filter  $2.5 \times 10^6 \text{ m}^3$  of water per hour. With an average phytoplankton concentration of 8,000 cells/mL and average PON associated with phytoplankton of 67.2  $\mu\text{g/L}$ , the bay-wide filtration of PON by quahogs is 14.6 kg/hr. Likewise, with average detritus PON of 17.8  $\mu\text{g/L}$ , the bay-wide population filtration is 3.88 kg/h. Thus, by way of comparison, the bulk of the organic nitrogen removal by qua-

TABLE I.  
Dissolved Free Amino Acids in Narragansett Bay in nmoles/L.

Amino Acid	Wickford	Greenwich Bay	South Ferry	Jamestown Harbor	Conimicut Point	Mean Conc.	SD
Aspartate	149	103	91	88	152	116	31.4
Glutamate	131	87	82	65	135	100	31.2
Asparagine	9	3	3	4	5	4.8	2.48
Serine	201	173	171	118	198	172	33.3
Glutamine	3	1	1	1	2	1.6	0.89
Histidine	1	1	0	0	1	0.6	0.54
Glycine	107	95	74	69	113	91	19.5
Arginine	62	48	31	18	56	43	18.2
Alanine	175	102	101	91	165	131	36.3
Tyrosine	2	1	1	1	1	1.2	0.45
Methionine	1	0	0	1	1	0.6	0.54
Valine	3	1	1	1	1	1.4	0.89
Phenylalanine	3	1	0	1	1	1.2	1.1
Isoleucine	2	1	0	0	1	0.8	0.84
Leucine	2	1	1	1	0	1.0	0.70
Lysine	1	0	0	0	1	0.4	0.54
Totals	852	618	557	478	833	667.6	167.3

hogs is by means of particulates, mostly phytoplankton, but on average about 14% of the organic nitrogen taken in by quahogs may be in form of DFAA.

#### DISCUSSION

Most work on the heterotrophic uptake and assimilation of DFAA in estuarine and coastal waters has focused on marine bacteria (e.g., Jorgensen 1982, Carlucci et al. 1984, Carlucci et al. 1992, Keil and Kirchman 1991, Jorgensen et al. 1993). It is clear that uptake of DFAA by microheterotrophs is a major pathway for the removal of organic nitrogen from coastal waters, but dense assemblages of invertebrates, especially filter-feeding mollusks, are an important alternative pathway. It has been argued that microheterotrophs have a competitive advantage over invertebrates in competition for DFAA in the environment, because they possess transport mechanisms that operate very efficiently at low (submicromolar) concentrations of DFAA (e.g., Sepers 1977). However, three lines of evidence show that marine invertebrates can effectively compete with bacteria for available DFAA. First, kinetic studies have shown that environmentally realistic concentrations of DFAA in the hundreds of nanomolar to micromolar levels, oyster larvae can compete well against bacteria by virtue of higher mass-specific rates of transport at the higher DFAA concentrations (Manahan and Crisp 1982). Second, it has been demonstrated that amino acids are rapidly and efficiently removed in a single pass through the ctenidia of filter-feeding mollusks (Wright and Stephens 1978, Siebers and Winkler 1984). Finally, axenic suspensions of invertebrate larvae remove DFAA from seawater at rates comparable to those found in nonaxenic suspensions (Manahan et al. 1983a, Davis and Stephens 1984).

The data (Fig. 2) showing aspartic acid uptake rates from 1 mmol/L in the range of 1 to 2  $\mu\text{mol/h}$  by intact *M. mercenaria* correspond to dry weight specific rates of approximately 400–800 nmol/g\*h. These data are in line with amino acid transport rates from such other bivalves as mytilids and oysters that have been more extensively studied (see review by Wright 1982). In a previous study (Rice and Stephens 1988), we showed alanine trans-

port by *M. mercenaria* occurring at only 22.9 nmol/g\*h. This discrepancy in results between the experiments is a result of differences of their methods. In the present study, the quahogs were allowed to begin actively pumping before the uptake experiments commenced, and nonpumping quahogs were eliminated from the experiment. However, in the former study, a procedure of perfusing the mantle cavity was used to provide a steady water flow through the animals, but water was not necessarily passing through the ctenidia.

The use of radiolabel to monitor the influx rates of amino acids into invertebrates is not an indicator of net amino acid flux (Manahan et al. 1983b). There is a possibility of efflux of amino acids from the animal that can only be monitored by chemical determination of the concentration of amino acids in the medium by HPLC or other means. In a number of early studies (e.g., Lum and Hammen 1964, Hammen et al. 1966, Bayne and Scullard 1977) it was reported that bivalves can excrete nitrogen in the form of amino acids. In these early studies, bivalves were confined in vessels for several hours to obtain enough excretory products for adequate analytical detection and quantification. Later studies using shorter incubation times or flow-through systems along with analytical techniques with greater sensitivity have not shown excretion of DFAA or any other DON (e.g., Jordan and Valiela 1982, Manahan et al. 1982, Siebers and Winkler 1984). Other studies have shown that excretion of amino acids occurs only in conditions of hyposmotic adjustment (Heavers and Hammen 1985, Rice and Stephens 1988). In the current study, I did not monitor the net flux of aspartic acid by directly following its change in concentration in the medium with time. However, I am confident that the influx of aspartic acid as represented by disappearance of radioactivity from the medium and its appearance in *M. mercenaria* soft tissues adequately reflects the net influx of aspartic acid. This is because we previously showed that influx of alanine as measured by depletion of radiolabel from the medium is tightly coupled with net flux of alanine as measured by HPLC when the quahogs are held in osmotically stable conditions with salinities above 17 ppt (Rice and Stephens 1988).



TABLE 2.  
Spreadsheet Model of DFAA Uptake and PON Filtration by Northern Quahogs.

Geographic Segments	Area <sup>a</sup> (sq. km)	Quahogs <sup>b</sup> (ind/sq m)	Est.		DFAA Uptake <sup>d</sup> (pmol/ind/hr)	Pop. Uptake <sup>e</sup> (kg/DON/hr)	Mean FR <sup>f</sup> (L/ind/hr)	Pop FR <sup>g</sup> (kg-phyto-N/hr)	Pop FR <sup>h</sup> (kg detrit-N/hr)
			Standing Crop Totals	Mean Size <sup>c</sup> (mm)					
Seekonk River	2.6	0	0	0	0	0	0	0	0
Providence River	21.2	12.5	2.65E+08	72	1.9	0.73	2.1	3.68	0.98
Upper Narragansett Bay	56.7	5.2	2.95E+08	48	1.3	0.56	1.4	2.77	0.73
Taunton River	12.2	0.1	1.22E+06	96	2.5	0.01	2.7	0.02	0.01
Mount Hope Bay	39.0	5	1.95E+08	96	2.5	0.69	2.7	3.59	0.95
Greenwich Bay	13.0	5.4	7.01E+07	35	1.0	0.1	1.0	0.48	0.13
Upper West Passage	54.8	2.7	1.48E+08	55	1.5	0.31	1.6	1.59	0.42
Upper East Passage	36.1	2.5	9.03E+07	59	1.6	0.21	1.7	1.04	0.27
Lower West Passage	47.6	1.2	5.71E+07	62	1.7	0.14	1.7	0.69	0.18
Lower East Passage	53.4	0.1	5.35E+06	96	2.5	0.02	2.7	0.1	0.03
Sakonnet River	52.7	1.1	5.79E+07	62	1.7	0.14	1.8	0.7	0.18
Narragansett Bay									
Total	389.3	3.0	1.18E+09			2.91		14.6	3.88

<sup>a</sup> Data on areas of Narragansett Bay geographic segments courtesy of Dr. Peter August of the URI Environmental Data Center.

<sup>b</sup> Source of information on quahog populations include Stringer (1959), Saita et al. (1967), Pratt et al. (1992), Lazar et al. (1994).

<sup>c</sup> Mean quahog sizes based on length-frequency analyses at individual geographic areas or estimates based on level of fishing effort (Pratt et al. 1992).

<sup>d</sup> DFAA uptake rates calculated from data of present study.

<sup>e</sup> Calculation of DON uptake assumes 14 grams nitrogen per mole of DFAA.

<sup>f</sup> Filtration rate in L/h calculated from the allometric equation  $FR = 0.307L^{0.967}$  assuming valve length L in cm (Doering and Oviatt 1986).

<sup>g</sup> Calculation of PON filtration assumes average phytoplankton densities of 8,000 cells/mL, mean phytoplankton cell dry weight of 0.28 ng/cell and nitrogen making up 3% of the cell dry weight (Durbin and Durbin 1981). Thus, average phytoplankton PON in Narragansett Bay is 67.2  $\mu\text{g/L}$ .

<sup>h</sup> The calculation of rate of filtration of particulate organic nitrogen associated with detritus assumes an average detrital suspended particulate matter (SPM) of 3.7 mg/L (Morton 1972), organic dry weight comprising 12.7% of SPM (Oviatt and Nixon 1975), and nitrogen content comprising 3.8% of organic dry weight (Oviatt and Nixon 1975, Newell and Shumway 1993). Thus, average detrital PON in Narragansett Bay is 17.8  $\mu\text{g/L}$ .

The spring concentrations of DFAA measured in Narragansett Bay waters ranging from 478 to 852 nmol/L (Table 1) are well within range of the DFAA concentrations found in other estuaries. Jorgensen (1982) reported that there were seasonal fluctuations in DFAA concentrations in the Kysing Fiord of Denmark ranging from minima of 200 nmol/L in summer and winter and maxima in the 700–900 nmol/L range in the spring and autumn, corresponding to peak phytoplankton blooms. Likewise, Poulet et al. (1985) found a significant correlation between various measures of primary productivity and DFAA concentrations in the Morlaix Bay of Brittany, France, but DFAA concentrations were found to be highly variable and in the 1 to 4  $\mu\text{mol/L}$  range. Mopper and Lindroth (1982) sampled DFAA at an open water Baltic Sea station during the month of May and found a diel variation of DFAA concentrations ranging from 200 to 400 nmol/L in late evening and minimal concentrations of 30–40 nmol/L in the early day. Macko and Green (1982) carried out the most extensive study of DFAA in a New England estuary following the concentrations of 23 different amino acids for 1 year in the Damariscotta River estuary of Maine. That showed that DFAA concentrations fluctuated greatly throughout the year, but with a yearly average was 208 nmol/L, the dominant three amino acids being alanine, glycine, and serine. Based on results from these other estuaries, the May sampling in of Narragansett Bay reported in this study may represent maximum DFAA levels, but this speculation awaits resolution pending more thorough sampling.

The spreadsheet model shows that the uptake of DFAA by *M. mercenaria* may be nearly as important as filtered PON associated

with detritus, which has been recognized as an important nutrition source for some bivalves (Newell and Shumway 1993). A weakness of this spreadsheet model is that it relies heavily on annual average estimates of PON and DFAA measurements taken during a single month. It is likely that, during different times of the year, the relative proportion of organic nitrogen removal in dissolved and particulate forms will exhibit strong seasonality. In addition, the relative magnitude of total organic nitrogen uptake will show strong seasonality because of the temperature dependency of filter feeding (e.g., Doering and Oviatt 1986).

The total amount of organic nitrogen uptake by the population of *M. mercenaria* in Narragansett Bay should be viewed with caution. Although *M. mercenaria* are dominant filter feeders in Narragansett Bay, they are not the only ones. Other filter feeders in Narragansett Bay include such other bivalves as *Mytilus edulis* and *Crassostrea virginica*, the filter-feeding gastropod *Crepidula fornicata*, spionid polychaetes, ascidians, and others. However, the model is illustrative that uptake of DFAA by benthic filter feeders may be a pathway of benthic–pelagic nutrient coupling that is often overlooked.

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## PERFORMANCE OF A TIDAL-POWERED UPWELLING NURSERY SYSTEM FOR NORTHERN QUAHOGS (HARDCLAMS) (*MERCENARIA MERCENARIA*) IN SOUTH CAROLINA

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**ABSTRACT** Entry into the hard clam aquaculture industry on a small scale has been limited by the cost of plantable seed. Growers must either purchase expensive seed at a suitable planting size (usually 8 mm SL or larger) or raise small seed to this size in a nursery. Land-based nurseries foster high survival and rapid growth, but require expensive waterfront property and are energy- and labor-intensive to operate. Field-based nurseries are inexpensive to operate, but seed survival is often very low and success is site-specific. Floating upwelling systems (FLUPSYs) combine many of the advantages of land-based systems (high survival, rapid growth) with those of field-based systems (inexpensive operation). One particular type of FLUPSY, a tidal-powered upwelling system (TPU), is described here. TPU performance was tested in South Carolina over a 5-year period. Tidal currents averaging 0.53 m/s produced flow rates of 52 Lpm through upwelling bins. The TPU requires a mean current velocity of 0.26 m/s to produce a flow rate of 26 Lpm through the upwelling units, which is comparable to flow that land-based upwellers provide. Daily growth rates as high as 15% were observed. Growth in the TPU was more rapid than published reports of growth rates in land-based systems in South Carolina. The TPU described here can produce 122,000 (12 mm) seed, starting from 1-mm seed, in a 32-week growing season (March through October). Capacity is considerably higher (up to 1,074,000) if seed are stocked at a larger size and/or harvested at a smaller size. The described system, built for \$4,500 and with annual operating costs of less than \$5,000, is a cost-effective nursery system that small-scale growers could implement to produce field-plantable seed.

**KEY WORDS:** *Mercenaria mercenaria*, nursery culture, seed production, quahog, hardclam, aquaculture, FLUPSY

### INTRODUCTION

The growout of clams to market size is a field-based operation that relies on the natural productivity of coastal waters to meet the high feeding requirements necessary to foster rapid growth. However, to achieve high survival in the growout phase, the seed must be relatively large (8–12 mm) when planted. Nursery systems are used to raise hatchery-produced seed to the larger sizes needed for field growout. Bivalve nurseries have traditionally been located on waterfront property allowing seawater to be pumped to raceways or upwelling systems that contain the seed clams. Such land-based nurseries are expensive to operate and require access to ever scarcer and more expensive waterfront property. Alternatives to land-based systems include pond or impoundment-based nurseries (Manzi et al. 1988, Battey and Manzi 1990, Hopkins et al. 1993, Bayes 1981) and field nurseries (Vaughan 1988, Castagna 1984, Kraeuter et al. 1998, Flimlin and Kraeuter 1997, Walker et al. 1995). Pond-based nurseries suffer from many of the same problems as land-based nurseries, in that they must be located near a source of clean, high-salinity seawater. Additionally, considerable expertise is required to maintain dense phytoplankton blooms required for rapid growth. Field nurseries place the seed in naturally productive waters under more protected conditions than are required for growout. Such nurseries employ various types of protective devices (nets, wire cages, sand trays, soft bags, etc.) to deter predators. These devices require frequent maintenance to remove fouling organisms and silt (Manzi and Castagna 1989, Kraeuter et al. 1998), and even under optimal conditions they must be stocked

at relatively low densities to achieve acceptable growth and survival.

Floating or suspended culture has been used for many years, usually in the form of suspended trays or nets (Manzi and Castagna 1989). Bayes (1981) described an upwelling system nursery located in an impoundment that offered protected conditions and rapid growth. Recently, floating upwelling systems (FLUPSYs) have been developed to capitalize on the features of land-based upwellers while avoiding the expense of waterfront property. A number of powered FLUPSYs have been designed and tested in recent years (Rivara and Bavaro 1997, Bishop 1998; RaLonde 1994, K. Brown, pers. comm.). These systems use airlifts, pumps, water wheels, or other electrical devices to move water through the system. However, one system, in use at Mook Seafarms, Damariscotta, Maine, uses tidal currents to provide water flow through the upwelling units (Mook 1988, Mook and Johnson 1988). Our tidal-powered upwelling (TPU) system was modeled after the Mook Seafarms system (Baldwin et al. 1994). This paper describes the performance of the South Carolina TPU, including flow rates, seed stocking densities, nursery rates, production capacity and comparisons with other nursery systems.

### METHODS

#### System Description

A tidal powered upwelling system (TPU) was constructed following plans drawn by Mook (1988), modified to have a more efficient scoop and shallower draft. The system construction is described in detail elsewhere (Baldwin et al. 1994). The TPU consists of a raft or floating dock (6 m × 3.6 m) with a tank in the center (4.8 m × 1.2 m) that houses 16 square upwelling bins, each

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providing bottom area of 0.23 m<sup>2</sup> (Fig. 1). Each bin is 0.46-m deep, with window screen or other appropriate mesh attached to the bottom. The front of the raft has an open scoop that traps tidally-driven water and directs it into the tank. The water rises through the screens of the upwelling bins (which retain the seed clams) and exits through 7.6-cm PVC pipe located below the water line. The bins are arranged in 2 rows of 8 discharging into a common trough. The TPU has a single point mooring on the scoop end of the raft so that the scoop always faces the direction of tidal flow. The system was deployed in a tidal creek in the Cape Romaine Wildlife Refuge near McClellanville, South Carolina.

**Tidal Current Velocity and Flow Rate Determination**

Stocking densities for upwelling systems are expressed in terms of flow ratio: the volumetric water flow rate past a given volume of seed clams, usually expressed as liters of water per minute per liter of clams (Lpm·L<sup>-1</sup>). The actual quantity of seed that can be supported in an upwelling system is a function of the available water supply, and also varies with seed size, smaller seed requiring a greater flow ratio than larger seed. In land-based systems, maximum water supply is a fixed quantity, and can only be altered by increasing the size of the pumps used. In tidal-powered systems, water supply is determined by the tidal currents at the deployment site and is not under operator control, except by relocating the system. Thus, in both types of upwelling system, higher flow ratios can be attained only by reducing the stocking density of seed clams. Consequently, before determining appropriate stocking densities for the tidal upweller, it was necessary to determine tidal current velocity at the deployment site and the resulting water flow rate through the upwelling bins.

Tidal current velocity measurements were made with a Montedoro-Whitney digital current meter reading in ft/sec. These measurements were supplemented with additional estimates attained by timing the passage of a weighted cork past the length of the raft. Current speed was measured at 15-minute intervals over several tidal cycles on different days and plotted against tidal stage, expressed in hours from slack tide. Mean tidal current velocity was estimated from this plot.

Water flow rates through the upwelling system were calculated from measurements of effluent current velocities. It was not possible to measure currents exiting from individual bins because of space constrictions. Therefore effluent current velocity was measured in the common exit trough. These measurements, made concurrently with tidal velocity measurements, were then regressed against tidal current velocity with the intercept forced through the

origin. This regression equation was then used to predict mean effluent velocity from the previously estimated mean tidal current velocity. Mean effluent velocity was multiplied by cross-sectional area of the effluent trough to yield mean total effluent flow volume (Lpm) that was divided by the number of upwelling bins contributing to the trough (8) to estimate volumetric flow rate through each bin.

**Stocking Densities**

A series of experiments was conducted to determine appropriate stocking densities for seed clams from 1 to 8 mm shell length. Two experiments were conducted in fall 1992, 4 between April and September 1993, 2 in summer 1996, and 6 between June and October 1997. Some of these experiments were conducted in one-quarter-scale experimental upwelling bins with discharge pipes constricted to provide water flow per unit area comparable to the production bins. A comparison of the experimental and production bins was made to verify that growth determined in the scaled-down version could be extrapolated to that in the production version (Fig. 2). For each of the seed sizes tested, replicate bins were stocked with a predetermined volume of seed to yield a desired flow ratio (Lpm·L<sup>-1</sup>). Target flow ratios were based on published requirements for a land-based upwelling nursery in South Carolina (Manzi et al. 1984). For each seed size, 3 flow ratios were tested. Biovolume was determined at 2-week intervals and, if volume had doubled in any of the treatments, the seed were sieved and the upwellers were restocked with uniformly sized seed at a new density appropriate to that seed size. A subsample of seed from each density was measured before sieving and a subsample of the pooled sieved individuals was measured before restocking. If seed had not doubled in any of the flow treatments, the experiment was continued for an additional week.

Biovolume increases were converted to daily growth rates by the formula:

$$DGR = (Fin\ vol/Init\ vol)^{1/n-1} - 1 \quad (1)$$

Daily growth rates from all 16 experiments were regressed against log-transformed flow ratios (Lpm·L<sup>-1</sup>). Within size classes, growth rate at different flow ratios was compared by ANOVA.

**Growth Determinations and Growth Comparisons**

Growth of different size seed at appropriate predetermined stocking densities was determined in multiple growth trials spanning different seasons and years (1992 to 1997). Growth of 4-mm

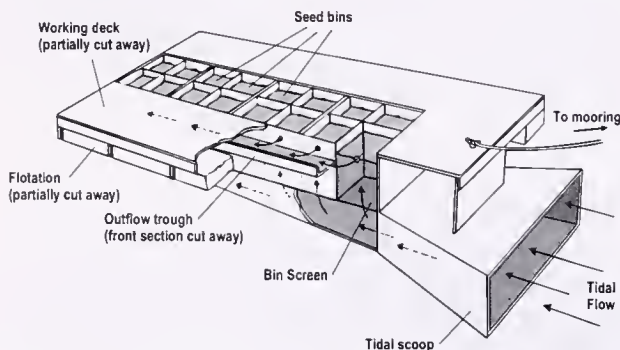


Figure 1. Diagrammatic representation of the tidal-powered upwelling system (from Hadley et al. 1997).

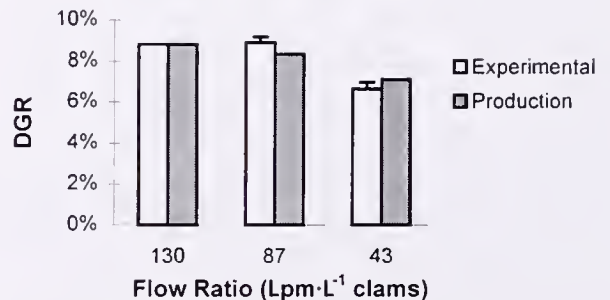


Figure 2. Daily growth rate (DGR) of 4-mm clams at 3 flow ratios in experimental (0.05 m<sup>2</sup>) and production (0.23 m<sup>2</sup>) bins.

seed was tested in each year and used as a baseline for adjusting for interannual variation in growing conditions.

Growth of different size seed in the TPU was compared to growth in bottom trays (0.6 m × 1.2 m), landbased upwellers (Manzi et al. 1984, Manzi et al. 1986), and land-based raceways (Hadley and Manzi 1984). Biomass increases were converted to daily growth rates (DGR) to allow comparisons between trials of different duration. Where necessary, shell lengths for the published data sets were estimated by the formula:

$$\text{Volume (mL)} = (0.072 \times \text{SL (mm)})^3 \quad (2)$$

Bottom-tray experiments were deployed concurrently with TPU trials and were analyzed by the paired comparisons test, treating dates as blocks. Comparison with land-based nurseries was based on published data (Manzi et al. 1984, Hadley and Manzi 1984, Manzi et al. 1986). Because of the large number of assumptions that had to be made to compare these data sets, statistical analysis was not attempted.

#### Production Capacity

A model was developed to project seasonal production of the tidal-powered upweller (as built) for two stocking sizes (1 mm, 4 mm) and 3 harvest sizes (8 mm, 10 mm, and 12 mm). The computer model assumes a biweekly sieving and redistribution and factors in expected daily growth rates and expected mortality drawn from empirical observations. At each 2-week interval, the proportion of individuals expected in each size class produced by sieving is calculated from the properties of the normal distribution, using empirically determined standard deviations of different size classes. Our data sets, spanning many cohorts, seasons, years, and sizes, indicate that individual shell lengths for sieved populations were normally distributed in all size classes tested (4 mm to 12 mm SL). The production model assumes a 32-week growing season, and that multiple cohorts are stocked as space becomes available.

## RESULTS

#### Tidal Current Velocity and Flow Rate Determination

When plotted against tidal stage, tidal current velocity closely resembled a sine wave. Mean tidal velocity was therefore calculated using the formula for the average value of the sine function:

$$2/\pi \times V_{\max} \quad (3)$$

where  $V_{\max}$  is the maximum velocity. Maximum tidal current velocity measured in this tidal creek was 0.84 m/s, yielding a mean tidal current velocity of 0.53 m/s.

Flow rates through the upwelling system were more problematic to determine. The weight of a person on the raft causes it to tilt enough to change the head pressure and thus the flow. Wave action also causes the flow rate to vary. Friction in the relatively small effluent canal also appeared to influence current meter readings. Nonetheless, effluent flow rate regressed against tidal current velocity (measured simultaneously) was significant ( $r^2 = 0.78$ ,  $P < .01$ ):

$$\text{Effluent flow rate (m/s)} = \text{Tidal current velocity (m/s)} \times 0.3785 \quad (4)$$

Substituting the previously determined mean tidal current velocity (0.53 m/s) in equation 4 yields a mean effluent flow rate of 0.20 m/s. Multiplying by the cross-sectional area of the effluent canal (0.033 m<sup>2</sup>) and converting to liters per minute yields a cu-

mulative flow through the effluent canal of 416 liters per minute (Lpm), or approximately 52 Lpm through each of the 8 upwelling bins. Actual flow rates measured were generally higher than this, so this may be considered a conservative estimate. Expected volumetric flow rates for this system at various tidal current velocities are listed in Table 1. Each TPU will have unique characteristics that will influence the relationship between tidal current velocity and flow rate through the upweller system.

#### Stocking Densities

For each size class, 3 flow ratios were tested, beginning with the recommended flow for a land-based upweller and increasing that by 50% and 100%. The results of these experiments are summarized in Figure 3. Regression of DGR on log-transformed flow ratios was significant:

$$\begin{aligned} \text{DGR} &= 0.0284 \text{Ln}(\text{Flow ratio}) - 0.0673 \quad (R^2 \\ &= 0.7334, P < .001) \end{aligned} \quad (5)$$

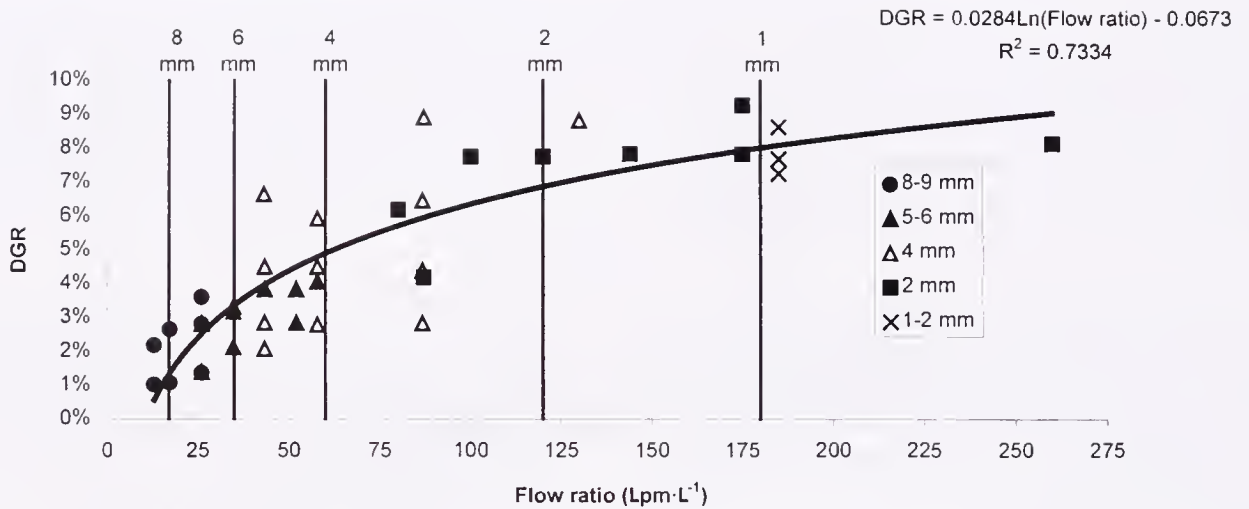
At flow ratios equivalent to those recommended for land-based upwellers, growth was similar to published reports for land-based nurseries in South Carolina. However, increasing the flow ratio by 50% (to 1.5 times the ratio used in land-based nurseries) resulted in a substantial growth increase. Increasing the flow ratio by 100% sometimes but not always resulted in a further growth increase. To achieve this highest flow ratio, stocking densities must be low (about 80% of the stocking density for a land-based upweller) and the faster growth does not compensate for the reduced carrying capacity. Thus, we selected the middle flow ratio (50% higher than that recommended for land-based nurseries) to use in our growth trials and our production model. Recommended flow ratios for different size seed in a tidal upweller and land-based upweller (Manzi et al. 1984) are presented in Table 2.

#### Growth Rate Determination and Comparisons with Other Nursery Systems

Growth rates for different size seed varied between seasons as well as between years. Mean growth rates from different experiments and the grand mean for each size class are given in Table 3. In 1997, growth of 4-mm seed was 40% below the average for all other years. For the purposes of modeling production capacity,

TABLE 1.  
Expected mean flow rate (Lpm) through each upwelling bin of the TPU at different tidal current velocities.

Average Current Velocity			Flow Rate (Lpm)
(m/s)	(f/s)	(knots)	
0.15	0.50	0.30	9.60
0.23	0.75	0.44	14.39
0.30	1.00	0.59	19.19
0.38	1.25	0.74	23.99
0.46	1.50	0.89	28.79
0.53	1.75	1.04	33.59
0.61	2.00	1.18	38.39
0.69	2.25	1.33	43.18
0.76	2.50	1.48	47.98
0.84	2.75	1.63	52.78
0.91	3.00	1.78	57.58
0.99	3.25	1.93	62.38



**Figure 3.** Daily growth rate (biomass increase per day, DGR) of different size seed clams in a tidal-powered upweller as a function of flow ratio. Vertical lines indicate recommended flow ratios for indicated seed size.

1997 growth rates were adjusted upward by 40%. The original and adjusted growth rates are shown in Table 3.

**Tidal Upweller vs. Bottom Trays**

Growth rate comparisons between the TPU and bottom trays are summarized in Table 4. Seed grew significantly faster in the bottom trays ( $P = .048$ ) but growth in both systems was remarkably fast, with daily growth rates as high as 18.9% in the bottom trays and 14.9% in the TPU for 1-mm seed. Growth rates of seed this size in land-based upwellers are estimated from published data to be no higher than 6.9%. For all size classes tested volume at least doubled in 2 weeks ( $DGR \geq 5.5\%$ ) as long as temperatures were higher than 20 °C. Seed produced in the upwellers tended to be more uniform in size than those produced in trays.

**Tidal Upweller vs. Land-based Nurseries**

At flow ratios recommended for land-based upwellers, clams grew faster in the TPU than in the land-based upwelling system and as fast as in land-based raceways (Table 5). At higher flow ratios, 50% higher than those used in land-based systems, even more rapid growth was obtained, with the exception of the 1-mm seed. The difference in the 1-mm seed growth rates was probably

seasonal. Growth rates in the TPU at the lower flow ratio ranged from 14.9% DGR for 1-mm seed to 2.5% DGR for 5–6 mm seed. Growth rates in a land-based upweller were calculated to be 6.9% for 1-mm seed and 1.4% for 3-mm seed, based on data in Manzi et al. (1984). At the higher flow rates, growth in the TPU ranged from 8.2% DGR for 1- and 2-mm seed to 3.0% DGR for 5–6 mm seed. Not enough information is available on the land-based tests to permit statistical comparisons.

**TABLE 2.**

**Recommended flow ratios (Lpm·L<sup>-1</sup> clams) for different size clam seed in tidal and land-based upwellers.**

Size (mm)	Tidal (Lpm·L <sup>-1</sup> )	Land <sup>a</sup> (Lpm·L <sup>-1</sup> )
1	180	120
2	120	80
3	100	70
4	60	40
5	45	30
6	35	23
7	27	18
8	18	12
9	12	9

<sup>a</sup> Data from Manzi et al. 1984

**TABLE 3.**

**Daily growth rates (DGR) (% biomass increase per day) of different size seed in the TPU at recommended flow ratios. For trials performed in 1997, adjusted growth rates appear in parentheses.**

Size	Date	DGR (%)	SE
1 mm	July 93	14.86	0.47
	March 94	7.68	0.34
	Sept 93	8.63	0.15
Grand mean		10.39	2.25
2 mm	July 93	7.74	0.00
	April 94	8.70	0.22
Grand mean		8.22	0.48
3 mm	Aug 93	7.74	0.50
	Oct 93	5.65	0.27
	Grand mean		6.69
4 mm	June 96	5.93	0.00
	Oct 93	1.67	0.04
	May 93	8.91	0.28
	June 97	4.49 (6.29)	0.20 (0.28)
	Aug 97	2.78 (3.90)	0.05 (0.07)
Grand mean		4.75 (5.34)	2.83 (1.22)
5–6 mm	June 96	3.84	0.21
	June 97	2.12 (2.97)	0.07 (0.10)
	Sept 97	3.18 (4.45)	0.03 (0.05)
Grand mean		3.04 (3.75)	0.05 (0.43)
8–9 mm	July 97	1.07 (1.50)	0.13 (0.19)
	Sept 97	2.65 (3.72)	0.22 (0.31)
Grand mean		1.86 (2.61)	0.79 (1.11)



### Production Capacity

Seasonal production capacity of the TPU for different size seed was estimated from a computer model using recommended flow ratios, empirically determined growth rates and standard deviations for different size seed, and estimates of mortality based on empirical observations. When stocked with 4-mm seed, the pilot TPU can theoretically produce 4 cohorts of 8-mm seed (totalling 1,074,000), 2 cohorts of 10-mm seed (459,000), or 2 cohorts of 12-mm seed (244,000) in a 32-week growing season (Table 6). When stocked with 1-mm seed, the system is projected to produce 2 cohorts of 8-mm seed (591,000), 2 cohorts of 10-mm seed (426,000), or a single cohort of 12-mm seed (122,000) in a growing season.

### DISCUSSION AND CONCLUSIONS

Mook (1988) reported maximum flow rates in his tidal powered upweller of 38 Lpm at a tidal current velocity of 0.38 m/s, and minimum flow rates of 14 Lpm at a current velocity of 0.1 m/s. Our system is predicted to have comparable flow rates at the higher current velocity, but slightly lower flow rates at the low current velocity (Table 1). Since currents as low as 0.1 m/s were not measured, and our regression forced a zero intercept, the predicted flow rates at low current speeds are conservative. The similarity in the flow rates of the 2 systems at operational current speeds suggests that flow rate for a TPU may be reliably predicted from tidal current velocity.

Flow rates comparable to a land-based nursery (30–35 Lpm) are predicted at mean tidal current velocities of 0.25–0.35 m/s (Table 1). Based on tidal current tables (International Marine 1998), it appears that velocities of this magnitude would be available along a large portion of the eastern seaboard. Only a few stations in the mid-Atlantic portion of the coast have maximum current velocities less than 0.4 m/s (the maximum current velocity needed to produce a mean current velocity of 0.25 m/s). A survey of regulatory agencies indicates that a tidal powered upweller would be permissible in all states where it would be operable (DeVoe and Nelson in prep.). Out of 18 coastal states surveyed, 15 indicated that a TPU would be allowed, although the number and type of permits required varied widely. The 3 states that would not allow a TPU also indicated that it would not be feasible because of slow current velocities.

Growth rates in the tidal upweller were not as good as in

bottom trays deployed in the same creek (Table 4). However, the bottom tray culture system is labor-intensive to operate, requires frequent cleaning, and has a much lower production capacity than the TPU. The more rapid growth in the bottom tray may be due to the very low stocking density. The bottom trays were stocked at their known optimal density, rather than at densities comparable to those used in the TPU.

The tidal upweller produced better growth rates than published reports for land-based upwellers (Manzi et al. 1984) and raceways (Hadley and Manzi 1984) in South Carolina (Table 5), particularly at the higher flow ratios recommended. Since the comparisons are made with data from previous studies, rather than with concurrent data, we cannot rule out the influence of interannual variation in growing conditions or the possibility that the clams used in the current study were naturally faster growing. The clams used in this study are descendants of those used in the published studies, which were hatchery reared seed bred for rapid growth. However, a number of generations separate the seed cohorts and additional selection may have occurred during this time span. It is interesting to note that the flow rate through the bins of the tidal powered upweller was approximately 50% higher than through the land-based nursery with which it was compared. Thus, using the 50% higher flow ratio actually results in stocking the same density of clams per bin as in the land-based system.

Other types of FLUPSYs used for growing bivalve seed range from small airlift-powered systems comparable in capacity to the TPU described here (RaLonde 1994, Rivara and Bavaro 1997), to large commercial facilities powered with pumps and having total flow rates of 6,000 gallons per minute (Bishop 1998). However, a TPU is the only type of FLUPSY which requires no external power source, so it can be located in remote areas where a powered system might not be feasible. Production data from other FLUPSYs have not been reported, so we are unable to compare the performance of the TPU with other FLUPSYs. Any performance differences would probably be attributable to the higher water flow that may be achieved with an externally powered system.

The system described here was built for a total cost of \$4,500 (1994 dollars) and operating costs were less than \$5,000 per year (Baldwin et al. 1994, Baldwin et al. 1995). This is considerably more economical, both in construction and operation, than traditional land-based systems (Baldwin et al. 1995) and compares

TABLE 4.

Daily growth rates (DGR) (% biomass increase per day) and final sizes of different size seed clams grown for 2 weeks in the TPU and in bottom trays.

Date	Initial Size (mm)	TPU		Bottom Tray	
		Final Size (mm)	DGR (%)	Final Size (mm)	DGR (%)
07/02/93	1.12	2.17	14.9	2.34	18.90
07/16/93	2.26	2.91	7.7	4.41	12.66
08/09/93	2.79	3.95	7.8	4.36	9.85
09/15/93	1.42	2.16	8.6	2.49	11.42
10/05/93	2.83	3.66	5.7	3.74	4.35
10/29/93	3.82	4.15	1.7	4.20	2.19
11/12/93	4.17	5.39	2.9	5.66	3.98

TABLE 5.

Daily growth rates (% biomass increase per day) of different size seed clams grown for 2 weeks in land-based upwellers (LBU), raceways, and the tidal-powered upweller operated at (A) the flow ratio of the LBU and (B) 1.5 times the flow ratio as the LBU. (Where more than one trial was conducted with seed of a given size, standard errors are given.)

Initial Size (mm)	LBU <sup>a</sup>		Raceways <sup>b</sup>		Tidal-A		Tidal-B	
	DGR (%)	SE	DGR (%)	SE	DGR (%)	SE	DGR (%)	SE
1	6.9	1.9	—	—	14.9	na	8.2	0.4
2	5.0	2.2	—	—	6.2	na	8.2	0.5
3	3.1	2.1	—	—	—	—	6.7	1.0
4	1.7	0.9	2.8	0.2	4.0	1.0	4.8	2.8
5–6	1.4	0.6	2.7	na	2.5	0.6	3.0	0.05

<sup>a</sup> Calculated from data in Manzi et al. 1984, Manzi et al. 1986

<sup>b</sup> Calculated from data in Hadley and Manzi 1984

TABLE 6.

Production capacity of the TPU (16 bins, 52 L/pm per bin). Seasonal production assumes a 32-week growing season.

Initial Size (mm)	Final Size (mm)	Days to First Harvest	Stocking Number Per Cohort (× 1000)	Harvest Number Per Cohort (× 1000)	Survival (%)	Cohorts Per Season	Total Stocked Per Season (× 1000)	Total Harvested Per Season (× 1000)
1	8	84	400	295	73.8	2.0	800	591
1	10	84	325	238	73.4	1.8	585	426
1	12	98	170	122	72.0	1.0	170	122
4	8	28	280	268	95.9	4.0	1,120	1,074
4	10	28	240	230	95.6	2.0	480	459
4	12	42	140	122	87.3	2.0	280	244

favorably with most field systems for which cost and production data are available (Rhodes et al. 1999). The TPU would therefore appear to be an efficient and economically attractive alternative to other nursery systems and may be suitable for small-scale growers who have access to suitable deployment sites.

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## MOLECULAR CHARACTERIZATION OF QPX (QUAHOG PARASITE UNKNOWN), A PATHOGEN OF *MERCENARIA MERCENARIA*

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**ABSTRACT** The taxonomic status of Quahog Parasite Unknown (QPX), a protist causing disease and high mortality in hardclams (*Mercenaria mercenaria*) from Canada to Virginia, has not been firmly established. The comparison of QPX 18s rDNA sequences with small-subunit rRNA (SSU rRNA) sequences available in the public domain places this organism firmly in the phylum Labyrinthulomycota. With the limited SSU data currently available for the order Labyrinthulida, placement within the family Thraustochytriidae is somewhat more tenuous. Morphological examination also suggests placement in the Labyrinthulomycota. The absence of sagenogenosomes and ectoplasmic nets suggests that QPX is a more primitive member of the phylum. The placement of QPX SSU sequence basal to all available Labyrinthulomycota SSU sequences other than that of *Labyrinthuloides minuta* tends to support this assessment.

**KEY WORDS:** 18s, SSU rRNA, *Mercenaria mercenaria*, disease, protist, hardclam, parasite, QPX

### INTRODUCTION

A protist causing disease and high mortality in hardclams (*Mercenaria mercenaria*) from Canada to Virginia has been observed since the early 1960s and is referred to as Quahog Parasite Unknown (QPX) (Drinnan and Henderson 1963, Whyte et al. 1994, Smolowitz et al. 1998, Ragone Calvo et al. 1998). The taxonomic status of this organism has yet to be firmly established. It was initially identified by Drinnan in the Gulf of St. Lawrence, Canada and morphologically classified as a phycomycete in the order Chytridiales (Drinnan and Henderson 1963). In the late 1980s, disease and resulting high mortality attributable to the same organism occurred in hardclams in a nursery located on Prince Edward Island, Canada (Whyte et al. 1994). Organisms named Quahog Parasite Unknown (QPX) from this outbreak were thought to be the same as those identified by Drinnan and were classified using culture characteristics and ultrastructural examination as members of the Thraustochytriidae or Labyrinthulidae, both of which are in the phylum Labyrinthulomycota in the kingdom Protoctista. In 1997, a parasite similar to QPX caused severe disease and mortality in hardclams from both Provincetown and Duxbury, MA, USA (Smolowitz et al. 1998). This organism continues to be a potent pathogen in hardclams in that area (Smolowitz unpublished data).

Light microscopic examination of infected tissues from diseased clams shows three basic forms in the parasite life cycle. These three forms (as well as zoospores) were identified in cultured QPX. The life cycle of the parasite in clam tissues and in culture has been described as follows (Smolowitz et al. 1998): thalli (single nucleated organisms, 2–10  $\mu\text{m}$  in diameter), sporan-

gia (10–25  $\mu\text{m}$  in diameter), and sporangia containing approximately 40 endospores (immature thalli). A well-defined, membrane-bound nucleus was not observable at the light microscopic level in sporangia until the endospore nuclei were developed. Lysis of the sporangia released endospores that formed new thalli. Organisms were adherent to one another by a mucoid material. Upon exposure to seawater, zoospores were released from the lysed sporangia (Kleinschuster et al. 1998). Although light microscopic examination of the organism in infected tissue and culture showed several forms that suggested placement in the Labyrinthulomycota, it did not suggest more specific taxonomic placement (Whyte et al. 1994; Smolowitz et al. 1998).

In this study, QPX organisms from infected hardclams cultured *in vitro* (Kleinschuster et al. 1998) have been used in a molecular genetic approach to refine the taxonomic placement of QPX. Small subunit ribosomal RNAs (SSU rRNA) have been used extensively for molecular systematic studies of protists (Woese 1987, Sogin 1989, Van de Peer and De Wachter 1997), and a large database of sequences is available from the following sources: Ribosomal Database Project (<http://www.cme.msu.edu/RDP/>; Maidak et al. 1999); RNA secondary structures (<http://pundit.icmb.utexas.edu/>; Gutell 1994); SSU rRNA database (<http://rma.uia.ac.be/ssu/>; Van de Peer et al. 1998). The general structure of rRNA is universally conserved across all taxa thus far examined (Woese 1987, Gutell 1994), and invariant sequence domains are interspersed among partially conserved and rapidly evolving regions (Sogin 1993). This makes the SSU useful for examining the relationships between the most divergent taxa as well as those within a single genus. Taken together, the availability of SSU rRNA sequences

from additional taxa and the existence of conserved and variable regions make it an ideal tool for molecular phylogenetic placement of the QPX organism.

## MATERIALS AND METHODS

### QPX Culture Methods

The *in vitro* propagation of pure cultures of QPX has been presented elsewhere (Kleinschuster et al. 1998). Briefly, cells of the parasite were obtained by aseptic dissection of infected clam tissue. Tissue samples were rinsed five times and minced into 1-mm<sup>3</sup> pieces. Minced samples were transferred to 15-mL centrifuge tubes containing 10 mL of sterile seawater and antimicrobics and incubated for 30 min at 25 °C under ambient air conditions. After this, the seawater was aspirated and replaced with culture medium consisting of MEM Eagle ( $\alpha$  modification, Sigma 0644), 5.1 g/L; CaCl<sub>2</sub> × 2H<sub>2</sub>O, 1.82 g/L; KCl, 0.68 g/L; MgCl<sub>2</sub> × 6 H<sub>2</sub>O, 4.36 g/L; NaCl, 24.26 g/L; MgSO<sub>4</sub> × 7 H<sub>2</sub>O, 3.16 g/L; HEPES, 5 g/L; glucose, 0.5 g/L; heat-inactivated fetal bovine serum (Hyclone), 10% by volume; penicillin, 100 U/mL; and streptomycin, 0.1 mg/mL. The pH of the final medium was adjusted to 7.2 and filter sterilized. The content of each centrifuge tube was transferred to a 25-cm<sup>2</sup> T-flask (Falcon) and incubated at 25 °C under ambient atmosphere. After the initial seeding of flasks, the medium was exchanged every 3–4 days (50%), and cells were routinely subcultured over a period of 6 months.

### Transmission Electron Microscopic Examination

Infected nodules were dissected from hardclam mantles and minced into pieces approximately 1-mm thin in one dimension. Cultured organisms were spun at 5000 X g for 5 min and washed 2 X in sterile sea water. Both QPX cultures and infected tissues were fixed with McDowell's and Trump's 4% formalin/1% glutaraldehyde (McDowell and Trump 1976) and processed for embedding in Spurr's epoxide resin (Dykstra 1993).

### DNA Extraction, PCR, and Sequencing

QPX organisms from two separate cultures (different isolates) were used. Cells were separated from the culture media by winding around a sterile pipette tip, homogenized in two volumes of proteinase K buffer (50 mM Tris-HCl, pH 8.0; 200 mM NaCl; 50 mM EDTA, pH 8.0; 1% SDS, pH 7.2; 1 mg/mL proteinase K; and 0.2% DTT), and incubated at 55 °C overnight (14 hrs). Nucleic acids were obtained by a phenol extraction and ethanol precipitation (Sambrook et al. 1989). Purified nucleic acids were rehydrated in 1X TE buffer (10 mM Tris-HCl, pH 7.5; 1 mM EDTA) to a final concentration of 100–500 ng/ $\mu$ l and stored for short periods at 4 °C and for longer periods at –20 °C.

An approximately 1.7 kb region of 18 S rDNA was amplified using primers 18 e (Hillis and Dixon 1991) and 18 p (Halanych et al., 1995, Halanych et al. 1998). The 50  $\mu$ l amplification reaction contained 100–500 ng template DNA, 2.5 mM MgCl<sub>2</sub>, 20  $\mu$ M dNTP (5  $\mu$ M each nucleotide), 0.4  $\mu$ M of each primer, 5  $\mu$ l 10 × buffer, and 1.5 units Taq polymerase (Promega, Madison, WI). The PCR profile (95 °C/1 min, 47 °C/2 min, 72 °C/2 min) continued for 30 cycles, with an initial denaturation at 95 °C/2 min and a final extension at 72 °C/10 min. Standard negative controls were included with each set of amplifications.

The appropriate PCR product was gel-isolated by the phenol-freeze-fracture method (Bewsey et al. 1991) and precipitated with

1/10 volume NaOAC, pH 5.6 and two volumes 100% ethanol. Sequencing reactions were performed with the original primers and five additional primers, 18 h (Hillis and Dixon 1991), 18 l, 18 m<sub>a</sub>, 18 m, and 18 r (Halanych et al. 1995; Halanych et al. 1998). 120 ng of template DNA was utilized in a 10  $\mu$ l sequencing reaction containing 4.25  $\mu$ l FS dye-deoxy terminator mix (Applied Biosystems, Foster City, CA), 0.33  $\mu$ M primer, and sterile distilled H<sub>2</sub>O. The cycle-sequencing profile (95 °C/30 s, 50 °C/15 s, 60 °C/4 min) continued for 25 cycles. Sequencing reactions were electrophoresed on an ABI 373 automated sequencer (Applied Biosystems, Foster City, CA) following standard protocols. Minimal editing of the 1,780 bp of SSU sequence was performed in Auto Assembler (v.1.4.0, Applied Biosystems). Of the 1,408 bp used for subsequent analyses, 1,187 bp was confirmed within an isolate with sequence from both strands. Sequences from the two isolates were compared in Sequence Navigator (v.1.0.1, Applied Biosystems), where no differences were found between cultures; thus, a single consensus sequence was generated. The sequence has been deposited in GenBank under the accession number AF155209.

### Sequence Alignment and Molecular Phylogenetic Analysis

Sequences for additional taxa were obtained from the small-subunit (SSU) rRNA database (Van de Peer et al. 1998) and GenBank (Benson et al. 1998) (Table 1). Because of controversy concerning the classification of the Thraustochytridae and Labyrinthulidae (Bower 1987, Porter 1990, Azevedo and Corral 1997), as well as protists in general (Margulis et al. 1993), all four SSU rRNA sequences available for the order Labyrinthulida were used

TABLE 1.

Taxa utilized in the phylogenetic analysis of SSU rRNA. Gross taxonomic classification after Margulis et al. (1990); within phylum Labyrinthulomycota after Porter (1990).

	Taxonomic Classification	GenBank #
Kingdom	Protoctista	
Phylum	Labyrinthulomycota	
Class	Labyrinthulea	
Order	Labyrinthulida	
Family	Labyrinthuliidae	
	None available	
Family	Thraustochytridae	
	<i>Labyrinthuloides haliotidis</i>	U21338
	<i>Labyrinthuloides minuta</i>	L27634
	<i>Thraustochytrium kinnei</i>	L34668
	<i>Ulkenia profunda</i>	L34054
	QPX	AF155209
Phylum	Oomycota	
Class	Peronosporomycetidae	
Order	Phythiales	
Family	Lagenidiaceae	
	<i>Lagenidium giganteum</i>	X54266, M54939
Family	Pythiaceae	
	<i>Phytophthora megasperma</i>	X54265, M54938
Class	Saprolegniomycetidae	
Order	Saprolegniales	
	<i>Achlya bisexualis</i>	M32705, J02951
Phylum	Zoomastigina	
Class	Bicoecids	
	<i>Cafeteria roenbergensis</i>	L27633

in the present analysis. In addition, a bicoecid and oomycetes from the orders Phythiales and Saprolegniales were used as outgroups. Following the secondary structure alignment protocol of Kjer (1995), structural information was applied to the raw data, and secondary structure was aligned accordingly. Because only homologous characters are meaningful in a phylogenetic analysis, regions with ambiguous alignment were removed from the data, yielding 1,408 bp of SSU rDNA sequence for all subsequent analyses. Aligned sequences with secondary structure annotations for all taxa used in these analyses are available from RS.

Phylogenetic analyses were performed in PAUP\* (version 4.0, Swofford 1998) and MEGA (version 1.0, Kumar et al. 1993). In the maximum parsimony analyses, all informative nucleotide characters and single base pair indels were equally weighted. As in Kjer (1995), eight multiple base pair insertion/deletion events were treated as binary characters. Insertions of variable length between taxa, or with variable sequence between taxa, were coded and then down-weighted so that each indel was equivalent to a single character. For distance-based methods, indels were treated as missing data. PAUP\* (version 4.0, Swofford 1998) was used to employ an exhaustive search in a maximum parsimony analysis to find minimum length trees. A bootstrap 50% majority rule consensus tree was constructed with a heuristic search and 1,000 replicates. A maximum likelihood tree was constructed under the Hasegawa-Kishino-Yano (1985) model, with nucleotide frequencies and transition/transversion ratios estimated from the data and a substitution rate following a gamma distribution with shape parameter estimated from the data. A bootstrap 50% majority rule consensus tree was constructed with a heuristic search and 100 replicates. MEGA was used to construct a neighbor-joining tree with genetic distances estimated under the Kimura 2-parameter model (Kimura 1980). Bootstrap values were calculated with 1,000 replicates.

## RESULTS

### Transmission Microscopy

Electron microscopic examination of cultured QPX and QPX-infected clam tissues showed that thalli and early sporangia contained electron-dense lipid bodies, mitochondria with tubular cristae, large vacuoles, and a perinuclear golgi apparatus. Membrane-bound granular inclusions often contained dark lipid-like material. Endospores and thalli contained distinct nuclei with prominent nucleoli. Sporangia contained numerous nuclei with prominent nucleoli and less distinct chromatin (Fig. 1). The cell wall of some endospores and occasional thalli, showed scale-like, laminated walls. Scaled walls were not seen in medium to large thalli or sporangia. Sagenogenetosomes and ectoplasmic nets were not identified in any form.

### SSU rDNA Sequence Analysis

An exhaustive parsimony search resulted in a single most-parsimonious tree (Fig. 2a). Distance and maximum likelihood methods produced trees with similar topologies (Fig. 2b and c, respectively). With all three methods, the four Thraustochytriidae and QPX formed a monophyletic group with a high bootstrap value (99%, 100%, 100%). *Labyrinthuloides minuta* was basal within the group, and QPX was the next most basal taxa, with a 100% bootstrap confidence. The branch length for *T. kinnei* was fairly long compared to all others in the family. Genetic distances for the Thraustochytriidae are reported in Table 2. QPX SSU

rDNA sequence is most divergent from that of *T. kinnei* (15.2% sequence divergence) and least divergent from that of *U. profunda* and *L. minuta* (10.7%).

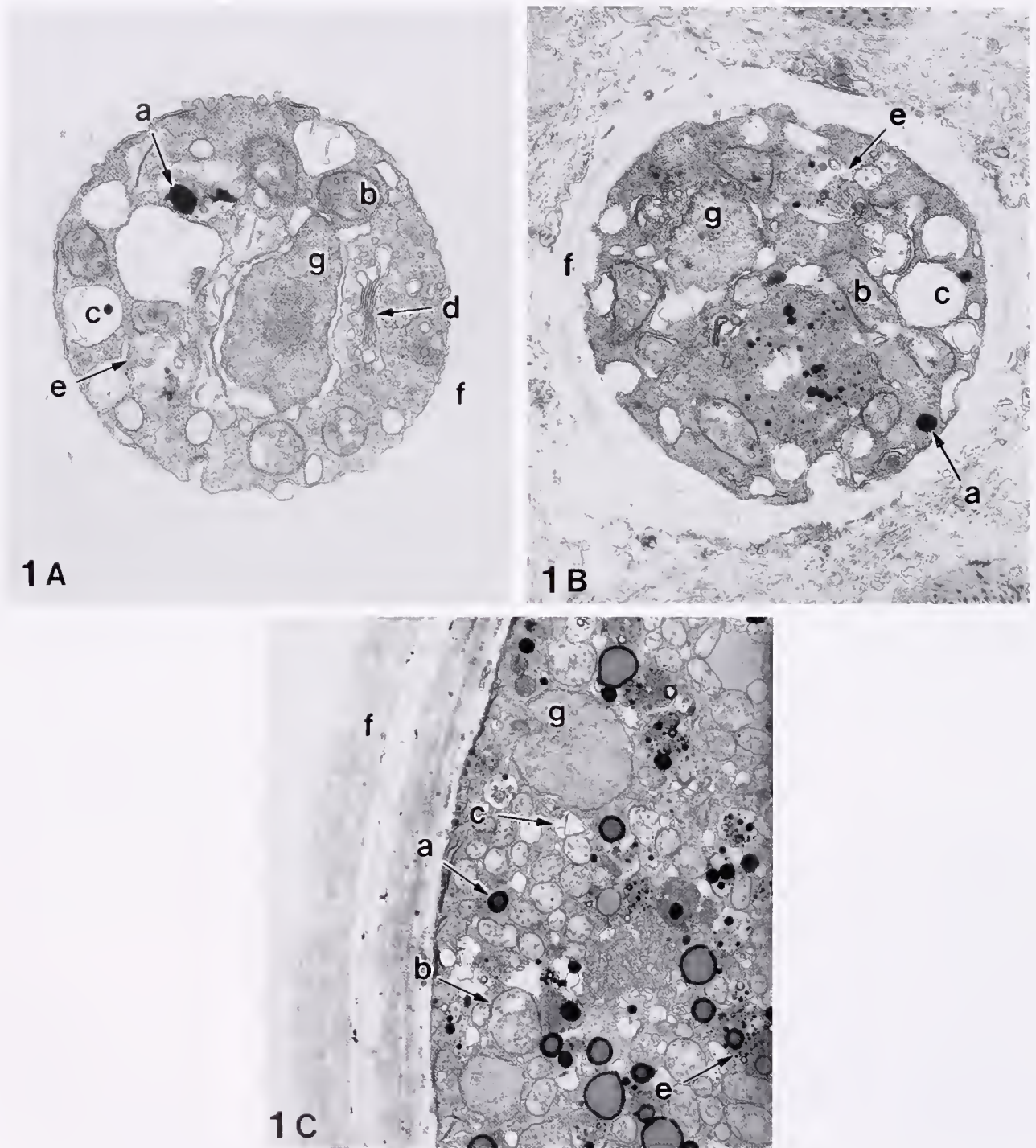
## DISCUSSION

Thraustochytrid-like organisms are common in the marine environment and in the palatal cavity of bivalves. The high concentration of QPX organisms within the infected nodules of the clam's mantle tissue greatly facilitated the extraction of QPX from the clams and the subsequent development of pure cultures of QPX. In addition, the genetic sequence obtained directly from PCR product was clearly from a single source, with no evidence for contamination of the cultures. The observation that the sequences from both isolates were identical is further evidence that the SSU rDNA sequence obtained is that of QPX. It is likely that the QPX organisms isolated from diseased Massachusetts clams were the same as those identified in diseased Canadian hardclams. However, there are some differences. Grossly, infected Massachusetts clams often display obvious large inflammatory nodules in the mantle tissue not seen in the Canadian clams. Sagenogenetosomes, commonly seen at the ultrastructural level in preparations of Labyrinthulomycota (Porter 1990), have not been identified in Massachusetts QPX organisms. Other differences include the lack of growth on the fungal medium PDA and slightly different staining characteristics using various histological stains (Whyte et al. 1994; Smolowitz et al. 1998). These findings indicate that the Massachusetts QPX may be a slightly different organism from the one seen in Canada. Alternatively, these differences may reflect only strain differences or variation in methods between laboratories.

Another QPX-like organism was identified in diseased hardclams in Virginia, U.S.A. in 1998 (Ragone Calvo et al. 1998). Morphologically, this organism seems similar to that seen in Massachusetts and Canada. Gross lesions (nodules in the clam mantles) were not identified in infected clams from Virginia. Mortality resulting from infections did occur in groups with a high prevalence of infection (Ragone Calvo et al. 1998), indicating that this is a pathogenic organism. The relationship of the organism identified in Virginia to the organism identified in Massachusetts and Canada is being investigated.

Most morphological features identified ultrastructurally in cultured QPX organisms and in clam tissues infected with the organism were similar to those seen by Whyte et al. (1994) (Kleinschuster et al. 1998; Smolowitz et al. 1998) and are similar to those identified in other members of the phylum Labyrinthulomycota. Interestingly, cell walls varied in diameter between different QPX organisms in the same culture as seen by Whyte et al. (1994). In addition, at an ultrastructural level, the walls of most thalli and sporangia of QPX from Massachusetts appeared to be fibrogranular; thin compacted scaled walls were noted only in some endospores. Such findings are at odds with descriptions of other organisms in the phylum Labyrinthulomycota (Porter 1990).

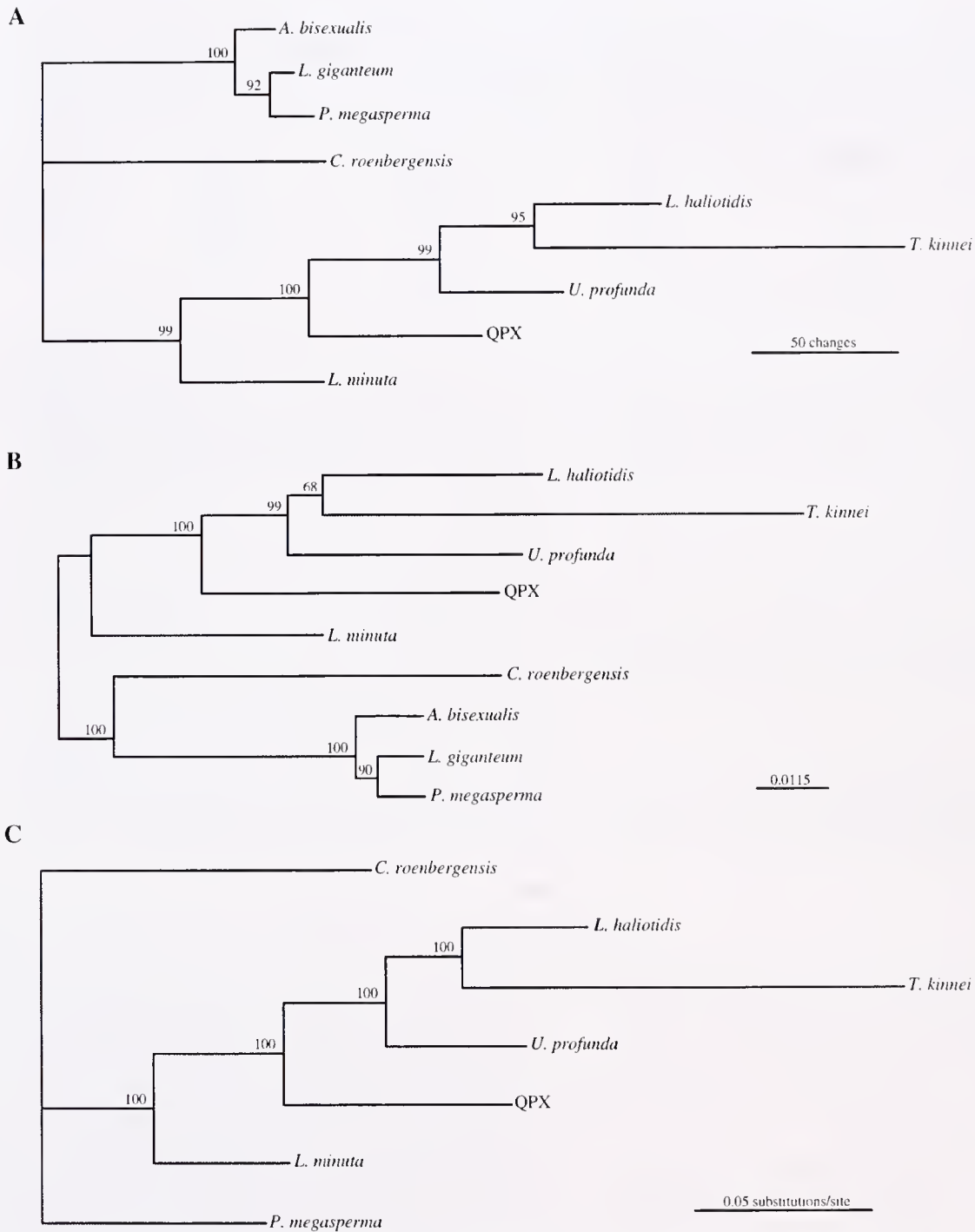
Sagenogenetosomes and ectoplasmic nets were not identified in cultured QPX organisms or in the infected tissues. However, production of mucoid material, which bound thalli and sporangia of all sizes together, was seen commonly both in cultured organisms and in infected tissues (Smolowitz et al. 1998). The occurrence of sagenogenetosomes and the production of ectoplasmic nets is reportedly common to all members of the phylum Labyrinthulomycota, except for the genus *Althornia* (Porter 1990), which does not



**Figure 1.** Electron micrographs of Quahog Parasite Unknown (QPX) organisms. **A.** A cultured QPX cell (23,800X). **B.** A QPX cell in infected clam tissue (15,600X). **C.** A portion of a young cultured QPX sporangium (8,500X). (a. lipid bodies, b. mitochondria with tubular cristae, c. vacuoles, d. perinuclear golgi, e. granular inclusions, f. fibrogranular cell wall, g. nuclei).

form sagengenosomes, and a thraustochytrid isolated from the clam *Ruditapes decussatus*, in which neither sagengenosomes nor ectoplasmic nets were observed (Azevedo and Corral 1997). Porter (1990) suggested that *Althornia* may be a primitive member of the Labyrinthulomycota; whereas, Azevedo and Corral (1997) suggest that their unnamed species be classified as a thraustochytrid within the Labyrinthulomycota without commitment to a generic designation.

The molecular sequence analyses performed here clearly places QPX within the Labyrinthulomycota. With all three phylogenetic methods, the placement of QPX is strongly supported (100%) by bootstrap analyses. Because SSU rRNA sequences from the family Labyrinthuliidae are unavailable, we cannot unequivocally place QPX within the Thraustochytriidae. The tree topologies are similar to that derived in Leipe et al. (1996), where *Labyrinthuloides minuta* is basal to the other member of the genus, *Labyrinthuloides*



**Figure 2.** Phylogenetic placement of QPX in relation to other Thraustochytriidae based on SSU rDNA nucleotide sequence data. **A.** Maximum parsimony analysis. Branch lengths represent number of changes, bootstrap values for 1,000 replicates over nodes. Outgroups: *Cafeteria roenbergensis*, *Achlya bisexualis*, *Phytophthora megasperma*, *Lagenidium giganteum*. **B.** Distance analysis. Neighbor-joining algorithm where branch lengths represent nucleotide divergence under the Kimura two-parameter model, bootstrap values for 1,000 replicates over nodes. **C.** Maximum likelihood analysis. Branch lengths as a percentage probability of change, bootstrap values for 100 replicates over nodes. Outgroups as in A.

*haliotidis* and all the other established members of the Thraustochytriidae. They suggest that *L. minuta* may represent a species of the genus *Labyrinthula*, in the family Labyrinthuliidae, as suggested in the original description (Watson and Raper 1957). The similar genetic distances between *L. minuta*, QPX, and *U. profunda* (roughly 10.7%, Table 2) do not clarify the family place-

ments. Because assignment of *L. minuta* to the family Thraustochytriidae on the basis of molecular data is somewhat tenuous, the positioning of QPX within the family also awaits the determination of SSU rDNA sequences for other species within the Labyrinthulomycota.

Morphological examination of Massachusetts QPX suggests,

TABLE 2.

Genetic distances and standard errors between members of the Thraustochytridae from SSU rDNA sequence data. Distances, estimated under the Kimura 2-parameter algorithm (Kimura 1980), in bold type in upper right hand corner of matrix, standard errors in plain text in lower left corner.

	<i>L. haliotidis</i>	<i>T. kinnei</i>	<i>U. profunda</i>	<i>L. minuta</i>	QPX
<i>L. haliotidis</i>		<b>0.1227</b>	<b>0.0814</b>	<b>0.1265</b>	<b>0.1180</b>
<i>T. kinnei</i>	0.0101		<b>0.1355</b>	<b>0.1684</b>	<b>0.1515</b>
<i>U. profunda</i>	0.0080	0.0106		<b>0.1076</b>	<b>0.1070</b>
<i>L. minuta</i>	0.0103	0.0121	0.0093		<b>0.1068</b>
QPX	0.0099	0.0114	0.0093	0.0093	

but does not verify, membership in the phylum Labyrinthulomycota. In addition, the lack of sagenogenetosomes, the unusual cell wall morphology, and the lack of ectoplasmic nets make placement of this organism into this phylum difficult. On the other hand, rDNA identification strongly suggests inclusion of QPX in the phylum Labyrinthulomycota and even suggests a relationship to members of the family Thraustochytridae. The unusual morphological findings in QPX organisms, taken together with the distinctive rDNA results, indicate QPX may represent a primitive form of the Labyrinthulomycota. Certainly, much more work needs to be done on the rDNA identification of members of this phylum. As in this study, the correlation of rDNA finding with morphological descriptions will help define this group of organisms, especially those responsible for important diseases, more thoroughly.

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## SEASONAL VARIATION OF TRACE ELEMENT AND ISOTOPIC COMPOSITION IN THE SHELL OF A COASTAL MOLLUSK, *MACTRA ISABELLEANA*

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**ABSTRACT** Shells of the bivalve *Mactra isabelleana* taken from marine and estuarine environments along the coast of Argentina, show a cyclicity in carbon and oxygen isotope ratios from analysis of incrementally grown shell carbonate that reflect seasonal changes in temperature and salinity in the coastal waters. Trends in trace element data, from the same sample intervals, can be compared to variations in the isotope data. In the estuarine shell, there are cyclic variations in strontium, magnesium, and barium (Sr, Mg, and Ba) that match closely the variation in  $\delta^{18}\text{O}$  and  $\delta^{13}\text{C}$ . The highest concentration of these elements are coincident with  $\delta^{18}\text{O}$  peaks and approach mean values for both isotopes and trace elements from the marine shell. However, there is a lack of statistically significant correlations between the trace element and isotope data, which suggests that the patterns of trace element variations cannot be only related to environmental parameters, although predominantly controlled by them. There is no correlation between isotopes and trace elements in the marine shell, which suggests physiological effects, rather than environmental, control the incorporation of the trace elements into the shell carbonate.

**KEY WORDS:** *Mactra*, trace elements, stable isotopes, Argentina

### INTRODUCTION

Two methods can be employed to determine water temperature and salinity variations from mollusk shells. One is to study species assemblages, the ratios of number of species to number of specimens, but this technique is restricted to species that are limited to a clearly defined temperature and salinity range. The second method is to study the chemical composition of the shells themselves, which is equally valid for both euhaline and euryhaline species. Here, we have adopted the second approach and compared chemical data from incrementally grown shell carbonate from the modern marine bivalve *Mactra isabelleana* to investigate the sensitivity of this species as an environmental indicator. *Mactra* is highly adaptable in a range of seawater conditions and occurs throughout late Quaternary coastal deposits along part of the Argentinean coast (Aguirre et al. 1995), although it has a much wider modern day distribution between Rio de Janeiro and Golfo San Matias, Argentina (Rios 1994). It has great potential as a palaeosalinity indicator if the conditions in the organism's environment are recorded in the chemical composition of the shell. To test this, we have taken samples from two different near-shore environments, an open marine, euhaline coastal zone and a typically mixed, poly-euhaline, littoral area that forms part of a major estuary and is influenced by both fresh and marine water. Seasonal variations (salinity and temperature) in these two environments are reflected in changes in  $^{18}\text{O}/^{16}\text{O}$  and  $^{13}\text{C}/^{12}\text{C}$  isotope ratios along the growth axis of the shells (Aguirre et al. 1998), which are compared here to trace element data (strontium, magnesium, barium, manganese, zinc, lead, and cadmium [Sr, Mg, Ba, Mn, Zn, Pb, Cd]) from the same samples.

### STUDY AREA AND FAUNA

*Mactra* is found in modern accumulations along a 350 km stretch of coast between La Plata and Mar del Plata, the northeastern coastal area of Buenos Aires Province (Argentina) (Fig. 1).

The oceanic littoral area from Punta Rasa to Mar Chiquita forms part of the Argentine Zoogeographic Province, which extends from Rio Grande do Sul (Brazil, 28 °S) to Golfo Nuevo (Argentina, ca. 43 °S). In the modern environment, *Mactra* lives in the near-shore littoral zone including the Río de la Plata estuary and in the oceanic sector beyond Punta Rasa. Water salinity corresponds mostly to the fluviomarine (mixohaline) zone in the estuary (8–18‰) and grades to polyhaline (18–30‰) toward the coast. At Punta Rasa, the salinity is mainly polyeuhaline (24–30‰). Salinity reaches oceanic values of 35‰ off Mar del Plata (Aguirre 1990).

*Mactra* is common in soft sediments along the shallow marine infralittoral zone where salinity is variable. It is relatively immobile, but can burrow rapidly into soft sandy and silty sediment. Large numbers of *Mactra* live in the polyhaline zones of the Río de la Plata estuary where the largest shells occur, although it is also a component of the assemblages south of Punta Rasa (Aguirre 1990; Aguirre and Whatley 1995). Modern shells were collected from two different near-shore environments: an open marine, euhaline to polyeuhaline coastal zone off Mar del Plata and a typically mixed, polyeuhaline, littoral area off Punta Rasa (Fig. 1).

The shells from these environments are noticeably different in size. Shells from the estuary are on average much larger, typically 6–7 cm from the apex along the growth axis, as opposed to 2–3 cm in shells from the open marine site. The shells are aragonite. Acetate peels of polished sections show that the shell consists of an outer crossed-lamellar unit, composed of concentric layers that extend into the umbonal and hinge areas, and an inner complex crossed-lamellar unit, composed of layers generally parallel to the shell surface and extending to the pallial line. The pallial myostracum separating these layers is absent in *M. isabelleana* or is very thin and indistinct, as in other species of the same genus (Taylor et al. 1969). Accretionary shell growth takes place by addition of calcium carbonate to the shell margin outside the pallial line and simultaneous shell thickening within the pallial line. This produces fine growth ribs. Macroscopic banding can be seen in radial shell

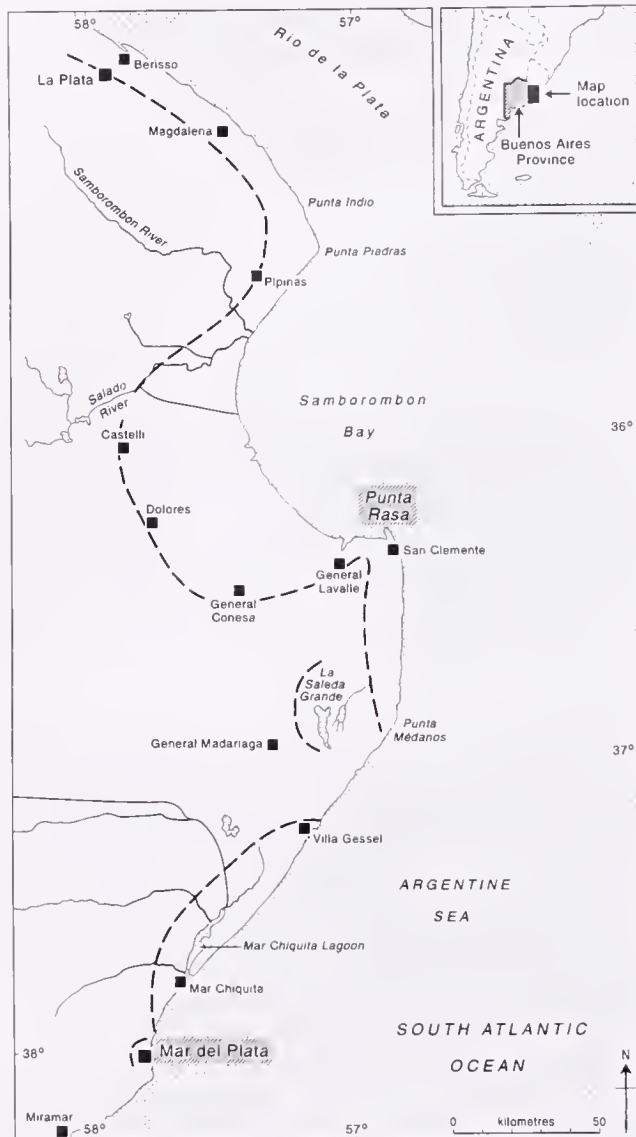


Figure 1. Location map showing the position of two collection localities: Punta Rasa in the Río de la Plata estuary and an open marine site off Mar del Plata, Buenos Aires Province, Argentina.

cross section and on the interior surface of the shell. The banding forms alternations of brown (translucent in transmitted light) and white (opaque in transmitted light) layers. Each couplet of bands is thought to represent 1 year's shell growth (Jones et al. 1983; Lutz and Rhoads 1980). In *M. isabelleana*, the brown bands are related to periods of slow shell growth during cold (winter) temperatures, and the white bands represent warmer (summer) temperatures.

#### SAMPLING AND ANALYSIS

Pristine shells, showing no signs of alteration, from the two environments were collected after death and were analyzed for  $^{18}\text{O}/^{16}\text{O}$  and  $^{13}\text{C}/^{12}\text{C}$  ratios as well as Sr, Mg, Ba, Mn, Zn, Pb, and Cd concentration variations. For the isotope measurements, samples were taken at 1-mm intervals along the exterior surface from the umbonal area toward the ventral margin using a 1-mm diameter drill. Material from the first few revolutions of the drill was discarded to avoid the periostracum, and drilling stopped before encountering the inner nacreous layer. The sampled mate-

rial was roasted *in vacuo* to remove organic material, and ca. 100  $\mu\text{g}$  portions analyzed in a VG Isocarb + Optima mass spectrometer together with similarly sized samples of a laboratory calcite standard. Results were corrected for isobaric effects and are reported in the usual  $\delta^{13}\text{C}$  and  $\delta^{18}\text{O}$  notation in per mil (‰) deviation from VPDB, based on calibration of the laboratory standard against NBS-19. Analytical precision (1 SD), based on the laboratory standard, for this method is typically  $< 0.07\%$  for both  $\delta^{13}\text{C}$  and  $\delta^{18}\text{O}$ .

Trace elements concentrations were determined in the shells by laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS). Individual laser ablation craters, approximately 20  $\mu\text{m}$  in diameter, are considerably smaller than the holes drilled for the isotope analysis. Thus, three LA-ICP-MS analyses, spaced at approximately 0.10 mm, were taken from a trough cut parallel to the isotope drill holes, which were then averaged to give a spatial resolution similar to the isotope analyses.

LA-ICP-MS was performed using a frequency quadrupled Nd:YAG laser, operating at 266 nm (ultraviolet), coupled to a VG PlasmaQuad II + ICP-MS with a modified vacuum system similar to the commercially available "S"-option. During the analysis of both samples and reference materials, the laser was fired at 5 Hz with a power of approximately 1 mJ, and each acquisition took approximately 30 seconds. The spectrometer was operated in peak jumping mode, with short dwell times being used for the more abundant isotopes of Ca and Sr.

Calibration was achieved using the NIST SRM 610 soda-line glass taking certified values for Sr and concentrations for the remaining elements from Pearce et al. (1997). The minor  $^{44}\text{Ca}$  isotope was used as the internal standard, and analyte isotopes were  $^{25}\text{Mg}$ ,  $^{88}\text{Sr}$  and  $^{138}\text{Ba}$ . CaO was assumed to be present in the shells at a constant 55.5 weight percent for internal standardization, which compensates for any variation in the ablation yield between different analyses. Calibration and calculation of concentration in the unknowns was done off line. Full details of the instrumentation and calibration methods are given in Perkins and Pearce (1995) and Perkins et al. (1997). Lower limits of detection (at three standard deviations on the background) were calculated at Mg = 1.4 ppm, Sr = 0.31 ppm, Ba = 0.09 ppm, Mn = 0.5 ppm, Cd = 1 ppm, Pb = 0.2 ppm, and Zn = 1 ppm. Accuracy and precision are both typically around  $\pm 5\%$  (Pearce et al. 1992).

#### RESULTS

The time series  $\delta^{13}\text{C}$ ,  $\delta^{18}\text{O}$ , Sr, Mg, Ba, Mn values are plotted against sample distance in mm from the umbonal area toward the ventral margin in Figures 2a,b. The distance of each sample from the umbo is related to time (i.e., the mollusk started growing at 0 mm). The age of each *Macrta* specimen can be estimated from growth increments of the shell identified by pairs of brown and white bands, which can only be seen in transmitted light. Each pair of bands represents 1 year's growth. The position of these bands corresponds to zones marked on Figures 2a,b and labeled 1–9 (representing 4 or 5 years) from the estuary (Fig. 2a) and 1–3 (representing 2 years) from the open marine site (Fig. 2b). The juvenile stage in both specimens, represented by zone 1, is where the most rapid growth occurred. In the estuarine shell, this is represented by 15 mm of material, as opposed to less than 7 mm in the subsequent bands.

##### The Estuarine Shell

The isotope values and some of the trace element concentrations (Sr, Mg, Ba) in the shell from the estuary exhibit cyclic

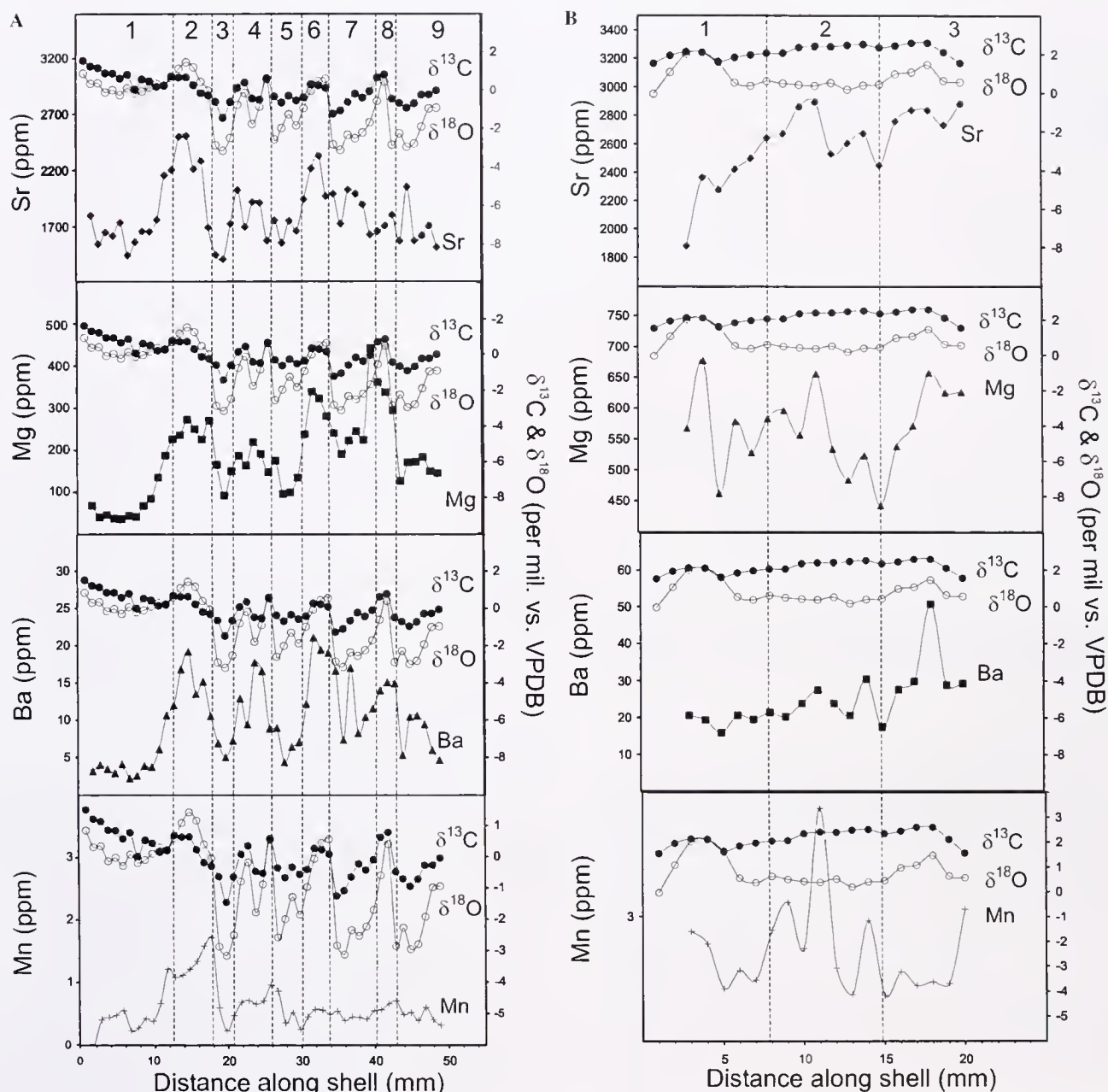


Figure 2.  $\delta^{13}\text{C}$  and  $\delta^{18}\text{O}$  values and Sr, Mg, Ba, Mn concentrations measured at intervals of 1 mm along the external surface from the umbonal area toward the ventral margin of two shells from estuarine (a) and marine (b) settings. The zones marked on Figures 2a and b correspond directly to the brown and white bands on the shells (i.e., in Figure 2a zones 1, 3, 5, 7, and 9 correspond with the brown bands and zones 2, 4, 6, 8 to the white; in Figure 2b zones 1 and 3 correspond with the white bands and zone 2 to the brown band). Each couplet of bands represents one years growth with the brown bands representing the cold (winter) temperatures and the white bands representing the warmer (summer) temperatures.

patterns of variation (Fig. 2a). The cyclicity in the  $\delta^{13}\text{C}$  and  $\delta^{18}\text{O}$  values is synchronous ( $R^2 = 0.70$ ). The isotope data form nine peaks and troughs (over 49 mm), which correspond directly to the spatial distribution of the banding in the shell. Zone 1 represents the juvenile stage from 0 to 12 mm and contains only one, low-amplitude cycle. Zones 3, 5, 7, and 9 have low  $\delta^{13}\text{C}$  and  $\delta^{18}\text{O}$  values and coincide with the brown (cold season) bands. The intervening zones (2, 4, 6, and 8) correspond to the white (warm season) bands. Each pair of zones (e.g., 2 and 3, etc.) forms a cycle. The  $\delta^{18}\text{O}$  cycles have amplitudes of up to 4.5‰ and take the form of gradual increases followed by a sudden decrease in  $\delta^{18}\text{O}$ .

The most obvious example is between zones 6 and 7, the fall from the maximum  $\delta^{18}\text{O}$  value, to the minimum occurs over 1 mm, and the subsequent increase to the next maximum occurs over 7 mm. The  $\delta^{13}\text{C}$  cycles have amplitudes of about 1‰. There is no statistically significant correlation between trace element concentrations and the isotope data (e.g., Fig. 3), although there seems to be a clear visual match. For example, peaks in the Sr, Mg, Ba data at 15, 23, 34, and 42 mm approximately coincide with maximum  $\delta^{18}\text{O}$  values in zones 2, 4, 6, and 8. The maximum concentrations of Sr, Mg, and Ba reach 2506, 339, and 21 ppm, respectively (Table 1).

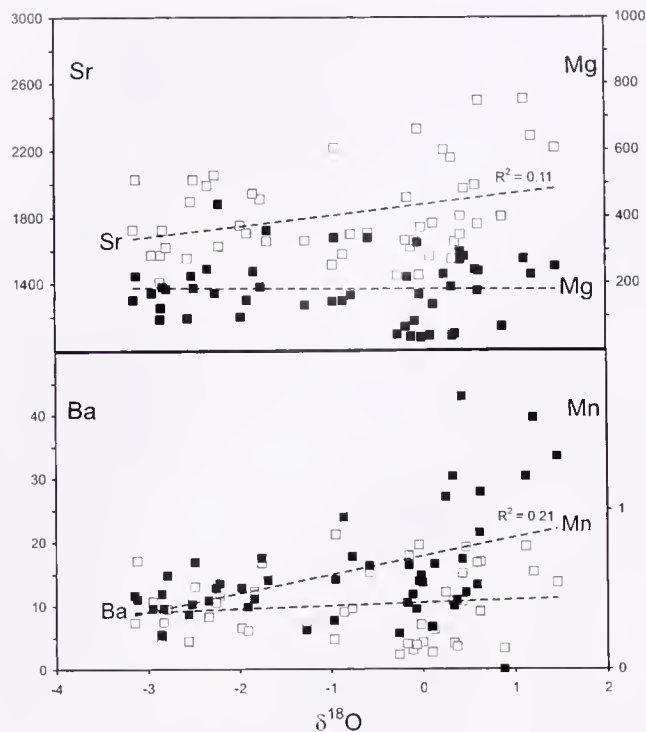


Figure 3. Sr, Mg, Ba and Mn concentrations versus  $\delta^{18}\text{O}$  from the estuarine shell.

#### The Marine Shell

The shell used here shows a weak cyclic pattern of variation in  $\delta^{13}\text{C}$  and  $\delta^{18}\text{O}$  values. Zone 1 coincides with the juvenile stage from 0–8 mm and contains the most positive  $\delta^{18}\text{O}$  values. Zone 2 (translucent) and zone 3 (opaque) represent one poorly defined  $\delta^{18}\text{O}$  cycle. The variations are less distinctive than in the shell from the estuary, the amplitude of the cycles is lower in both  $\delta^{13}\text{C}$  (1‰), and  $\delta^{18}\text{O}$  (2‰), and all values are positive. There is no statistical or visual match between the isotope and trace element data in this shell. Sr shows an increase from 1,800 ppm in the juvenile portion of the shell to values exceeding 2,600 ppm. Mg, Ba, and Mn show irregular patterns, although it is notable that the average concentration data (with the exception of Zn and Cd) in the marine shell are higher than the data for the estuarine shell (Table 1). The means of the peak values for Sr, Mg, and Ba (approximately at 15, 23, 34, and 42 mm) in the estuarine shell are only slightly lower than for the means of the whole marine shell.

### INTERPRETATION

#### Isotopes

The  $^{18}\text{O}/^{16}\text{O}$  ratio in the shell carbonate is dependent on the temperature of precipitation and the  $\delta^{18}\text{O}$  value of the water in which the organism lived (Mitchell et al. 1994, Perrier et al. 1995, Cohen and Tyson 1995, Jones and Quitmyer 1996, Marshall et al. 1996, Hendry and Kalin 1997). The effects of temperature changes on the isotopic fractionation associated with the formation of the shell aragonite may be most important in the marine shell. The temperature of seawater in the area varies between 19–22 °C (warm season) and ~10 °C (cold season). Assuming the shell aragonite formed in isotopic equilibrium with seawater, the difference in the  $\delta^{18}\text{O}$  values of the shell between seasons would be ~2–3‰

(e.g., using the equation of Craig [1965]) this is within the range of values observed in the shell from the open marine site (~2‰), but smaller than the amplitude of the largest  $\delta^{18}\text{O}$  cycle of the shell from the estuary (~4.5‰), suggesting that temperature variations alone cannot account for the cycles in the estuarine shell.

The second major source of  $\delta^{18}\text{O}$  variation is mixing of seawater with freshwater. Great variability occurs in the composition of coastal marine waters, which are commonly around 0–2‰ relative to SMOW, although can reach +4‰ in hypersaline areas (Lloyd 1969) because of evaporative loss of  $\text{H}_2^{16}\text{O}$ . Depletion (of  $^{18}\text{O}$ ) occurs because of the mixing of coastal precipitation and continental runoff waters and  $\delta^{18}\text{O}$  values can be as low as -6‰ (Lloyd 1964). Seasonal mixing of seawater and freshwater will cause depletion in  $\delta^{18}\text{O}$  in the estuarine shell during annual floods. Peaks and troughs in the  $\delta^{18}\text{O}$  data match with the color banding that has been interpreted as caused by seasonal differences (Aguirre et al. 1998). The majority of the  $\delta^{18}\text{O}$  cycles in Figure 2a are characterized by an abrupt decrease in  $\delta^{18}\text{O}$  within a 1-mm interval between two adjacent samples. This must reflect an abrupt change in environmental conditions from summer (warm season) to winter (cold season), perhaps representing the autumn flood or differential seasonal growth rates. The subsequent gradual rise in  $\delta^{18}\text{O}$  values probably reflects the general decrease in supply of freshwater through the winter. It is interesting to note that the average  $\delta^{13}\text{C}$  and  $\delta^{18}\text{O}$  of the two shells are significantly higher in the marine specimen. It seems likely that the most enriched  $\delta^{18}\text{O}$  values from the estuarine shell (maximum  $\delta^{18}\text{O}$  for the five peaks in zones 2, 4, 6, 8, and 9 =  $0.6 \pm 0.6\text{‰}$ ) represents a return to more typical marine conditions, because the mean  $\delta^{18}\text{O}$  for the marine shell is within error at 0.8‰.

The variation in  $^{13}\text{C}/^{12}\text{C}$  ratios in shell carbonate is dependent on the source and amount of marine bicarbonate. Bicarbonate is derived from photosynthesis and respiration by marine organisms, the addition of terrestrial organic material, and exchange with atmospheric  $\text{CO}_2$ .  $\delta^{13}\text{C}$  is relatively insensitive to temperature changes. Typical  $\delta^{13}\text{C}$  values for coastal marine carbonate precipitated in isotopic equilibrium with ambient seawater generally fall between -2 and +4‰. In the estuarine shell, depleted  $\delta^{13}\text{C}$  values are coincident with depleted  $\delta^{18}\text{O}$  values and are directly related to freshwater input, which brings a greater amount of  $^{12}\text{C}$  from terrestrial plant material, via the Río de la Plata river during winter flood events. This is consistent with other studies (e.g., Klein et al. 1996), which have shown that the  $^{13}\text{C}$  content of total dissolved inorganic carbonate (TDIC) of marine water in coastal settings is directly related to salinity. The mean  $\delta^{13}\text{C}$  value from the marine shell (= 2.1‰) is typical of coastal marine waters.

#### Trace Elements

In most calcareous shells, it is unclear where the trace elements reside or in what form they are present (Rosenberg 1980). They could occur in the mineral or organic portions, in shell inclusions associated with detrital clay particles (Eisma et al. 1976), or absorbed onto the shell surface (Foster and Chacko 1995). The amount of elemental incorporation into shells is dependent on a variety of factors, including such environmental effects as salinity and temperature, although studies by Lorens and Bender (1980), Lorens (1981), and Mucci (1986), among others, have shown that kinetic and metabolic effects can exert a greater influence. For example, fast shell growth can result in Sr/Ca ratios that exceed

TABLE 1.

Mean isotope and trace element concentration data from the estuarine and marine *Macra* shell.

Mean Value	Marine Mean Value (1 SD)	Estuarine Mean Value (1 SD)	Estuarine Max. Value	Estuarine Min. Value
$\delta^{18}\text{O}$ (‰)	0.8 ( $\pm 0.6$ )	-0.9 ( $\pm 1.4$ )	1.5	-2.9
$\delta^{13}\text{C}$ (‰)	2.1 ( $\pm 0.3$ )	0.0 ( $\pm 0.7$ )	1.6	-1.4
Sr (ppm)	2601	1819	2506	1444
Mg (ppm)	567	183	339	41
Ba (ppm)	25	10	21	2
Mn (ppm)	2.7	0.6	1.7	0
Zn (ppm)	17	31		
Pb (ppm)	2.2	1.5		
Cd (ppm)	0	0.5		

equilibrium values, and higher rates of metabolic pumping of Ca can result in lower than average Sr/Ca (Amiel et al. 1973).

In *Macra*, there is no statistically significant correlation between isotopes and concentration of trace elements in either the marine or estuarine shell along incremental growth intervals, suggesting that environmental influences are not the only cause of trace element variation. Interestingly, there is a good visual match between isotopes and Sr, Mg, and Ba in the estuarine shell. The mean trace element concentrations in the marine shell are all much higher than those of the estuarine shell. The lowest concentrations of trace elements in the estuarine shell coincide with the lowest  $\delta^{13}\text{C}$  and  $\delta^{18}\text{O}$  values, which suggests that addition of river water is the predominant, but probably not the only, environmental control. Therefore, troughs in the elemental data are purely a function of ion availability, because the concentration of these elements is much lower (e.g., two orders of magnitude for Sr) in freshwater. However, the mean trace element concentrations from the marine shell are all higher than the mean trace element concentration of the  $\delta^{18}\text{O}$  peaks (at 15, 23, 34, and 42 mm along the shell) in the estuarine shell (Table 1), despite the fact that the peaks in  $\delta^{18}\text{O}$  in the estuarine shell are thought to represent a change back to normal marine salinities.

It is less clear where the other elements (e.g., Mn, Zn, Pb, Cd) reside. Mn can partly replace Ca (White et al. 1977) in the aragonite lattice, although it equally can be found bound within pigments. In the estuarine shell, there are minor peaks in Mn in zones 2 and 4 (Fig. 2a), which coincide with summer conditions. Mn in the marine shell is highly variable, although the greatest peaks in

zone 2 occur during summer growth. Other trace elements are all in low concentrations, although Zn and Cd are higher in the estuarine shell; whereas, Pb is slightly higher in the marine sample.

### CONCLUSIONS

The two shells of *Macra*, taken from extremes in Argentinean near-shore environments, show a cyclicity in  $\delta^{13}\text{C}$  and  $\delta^{18}\text{O}$ , which reflects seasonal changes in the coastal waters. This conclusion is supported by the amplitude and form of the cyclicity in the isotope data. There are trends in the trace element concentration data that can be compared with variations in the isotope data. In the estuarine shell, there are cyclic variations in Sr, Mg, and Ba that visually match the variation in  $\delta^{18}\text{O}$  and  $\delta^{13}\text{C}$ . The highest concentrations of these elements are coincident with  $\delta^{18}\text{O}$  peaks and approach mean values for both isotopes and trace elements from the marine shell. The lack of statistically significant correlations between the trace elements and isotope data suggests that the patterns of trace element variations cannot be directly related to single environmental parameters, and variations are more likely to represent a combination of environmental and physiological variables. In the estuarine shell, the dominant effect seems to be dilution of marine water by river water, and this far outweighs other causes of variation that are seen in the marine shell.

*Macra isabelleana* shells, thus, have great potential as palaeosalinity indicators for the southern hemisphere, off the Atlantic coast of South America, which is especially significant, because palaeoclimate records tend to be scarce or contradictory in this area (Aguirre et al. 1998).

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## THE ROLE OF BIOTIC INTERACTIONS IN JUVENILE MORTALITY OF THE COCKLE (*CERASTODERMA EDULE* L.): FIELD OBSERVATIONS AND EXPERIMENT

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**ABSTRACT** Recurrent drastic mortalities of juveniles in a population of the edible cockle, *Cerastoderma edule*, from Western Brittany, prompted a study of the role of biotic interactions. A predation hypothesis was tested through a field experiment using enclosure cages, in parallel with field observations of the natural population. Over 1 month, the juvenile mortality rate outside of the cages was more than 40%. Selective predation on juveniles below 11 mm in length was responsible for more than 85% of this mortality. Juveniles of the green crab, *Carcinus maenas*, were recognized as the most important predators of the young cockles. Intraspecific competition did not influence survival or growth of the cockle spat, despite the relatively high density of the experimental population i.e. more than 800 individuals.m<sup>-2</sup>.

**KEY WORDS:** *Cerastoderma edule*, cockle, spat, mortality, predation, competition

### INTRODUCTION

In temperate ecosystems, populations of the edible cockle, *Cerastoderma edule* (L.), are frequently affected by strong seasonal or interannual density fluctuations. During the benthic life span, critical stages may occur in all size classes, but the factors influencing the mortality of the early stages are major determinants of these fluctuations. Among the multiple sources of mortality, biotic interactions must be taken into account. These biological processes are related to competition and adult-larval interactions (André and Rosenberg 1991; Jensen 1993; Bachelet et al. 1992), or to predation (Hancock 1970; Brown and O'Connor 1974; Hylleberg et al. 1978; Sanchez-Salazar et al. 1987(a, b); Kamermans and Huitema 1994).

Critical stages affecting the population structure of the cockle were described as part of a concerted study of the main cockle beds from the French Channel and Atlantic coasts (Ducrotot et al. 1991). In Northern Brittany and Arcachon, research focused on the role of intraspecific competition (Bachelet et al. 1992). In situ experiments clearly showed that negative juvenile-adult interactions were unlikely to explain early benthic mortalities in Brittany because of a relatively low density of adult population. In this area, a long term survey revealed recurrent features in spat mortality (Guillou and Tartu 1994). Every year, the main peak of recruitment occurred in autumn, followed by a winter decrease in density. After a short pause, strong mortality resumed in spring and suddenly stopped in June.

This study aimed to investigate whether biotic interactions were responsible for the spring juvenile mortality through a field experiment involving predator enclosure cages and juvenile cockle density manipulations. Cage experiments and density manipulations have been widely used in experimental ecology (Peterson 1979; Reise 1985; Ejdung and Bonsdorff 1992; Kamermans 1992) and may reveal species interactions affecting survival and growth.

### MATERIALS AND METHODS

The cockle bed studied lies in the western part of the Bay of Morlaix, in Northern Brittany, in the intertidal area of the Penzé Estuary. The sampling station (48°40.2' N; 3°56.8' W) was lo-

cated just below the mid-tide level, where the cockle density was highest. The main physico-chemical and edaphical factors had already been described (Guillou et al. 1990) and revealed a primarily oceanic environment. This sheltered area supports weak hydrodynamical processes, both in swell and currents. The substrate is an heterogeneous muddy sand with 20 to 40% silt. In order to determine the influence of biotic interactions on cockle mortalities, a field experiment was carried out in parallel with a demographic survey of the natural population.

#### Field Experiment

Three replicates of a cage and an adjacent control were randomly located inside the cockle bed. The pairing (cage and control) reduced the bias of local heterogeneity. The design consisted of PVC cylinder (30 cm in diameter and 15 cm high). These tubes were embedded in the substrate until only 3 cm protruded. Control enclosures were wide open whereas the enclosure cages were protected by a 5 mm mesh size net.

Predators and bivalve competitors were removed from the cages and controls after sieving the upper 3 cm of sediment through a 5 mm mesh sieve and large individuals of the macrofauna (mainly adult cockles) were removed from the underlying substrate (10 cm depth).

The experiment was conducted from 3 May to 7 June 1993. The size of the recruits in the natural population during this period (mean size on 3 May: 6.4 ± 2.6 mm) was sufficient to minimize handling mortality. Sixty juvenile cockles were randomly put in each enclosure, corresponding to 845 individuals.m<sup>-2</sup>. This experimental density was more than 6 times the mean value in the natural environment at that time, but was similar to the highest density observed in this season during the long term survey i.e., 850 individuals.m<sup>-2</sup> in May 1990 (Guillou and Tartu 1994).

#### Natural Population Sampling

Analysis of the natural population of the juvenile cockles was performed between January and July 1993. The sampling frequency was monthly at the start of the study and twice a month during the critical period. The sampling was divided into 3 strata corresponding to the location of the 3 replicates in the experimental design. A core sampler (unit-area: 1/16 m<sup>2</sup>, 10 cm depth) was used on the minimal basis of six random unit samples per stratum. The samples were preserved in formalin. The sieving was done

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through 1 mm mesh size. Juveniles were measured along the antero-posterior axis to the nearest 0.1 mm.

#### Data Processing

A modal analysis was carried out on the size frequency distributions of the juvenile population in the natural environment using NORMSEP program (Abramson 1970). Mortality is expressed by a raw mortality rate which is the percentage of juveniles that died between two successive samplings:  $Q = [(N_1 - N_2) / N_1] \times 100$ ,  $N_1$  and  $N_2$  being the numbers of juveniles in the consecutive samplings. This ratio was standardized as a 10-day mortality rate:  $R = Q \div [(t_2 - t_1) / 10]$ ,  $t_1$  and  $t_2$  being the date of consecutive samplings. In the same way, growth was estimated by a 10-day growth rate:  $k = [(X_2 - X_1) \times 100 / X_1] \div [(t_2 - t_1) / 10]$ ,  $X_1$  and  $X_2$  being the mean body lengths in the consecutive samplings.

Statistical tests were carried out in the data analysis of population parameters and experiment. The experimental results on the mortality rate (heterogenous variances) were analysed using a  $\chi^2$  test ( $\sum[(\text{observed} - \text{expected})^2 / \text{expected}]$ ). Density and growth were analysed using a  $t$  test after checking homoscedasticity. The statistical analysis was performed with a minimum of 5% significance level.

## RESULTS

#### Natural Population

Analysis of the size-frequency distributions (Fig. 1) was performed on the newly recruited juveniles (G0) and on its cohort structure. The maximal density of juveniles (152 individuals.m<sup>-2</sup>) was reached at the end of March (Table 1). Significant decrease in the density could be observed from May. The 10-day mortality rates were not homogeneous after this time, the period of high mortality rate (21% from 3 to 24 May, 32% from 7 to 21 June) being interrupted by a notable pause from 24 May to 7 June (mortality=0). After 21 June, when mean size was 15.6 mm, density stabilized at a level of 40 individuals.m<sup>-2</sup>. This threshold of strong mortality was observed every year, density remaining at a nearly constant level over the following months (Guillou and Tartu 1994).

The growth of the newly recruited year class started slowly between January and April, the value of the winter 10-day growth rate being slightly biased by the underestimate of the mean size until March. In Spring, the growth rate increased and maximum

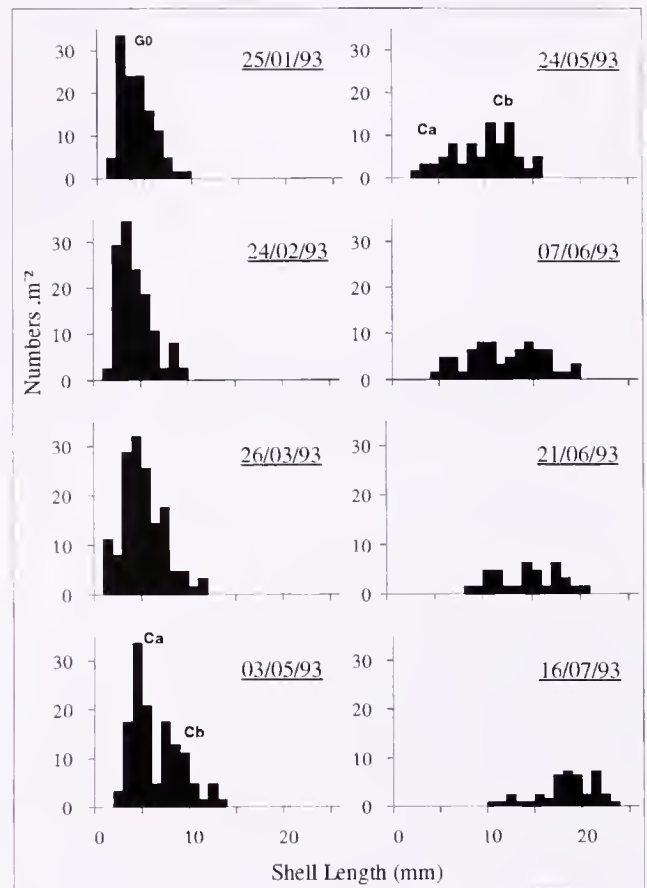


Figure 1. Size distributions of the juvenile cockles (sampled on 1 mm mesh size) recruited during the previous autumn and followed from January to July 1993. Ca and Cb are distinct cohorts of the year class (G0).

values were reached between May and June. This period of strong growth occurred during the high mortality stage. A discriminate analysis of the G0 group size-frequency distributions (Fig. 1) showed from 3 May a segregation into two cohorts (Ca and Cb) due to selective growth. The mean values of survival and growth rates of the cohorts were calculated during the critical period from 3 to 24 May (Table 2).

TABLE 1.

Mean density, 10-day mortality rate, mean size and 10-day growth rate of the juveniles in the natural population of the edible cockle.

Sampling date	Number of samples	Density (N. m <sup>-2</sup> ± s.d.)	10-day mortality rate (%)	Mean size (mm ± s.d.)	10-day growth rate (%)
25/01/93	6	122 ± 45		4.2 ± 3.0	
24/02/93	6	133 ± 53	-3	4.5 ± 2.4	2
26/03/93	6	152 ± 73	-5	5.1 ± 2.2	4
03/05/93	6	134 ± 26*	3	6.4 ± 2.6	7
24/05/93	6	75 ± 36*	21	9.2 ± 3.1	21
07/06/93	6	77 ± 30	-2	11.9 ± 4.4	21
21/06/93	6	43 ± 28*	32	15.6 ± 4.4	22
16/07/93	10	42 ± 20	1	18.4 ± 3.0	7

\* Significantly different from the previous sampling.

A selective mortality was observed inside the newly recruited year class. Between 3 and 24 May, the mortality rate in the Cb cohort was less than 25% of that in Ca which supported more than 80% of the global juvenile mortality.

**Field Experiment**

At the end of the experiment, a highly significant difference ( $X^2 = 37.21$ ,  $df = 2$ ,  $P < 0.05$ ) was revealed in the densities between the cages and the controls (Table 3). In all the cages, the mortality rates were very low, which demonstrated their protective effect from predation. A comparison of the cages and controls showed that 85% of the young cockle mortality was eliminated by the cages.

A possible bias could be expected from the experimental device (net covering the cages, fouling) at the level of the growth or the survival of the juvenile cockles. The mean mortality rate calculated over the 3 control replicates (10-day rate: 12.9%) was very close to the mortality rate in the natural environment (12.1%), showing that no significant disturbance resulted from the enclosure itself. Moreover, the mean size of the juveniles in the cages and in the controls showed no significant differences ( $t$  test,  $df = 266$ ,  $P$  value = 0.19α(0.05)), confirming that the net covering the cages did not inhibit growth.

**DISCUSSION**

These field observations and experiment in 1993 confirmed the recurrent spring mortality of the juvenile cockles in Northern Brittany, revealed by a long term survey between 1987 and 1992 (Guillou and Tartu 1994). From 26 March to 21 June 1993, the juvenile mortality in the natural population reached 72% of the cohort number despite a low initial density.

In the cage experiment, the presence of the net eliminated on average 85% of mortalities observed in the control, which emphasized the major role of predation. High predation mortality in juvenile cockles has often been described (Hancock and Urquhart 1965; Hancock 1970). The green crab, *Carcinus maenas*, is known as a voracious predator of cockles (Jensen and Jensen 1985; Sanchez-Salazar et al. 1987a). In the site from Northern Brittany, this species appeared to be the main predator of the juvenile cockles. More than 80% of the mortality affected the smallest cohort (6mm length), vs. less than 20% in the biggest (11mm length). This selective mortality is in accordance with the observations of Sanchez-Salazar et al. (1987a) concerning higher predation of the

**TABLE 2.**

Density, 10-day mortality rate, and mean size of the cohorts, Ca and Cb (see Fig. 1) in May 1993.

Sampling date G0 Cohorts	03/05/93		24/05/93	
	C.a	C.b	C.a	C.b
Density (N.m <sup>-2</sup> )	78	56	29	48
10-day mortality rate (%)			30	7
Mean length (mm)	4.8	8.4	6.3	11.3

**TABLE 3.**

10-day mortality rate and mean size of the juvenile cockles, in the field experiment and in the natural population (G0) from 3 May to 7 June 1993 (experimental period).

	Cage	Control	Natural pop. (G0)
10-day mortality rate (%)	1.9 ± 1.2	12.9 ± 6.6	12.1
Mean length (final) (mm ± s.d.)	13.5 ± 2.3	13.3 ± 2.2	11.9 ± 4.4

green crab on the cockle juveniles smaller than 10 mm. A refuge size was also defined by Jensen and Jensen (1985) beyond which the prey are safe from their predators. In Brittany, the long term survey showed that mortality strongly decreased every year once the cockle size was between 10 mm and 15 mm (Guillou and Tartu 1994). In the present study the size at which mortality decreased was about 11 mm which correlated with the size of the predator as only juveniles of *Carcinus* were observed on the site. This agrees with relationships established between the size of the crabs and the size of the cockles (Ropes 1969; Seed and Brown 1978; Sanchez-Salazar et al. 1987b), the escape from predation resulting from a strengthening of the shell and a deeper burrowing into the sediment. This selective mortality emphasized the role of predation in regulating and structuring populations from benthic communities (Virmstein 1977; Peterson, 1979; Holland et al. 1987).

Migrations have also been advanced to explain density fluctuations in bivalve populations (Hall et al. 1990; Commito et al. 1995). In this study, however, passive migration was quite unlikely as hydrodynamical processes are weak in this sheltered environment (Guillou et al. 1990). Active migration can take place in the early benthic stage of juvenile cockles (Baggerman 1953), but the experimental design (3 cm protruded controls) made it very improbable. Moreover, broken valves observed in the controls emphasized the role of predation.

The high initial densities of juveniles in this experiment could also be a source of mortality due to intraspecific competition. However, analysis of growth and survival did not support this. Although a negative influence of the high density of juveniles on their survival was expected, the difference in mortality between the controls and the natural environment was very low. Moreover, the size distributions of the juveniles throughout the experiment (Table 3) showed that the mean size of individuals in cages and controls was slightly greater than in the natural environment, and hence was contrary to the expected consequences of high density, indicating a lack of intraspecific competition. These conclusions were supported by the data from the long term survey (Guillou and Tartu 1994). Survival curves of the juveniles showed the same trend every year whatever the density of recruits that ranged from a scale 1 to 6 according to the year. The growth curves also showed the same trend throughout the multiyear survey, confirming that the carrying capacity in juveniles was certainly much higher than the experiment density (845 individuals.m<sup>-2</sup>).

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## UNIQUENESS OF THE GASTROPOD ACCESSORY BORING ORGAN (ABO): COMPARATIVE BIOLOGY, AN UPDATE

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**ABSTRACT** Unique among organs of invertebrate animals, the gastropod accessory boring organ is a compact, histologically, physiologically, biochemically complex mechanism by which boring predatory gastropods penetrate the calcareous armor of live molluscan prey to feed. The present review considers the close similarity of the ABO in naticoidean and muricoidean boring gastropod species, the worldwide, distribution of the ABO, its physiological–biochemical functions, and its possible evolutionary origin. The ABO occurs only in the midventral part of the foot of muricoidean neogastropods and under the tip of the proboscis of naticoidean mesogastropods. Many reports in the literature, and a new study of additional species from temperate and tropical regions by us, further confirm the quite remarkable similarity of the morphology of the ABO in a wide range of species from many different regions of the world. In 29 species and subspecies of live muricoidean snails ranging in shell height from 12.1 to 115.0 mm, the mean diameter of the relaxed ABO ranged from 0.9 to 4.4 mm, and the mean width of the radula, spread over the tip of the odontophore, from 0.12 to 1.40 mm. The fine structure and physiology of the secretory disc of the ABO is strikingly similar in the one naticoidean and four muricoidean species that have been studied in detail to date. The disc possesses features characteristic of highly active secretory cells. Cytologically, actively boring and inactive ABOs differ conspicuously. Active glands possess a thicker secretory epithelium, longer microvilli, more mitochondria, membrane-bound granules, vesicles, endoplasmic reticulum, and lysosomes, than inactive glands, and a denser concentration of hemocyanin molecules in the intercellular sinuses. Secretory granules probably pass to the surface of the ABO through interstitial ducts in the center of each secretory cell group. Glycogen is abundant in inactive ABOs and sparse in active glands. Shell dissolution in the borehole is primarily a chemical process involving enzymes (not yet identified), an inorganic acid (HCl), and chelating agents (not yet identified). Borehole size and shape mirror the external morphology of the extended ABO. Of the several enzymes that have been identified within the secretory disc, a prominent one is carbonic anhydrase. It plays a pivotal role in shell dissolution, but it does not function as a direct demineralization agent; it catalyzes the hydration of metabolic carbon dioxide producing hydrogen ions, which are selectively transported into the borehole for the release of calcium ions. These are transported into the ABO sinuses and thence into the circulatory system of the snail. The ABO develops early in the embryology of naticids as a patch of enlarged epidermal cells behind the mouth on the ventral side of the proboscis tip; the process has not yet been studied in muricids. Both naticid and muricid juveniles drill small prey upon hatching from the egg capsule. A dorid nudibranch, a tonnacean mesogastropod, and a marginallid neogastropod also drill round holes in the shell of molluscan prey, but lack of an ABO. Possible anatomical precursors suggesting the line of evolution of the ABO from its late Cretaceous origins were not found. Although the position of the muricoidean ABO in the foot and that of Naticoidea on the proboscis tip differs, the organs are essentially identical, and represent a striking case of convergent evolution.

**KEY WORDS:** Gastropoda, Naticoidea, Muricoidea, accessory boring organ, morphology, histology, physiology, biochemistry, distribution, evolution

### INTRODUCTION

The accessory boring organ (ABO) is unique among all organs of invertebrates examined to date. In the compact, mushroom-shaped organ there has evolved a physiological–biochemical mechanism that permits predatory gastropods to penetrate the external shell of their molluscan prey and within the protection of the shell, to feed in comparative safety on the flesh within.

Since Troschel's (1854) first reported discovery of the ABO (he thought it was a muscular sucker for holding prey during perforation) on the ventral tip of a naticid proboscis, the ABO has continued to excite interest among marine malacologists, especially about how the gland secretion weakens the shell at the surface of the borehole permitting the radula to rasp off and swallow the softened shell crystals. The similarity in form of the ABO in predatory gastropods as distantly related as naticoidean mesogastropods and muricoidean neogastropods has also raised the question as to whether the gland is homologous in the two taxa. To date the ABO has been found in no other invertebrates. The present review considers the similarity of the structure and ultrastructure of the gastropod ABO in naticoidean and muricoidean boring species, the physiological function of the ABO, and its evolutionary origin.

### MORPHOLOGY OF THE ABO

The ABO occurs only in the foot of muricoidean neogastropods and on the ventral tip of the proboscis of naticoidean mesogastropods (Carriker 1981). In all muricoidean males, the ABO is located deep in the midanterior ventral part of the foot (Fig. 1) within a vestibule out of which it balloons under blood pressure into the borehole during the boring process. In the retracted position, the ABO is tightly folded and lies snugly suspended within the vestibule. This chamber is formed by the invagination into a space in the pedal musculature of a thin area of the ventral pedal epithelium. Around the circumference of the secretory disc, the epithelium thins and turns dorsally to form the wall of the cylindrical peduncle. When retracted, the free surface of the disc is a much convoluted, thickened pad of specialized secretory tissue whose creases and folds run radially. In vivo, the everted disc appears translucent white, the color emanating from a white layer of tissue buried beneath the surface of the disc. The length and extensibility of the peduncle permit eversion of the ABO into deep boreholes in thick shell. From the inner dorsal surface of the ABO disc, a pair of large blood vessels, many fine nerves, and minute muscle and collagen fibers pass dorsally through the ABO sinus into the pedal musculature. When everted into the prey borehole, the ABO takes

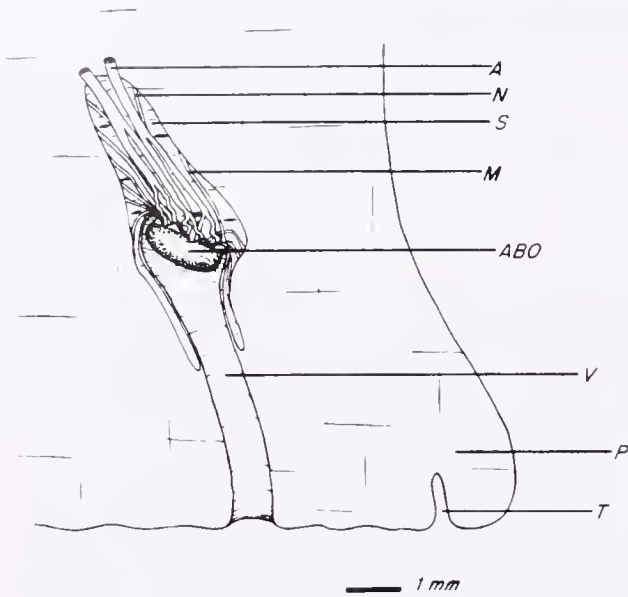


Figure 1. Drawing of sagittal section of anterior part of foot of a male muricoidean, *Rapana thomasiana*, through the accessory boring organ, ABO. S, ABO sinus containing arteries (A), nerves (N), and muscles (M) passing to back of ABO. V, ABO vestibule through which ABO is extended by blood pressure into borehole. P, propodium. T, transverse furrow of foot (from Carriker 1981).

the shape of a stout-stalked mushroom, its cushiony, translucent, glistening disc suspended at the end of the thin-wall peduncle (Fig. 2). The diameter of the ABO is approximately equal to that of the proboscis.

In females of some muricoidean species, the ABO is also located in the midanterior ventral region of the foot, but lodged anterior to, and against the ventral pedal gland (=egg capsule gland). Otherwise, its structure is identical to that of males (Fig. 3).

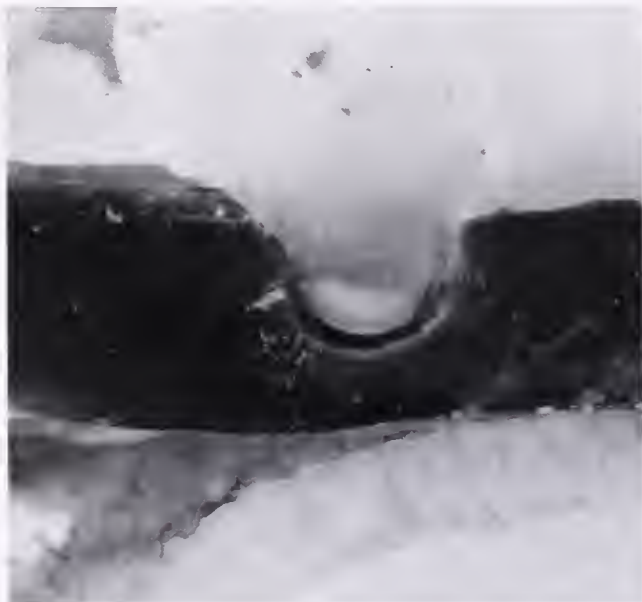


Figure 2. Accessory boring organ of *Urosalpinx cinerea follyensis* extended from foot into an incomplete borehole (1 mm diam.) in a glass-shell model. Light micrograph (from Carriker 1969).

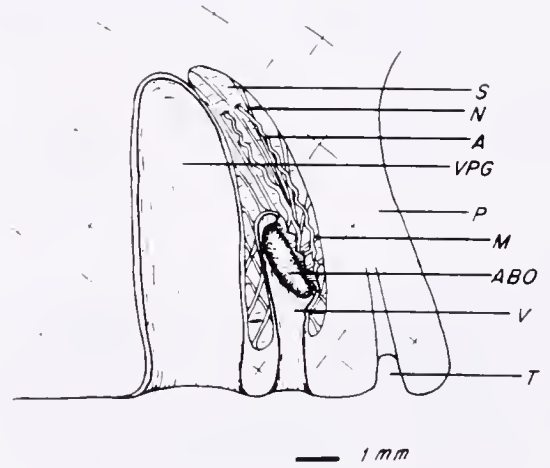


Figure 3. Drawing of sagittal section of anterior part of foot of a female muricoidean, *Urosalpinx cinerea follyensis*, through the accessory boring organ, ABO, and ventral pedal gland (VPG). S, ABO sinus. V, ABO vestibule. P, propodium. T, transverse furrow. N, nerve. A, artery. M, muscle (from Carriker 1981).

Curiously, in females of most species of the Rapaninae, the ABO within its vestibule rests atop (above), and its vestibule is continuous with the lumen of the ventral pedal gland (Fig. 4), so that a common duct serves the ABO and the egg capsule gland. Thus, during eversion, the ABO passes ventrally into the borehole through the lumen of the egg capsule gland. The structure of the ABO is identical to that of males. What evolutionary advantage the close proximity of the two glands provides the species, is unclear (Carriker 1981).

In both male and female naticoideans, by contrast, the ABO has

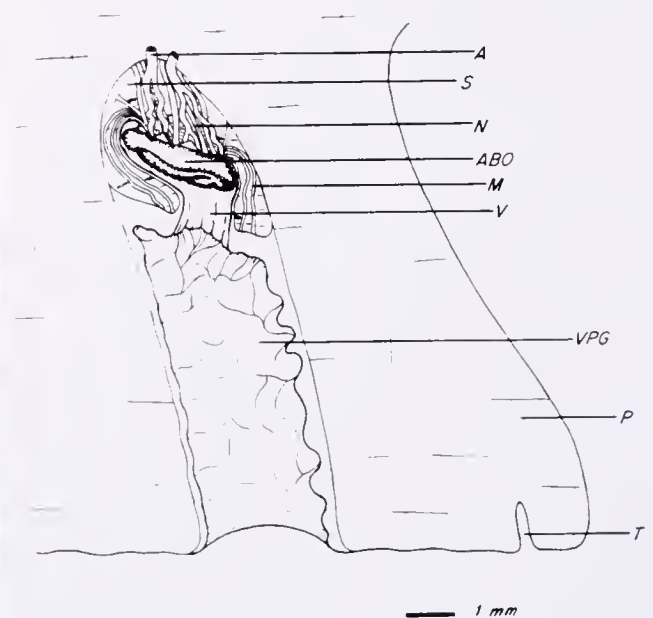


Figure 4. Drawing of sagittal section of anterior part of foot of a female muricoidean, *Rapana thomasiana*, through the accessory boring organ, ABO, and ventral pedal gland, VPG. The ABO is located atop the ventral pedal gland and in eversion passes through the lumen of the gland. S, ABO sinus. N, nerve. A, M, muscle. V, ABO vestibule. P, propodium. T, transverse furrow (from Carriker 1981).

evolved under the ventral lip of the cephalic proboscis close to the mouth and the radula (Fig. 5). In the retracted position, the naticid ABO resembles a somewhat wrinkled cushion of tissue suspended from the ventral end of the proboscis, and takes up a part of the lumen of the mouth. When everted into the borehole, the fully tumescent ABO resembles a fungiform papilla with a short peduncle and has a diameter equal to that of the proboscis. From the ventral wall of the proboscis extend small muscle and collagen fibers, nerves, connective tissue, sinuses, larger muscles, and a large branch of the buccal artery, to the dorsum of the ABO. The large artery branches into a rich network within the basal zone of the secretory disc (Bernard and Bagshaw 1969).

The naticoid ABO differs anatomically from that of muricoideans primarily in possessing a zone of subdermal mucocytes around the periphery of the secretory disc. The mucins are thought to provide a lubricant and "seal" for the ABO when applied to the boring site on the shell (Fretter and Graham 1994). Also, the peduncle is long in muricoideans, to accommodate the position of the gland deep within the foot and short in naticoid ABOs, because the gland is exposed on the lower tip of the proboscis.

#### ANATOMICAL UNIFORMITY OF ABO

Many reports in the literature confirm the quite remarkable similarity in the gross anatomy of the ABO in a wide range of species from many different regions of the world. Tables 1 and 2 summarize these reports. Table 1 records the anatomical location of the ABO in male and female muricoidean families.

In all muricoidean males, the ABO occurs in the same relative position in the midventral part of the foot. Of the 48 species and subspecies listed, the ABO is present atop the egg capsule gland in 28 taxa; and in the remaining, in front of the egg capsule gland.

In all the naticoid ABOs examined to date, the ABO occurs only on the ventral tip of the proboscis. The fact that these snails burrow through frequently abrasive sandy substrata, could have influenced the evolution of the ABO in the protected, retractable proboscis (Kool 1993a). In no boring gastropod species was a simple-structured, possible precursor gland to the ABO observed.

#### ABO-RADULA RELATIONSHIPS

Interested in the relationships among the variables of ABO diameter, radular width, and shell height in male and female boring

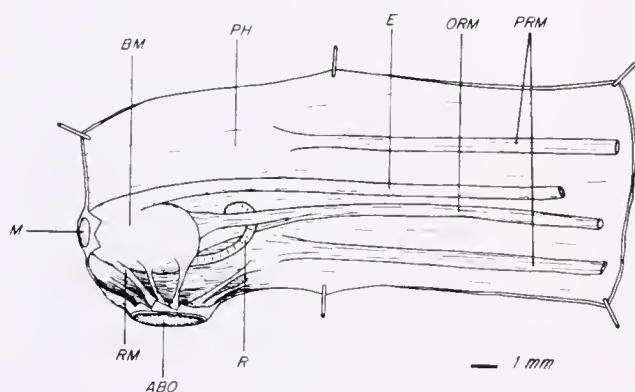


Figure 5. Drawing of left side of proboscis of a naticoid, *Polinices duplicatus*, opened laterally to show accessory boring organ, ABO; buccal mass, BM; and proboscis hemocoel, PH. M, mouth; R, radular sac; E, esophagus; ORM, odontophoral retractor muscle; PRM, proboscis retractor muscle (from Carriker 1981).

snails from widely different regions of the earth, we examined the boring mechanism of a large sample of muricoidean and naticoid gastropods. Snails were obtained by one of us collecting along the east coast of the United States (Carriker 1961) and by air shipments from colleagues abroad. Snails were placed in a little seawater in plastic bags supported in loose packing (see Acknowledgements section). For anatomical purposes, snails were relaxed in 10 ppm sevin in seawater, quickly frozen on dry ice, and stored frozen for at least 1 week (Carriker and Blake 1959). Dimensions of the ABO were obtained by excision of the organ from live snails and soaking the organ in distilled water for 3 minutes, causing it to swell to maximum size. Width of the radula was measured normally extended over the front tip of the exposed odontophore of live snails under a binocular microscope. Results of the measurements are recorded in Table 3 and Figures 6–8.

The height of the shell of muricoideans ranged from 18.5 mm (*Urosalpinx perrugata*) to 115.0 mm (*Murex fulvescens*). The mean diameter of the fresh, relaxed ABOs ranged from 0.9 mm (*Murex cellululosus*, *Urosalpinx tampaensis*) to 4.4 mm (*Murex pomum*); and radular width spread over the tip of the odontophore (in the rasping position), from 0.9 mm (*Urosalpinx tampaensis*) to 1.4 mm (*Rapana thomasiana*). Although, generally, the larger snails possessed larger ABOs and radulae, correlations were not close. Generally, the dimensions of the ABO and radula were smaller in males than in females, although, for the most part, this simply reflected smaller males than females.

Comparison of the sizes of the ABO and radula in normally feeding and starved (for 6 months) snails (*Urosalpinx cinerea follyensis*) showed negligible loss in size because of lack of food, suggesting that these organs are ready to function normally and at full capacity as soon as food is encountered.

Although, in a populational sense, the mean diameter of the ABO tended to increase slightly with increase in shell height, there was a wide range of ABO diameters within a given shell height, as, for example, in shell heights 18 and 23 mm (Table 3, Fig. 6). Larger snails, such as *Murex fulvescens*, with a shell height of 115 mm, would be expected to have a large ABO, in this case 4.2 mm, but one smaller species, *Murex pomum* (44.7 mm shell height), possessed the largest ABO (4.4 mm mean diameter) of all the species examined (Figs. 6, 8).

Mean radular width showed a general increase with increasing shell height (Fig. 7). However, the range of mean radular widths was only 0.12 to 1.40 mm, considerably smaller than the range of mean diameters of ABOs, 0.9 to 4.4 (Fig. 8).

Functionally, this size differential is important, because the radula evenly and completely scrapes around within the small space of the deepening borehole, softened by the ABO, during penetration of prey shell. A proportionally wider radula would be difficult for the odontophore to manipulate in producing the usually quite smooth, symmetrical boreholes characteristic of boring gastropods. This is demonstrated in Figure 8 by the proportionate increase in ABO and radular sizes as the shell increases in height. At the upper limit, were individuals of *Rapana thomasiana*, with a mean radular width of 1.4 mm and an ABO diameter of 2.7 (Fig. 8). In all species, the radula appears small enough to function efficiently within the confines of the forming borehole.

Anatomically, the ABOs of all species listed in Table 3 were remarkably similar, confirming the uniformity of ABOs recorded in Tables 1 and 2. No possible precursor stages of the ABO were identified. All were fully formed.

TABLE 1.

Anatomical location of the ABO in species of muricoidean gastropods from different regions of the world reported in the literature.

Subfamily and Species	Region	Reference		Location of ABO	
		Anatomy (Form)	Histology	Male	Female
<b>Muricinae</b>					
<i>Chicoreus brevifrons</i> (Lamarck)	Boqueron, Puerto Rico	Carriker 1961, Carriker 1981	Carriker 1961	S	T
<i>Chicoreus florifer</i> (A. Adams)	Florida, USA	Carriker 1961, Carriker 1981	Carriker 1961	S	T
<i>Favaria cellulosus</i> (Conrad)	Florida, USA	Carriker 1961, Carriker 1981	Carriker 1961	S	T
<i>Phylonotus pomum</i> (Gmelin)	Florida, USA	Carriker 1961, Carriker 1981	Carriker 1961	S	T
<b>Muricopsinae</b>					
<i>Muricopsis ostrearum</i> (Conrad)	Florida, USA	Carriker 1961, Carriker 1981	Carriker 1961	S	T
<b>Ocenebrinae</b>					
<i>Acanthina monodon</i> (Pallas)	Valparaiso, Chile	Kool 1993a Wu 1985		S	A
<i>Acanthina spirata</i> (de Blainville)	Tomales Bay, California, USA	Hemingway 1975		S	A
<i>Eupleura caudata</i> (Say)	North Carolina, USA	Carriker 1955, Carriker 1958, Carriker 1959, Carriker 1961, Carriker 1981	Carriker 1961	S	A
<i>Eupleura caudata etterae</i> (Baker)	Eastern Shore, Virginia, USA	Carriker 1959, Carriker, 1961, Carriker 1981; Provenza et al. 1966	Carriker 1961	S	A
<i>Eupleura sulcidentata</i> Dall	Florida, USA	Carriker 1961, Carriker 1981	Carriker 1961	S	A
<i>Forreria belcheri</i> (Hinds)	San Francisco, California, USA	Kool 1993a		S	A
<i>Haustrum haustorium</i> (Gmelin)	New Zealand	Kool 1993a		S	T
<i>Muricanthus fulvescens</i> (Sowerby)	Shackleford Banks, North Carolina, USA	Carriker 1961, Carriker 1981 Kool 1993a	Carriker 1961	S	T
<i>Nucella emarginata</i> (Deshayes)	Washington, USA	Carriker 1961, Carriker 1981	Carriker 1961	S	A
<i>Nucella lamellosa</i> (Gmelin)	Washington, USA	Carriker 1961, Carriker 1981	Carriker 1961	S	A
<i>Nucella lapillus</i> (Linnaeus)	Massachusetts, USA England, France, New Brunswick, Canada	Carriker 1961, Carriker 1981 Chetail and Binot 1967 Chetail et al. 1969 Chetail and Fournié 1970 Chetail et al. 1982 Derer 1975 Fretter 1941, Fretter 1946 Kool 1993a Webb and Saleuddin 1977	Carriker 1961 Chetail and Binot 1967 Chetail et al. 1968 Chetail and Fournié 1970 Chetail et al. 1982 Derer 1975 Fretter 1941, Fretter 1946 Webb and Saleuddin 1977	S	A
<i>Ocenebra erinacea</i> (Linnaeus)	England	Carriker 1961, Carriker 1981 Fretter 1941, Fretter 1946	Carriker 1961 Fretter 1941, Fretter 1946	S	A
<i>Ocenebra inornata</i> (Récluz)	Japan; Washington, USA	Carriker 1961, Carriker 1981	Carriker 1961	S	A
<i>Ocenebrina edwardsi</i> (Payr.)	Livorno, Italy	Bolognani-Fantin et al. 1981 Franchini et al. 1983	Bolognani-Fantin et al. 1981	S	A
<i>Pterorytis foliata</i> Gmelin	Washington, USA	Carriker 1961, Carriker 1981		S	A
<i>Urosalpinx cinerea</i> (Say)	England, Florida to Massachusetts, USA	Carriker 1943, Carriker 1955, Carriker 1958, Carriker 1959, Carriker 1961 Fretter 1941, Fretter 1946 Fretter and Graham 1994	Fretter 1941, Fretter 1946	S	A

continued on next page



TABLE I.  
continued

Subfamily and Species	Region	Reference		Location of ABO	
		Anatomy (Form)	Histology	Male	Female
<i>Urosalpinx c. follyensis</i> (B. Baker)	Eastern Shore, Maryland and Virginia, USA	Carriker 1943, Carriker 1959, Carriker 1961, Carriker 1963, Carriker 1966, Carriker 1969, Carriker 1978, Carriker 1981 Carriker and Van Zandt 1972a, Carriker and Van Zandt 1972b Carriker and Chauncey 1973 Carriker and Williams, 1978 Carriker et al. 1967 Carriker et al. 1978a, Carriker et al. 1978b Provenza et al. 1966 Person et al. 1967 Smarsh et al. 1969	Carriker 1961, Carriker 1981 Carriker 1961 Carriker 1961	S	A
<i>Urosalpinx perrugata</i> (Conrad)	Florida, USA	Carriker 1961, Carriker 1981	Carriker 1961	S	A
<i>Urosalpinx tampaensis</i> (Conrad)	Florida, USA	Carriker 1961, Carriker 1981	Carriker 1961	S	A
Ergalataxinae					
<i>Bedevea hanleyi</i> Angas	Port Jackson, Australia	Carriker 1961, Carriker 1981	Carriker 1961	S	T
Trophoninae					
<i>Trophon geversianus</i> (Pallas)	Argentina	Harasewych 1984		S	T
Rapaninae					
<i>Acanthais brevidentata</i> (Wood)	Eastern Pacific	Vermeij and Kool 1994		S	T
<i>Cochholepas choncholepas</i> (Bruguiere)	Chile	Arias 1983 Gruber and Carriker 1990 Kool 1993a	Arias 1983 Gruber and Carriker 1990	S	A
<i>Cromia amygdale</i> Kiener	Queensland, Australia	Kool 1993a		S	T
<i>Cymia tectu</i> (Wood)	Panama	Kool 1993a		S	A
<i>Dicathais orbita</i> (Gmelin)	New South Wales, Australia	Kool 1993a		S	T
<i>Drupa morum</i> Röding	Guam, USA	Kool 1993a		S	T
<i>Mancinella alouina</i> (Röding)	Queensland, Australia	Kool 1993a		S	T
<i>Mancinella deltoidea</i> (Lamarck)	Bimini, Bahamas	Carriker 1961, Carriker 1981	Carriker 1961	S	T
<i>Morula uva</i> (Röding)	Guam, USA	Kool 1993a		S	T
<i>Nasa sarta</i> (Bruguiere)	Guam, USA	Kool 1993a		S	T
<i>Neorapana muricata</i> (Broderip)	Sonora, Mexico	Kool 1993a		S	T
<i>Pinaxia versicolor</i> (Gray)	Madagascar	Kool 1993a		S	T
<i>Plicopurpura patula</i> (Linnaeus)	Florida, USA	Kool 1993a		S	T
<i>Purpura clavigera</i> Kuester	Japan	Carriker 1961, Carriker 1981 Lo et al. 1980	Carriker 1961 Lo et al. 1980	S	T
<i>Purpura persica</i> (Linnaeus)	Krakatoa, Indonesia	Kool 1993a		S	T
<i>Rapana rapiformis</i> (Born)	Mahe, Seychelles	Kool 1993a		S	T
<i>Rapana thomasi</i> (Grosse)	Japan	Carriker 1961, Carriker 1981		S	T
<i>Stramonita haemastoma</i> (Linnaeus)	Florida, USA Bimini, Bahamas	Carriker 1961, Carriker 1981 Kool 1993a	Carriker 1961	S	T
<i>Thais nodosa</i> (Linnaeus)	Ascension Island	Kool 1993a		S	T
<i>Tribulus planospira</i> (Lamarck)	Galapagos Islands, Ecuador	Kool 1993a		S	T
<i>Vasula melones</i> (Duclos)	Panama	Kool 1993a		S	T
<i>Vexilla vexillum</i> (Gmelin)	Oahu, Hawaii, USA	Kool 1993a (parasitic on urchins)			Absent

Note: Nomenclature based on Kool (1993a) and Vaught (1989), updated by J. Harasewych.

In all specimens, shape of the ABO was fungiform, and the histology (when reported) was typically muricid.

S = in sole of foot, in all males and in some females; A = anterior to the ventral pedal gland in sole of foot, in some females; T = atop the ventral pedal gland, in some females.

TABLE 2.

Anatomical location of the ABO in species of naticoid gastropods from different regions of the world reported in the literature.

Species	Region	Reference		Location of ABO	
		Anatomy (Form)	Histology	Male	Female
<i>Lumatia heros</i> (Say)	Massachusetts, USA	Carriker 1981		P	P
<i>Lumatia lewisii</i> (Gould)	Washington, USA	Carriker 1961, Carriker 1981		P	P
<i>Lumatia nitida</i> Donovan	Germany, Sweden	Ankel 1937, Ankel 1938 Ziegelmeier 1954	Ankel 1938 Ziegelmeier 1954	P	P
<i>Lumatia triseriata</i> (Say)	Massachusetts, USA	Carriker 1981		P	P
<i>Natica catena</i> da Costa	France	Fischer 1922	Fischer 1922	P	P
<i>Natica josephina</i> Risso	Naples, Italy	Schiemenz 1891 Simroth 1896-1907	Schiemenz 1891 Simroth 1896-1907	P	P
<i>Natica lineata</i> Chemnitz	Germany	Haller 1892		P	P
<i>Natica millepunctata</i> Lamarck	Naples, Italy	Troschel 1854 Schiemenz 1891 Hirsch 1915	Schiemenz 1891	P	P
<i>Natica severa</i> (Gould)	Korea	Carriker 1961, Carriker 1981	Carriker 1961	P	P
<i>Neverita didyma</i> (Roeding)	Korea	Carriker 1961, Carriker 1981	Carriker 1961	P	P
<i>Polinices duplicatus</i> (Say)	Beaufort, North Carolina, USA	Carriker 1961, Carriker 1981	Carriker 1961	P	P
<i>Polinices lewisii</i> (Gould) (see <i>L. lewisii</i> above)	Europe Washington, USA	Troschel 1854 Schiemenz 1891 Fischer 1922 Ziegelmeier 1954 Bernard and Bagshaw 1969 Carriker 1981 Page and Pedersen 1998	Bernard and Bagshaw 1969 Page and Pedersen 1998	P	P
<i>Sinum perspectivum</i> (Say)	Beaufort, North Carolina, USA	Carriker 1961, Carriker 1981	Carriker 1961	P	P

Species names reported in the cited references.

The shape of the ABO was a cylindrical pad on a short peduncle, and the histology (when reported) was similar to that of muricid ABOs.

P = under ventral tip of proboscis.

#### ABOs OF TROPICAL GASTROPODS

In a further search for possible precursor stages of the ABO, we examined additional naticoids and muricoids from Guaymas, Mexico; Santa Marta, Colombia; Oahu, Hawaii; and Marin-duque Island, The Philippines. Pedal cubes of tissue containing the ABO were processed by standard histological techniques, cut into sections 7- to 10- $\mu$ m thick, stained with a standard trichrome stain, and mounted on slides for microscopic examination. The number of tissues processed per species ranged from 1 to 5 (depending upon the snails available). Because tissues were fixed in the field for the most part, it was not possible to measure the diameter of the ABO.

Suffice it to say that all muricoid species and one species of Naticoidea examined possessed a typical accessory boring organ (Table 4)! The most interesting species was *Drupa ricina*. Wu (1965) noted that this species possesses the primitive features of the *Stenoglossa*, is not thought to be a typical predator of hard-shelled mollusks, and feeds on soft prey. However, we found that four individuals did possess a typical ABO. The tropical venture did add more species of gastropods that possess an ABO, but did not reveal any information on the possible early evolution of the group.

#### FINE STRUCTURE OF THE ABO

The fine structure of the secretory disc of the ABO is strikingly similar in the five species of boring gastropods studied to date:

- *Nucella lapillus* (Derer 1975, Webb and Saleuddin 1977, Ché-tail et al. 1982),
  - *Ocenebra erinacea* (Humphrey 1990),
  - *Ocenebrina edwardsi* (Franchini et al. 1983),
  - *Polinices lewisii* (Bernard and Bagshaw 1969),
  - *Urosalpinx cinerea* (Provenza et al. 1966, Nylén et al. 1969).
- The excellent, detailed studies of Nylén et al. (1969) on *U. cinerea* and Derer (1975) on *N. lapillus*, are especially informative.

In brief, the secretory disc consists solely of a single layer of very tall columnar cells, arranged in compact cell groups with blood spaces around them (Fig. 9). The distal ends of the cells form a continuum over the surface of the ABO. Blood flows into the intergroup spaces in elaborately branching arterioles and capillaries that pass through the ABO sinus. At high magnifications, the blood pigments, hemocyanin particles, are identifiable in the blood vessels and intergroups sinus spaces. Nerves and muscles accompany the vessels into the base of the disc and pass among the cell groups to the undersurface of the disc continuum. From the intergroup spaces, blood flows back into the open ABO sinus, and thence returns into the principal venous channels of the foot. This complex architecture is admirably suited to supply abundant oxygen and nutrients to all parts of the metabolically highly active, extended ABO during shell boring (Peson et al. 1967).

Longitudinally, the tall cell groups in the secretory epithelium are divisible into three distinct zones: the distal zone, bearing the brush border, and containing dense populations of long mitochondria; the middle zone, with relatively few organelles; and the basal

TABLE 3.

Mean diameter (mm) of relaxed ABO relative to mean width (mm) of radula spread over odontophore in species of boring gastropods from different temperate and tropical regions of the world.

Species	Region	Snail Sex	Snail Mean Height	ABO Mean Diam.	Radula Mean Width
<i>Bedevelia hanleyi</i> [5]	Port Jackson, Australia	F	19.4	1.2	0.17
Angas [3]		M	18.7	1.3	0.28
<i>Eupleura caudata</i> [6]	Virginia, USA	F	22.5	1.2	0.20
(Say) [3]		M	18.7	1.1	0.17
<i>Eupleura caudata</i> [6]	Virginia, USA	F	35.5	1.7	0.30
<i>etterae</i> (Baker) [6]		M	26.2	1.3	0.23
<i>Eupleura sulciden tata</i> Dall [1]	Florida, USA	F	23.0	1.1	0.15
<i>Murex brevifrons</i> Lamarck [2]	Puerto Rico, USA	F	94.1	3.4	0.95
<i>Murex cellulosis</i> Conrad [2]	Florida, USA	?	21.5	0.9	0.20
<i>Murex florifer</i> A. Adams [6]	Florida, USA	?	43.7	2.3	0.45
<i>Murex fulvescens</i> Sowerby [4]	North Carolina, USA	?	115.0	4.2	0.85
<i>Murex pomum</i> Gmelin [6]	Florida, USA	?	44.7	4.4	0.65
<i>Muricopsis ostrearum</i> (Conrad) [6]	Florida, USA	?	22.7	1.0	0.19
<i>Neverita didyma</i> (Roeding) [2]	Inchon, Korea	?	24.5	1.9	0.50
<i>Nucella lapillus</i> [3]	Plymouth, England	F	25.7	1.5	0.29
(Lionaeus) [3]		M	27.3	1.4	0.27
" [4]	Plymouth, Massachusetts, USA	F	23.3	1.2	0.32
[2]		M	22.9	1.1	0.30
" [2]	St. Andrews, New Brunswick, Canada	F	33.5	2.4	0.48
[2]		M	29.0	2.2	0.38
<i>Ocenebra erinacea</i> [3]	Plymouth, England	F	30.3	1.9	0.28
(Lionaeus) [3]		M	27.0	1.6	0.23
<i>Ocenebra inornata</i> [4]	Washington, USA	F	39.7	1.5	0.40
(Recluz) [2]		M	34.8	1.5	0.39
<i>Polinices duplicatus</i> (Say) [4]	North Carolina, USA	?	34.3	2.6	0.73
<i>Pterorytis foliata</i> (Gmelin) [3]	Washington, USA	?	64.7	2.2	0.60
<i>Purpura clavigera</i> Kuester [6]	Japan	?	43.0	1.8	0.63
<i>Rapana thomasi</i> Crosse [3]	Japan	?	78.5	2.7	1.40
<i>Sinum perspectivum</i> Say [1]	North Carolina, USA	?	25.0	1.6	0.63
<i>Thais deltoidea</i> [4]	Bimini, Bahamas	F	37.0	2.4	0.51
(Lamarck) [2]		M	34.5	2.4	0.48
<i>Thais emerginata</i> [4]	Washington, USA	F	29.3	1.6	0.35
Deshayes [2]		M	26.8	1.5	0.32
<i>Thais haemastoma</i> [4]	East Coast, Florida	F	57.3	2.4	0.65
<i>floridana</i> (Conrad) [6]		M	58.7	2.5	0.78
<i>Thais haemastoma</i> [3]	Pensacola, Florida, USA	F	50.0	2.2	0.54
<i>canaliculata</i> (Gray) [3]		M	41.0	1.9	0.47
<i>Thais haemastoma</i> [1]	Bimini, Bahamas	F	19.0	1.6	0.35
<i>floridana</i> (Conrad) [3]		M	20.0	1.5	0.40
<i>Thais lamellosa</i> [4]	Washington, USA	F	40.3	1.5	0.50
(Gmelin) [2]		M	36.5	1.4	0.42
<i>Urosalpinx cinerea</i> [3]	Florida, USA	F	26.7	1.6	0.28
(Say) [3]		M	21.7	1.3	0.23
" [6]	North Carolina, USA	F	26.2	1.5	0.26
[7]		M	22.7	1.7	0.27
" [7]	Massachusetts, USA	F	21.7	1.9	0.25
[5]		M	22.2	1.9	0.22
" [6]	Burnham, England	F	31.5	1.7	0.30
[6]		M	30.7	1.7	0.30
<i>Urosalpinx cinerea</i> [6]	Eastern Shore,	F	37.8	2.2	0.38
<i>follyensis</i> Baker [6]	Virginia, USA	M	30.0	1.8	0.31
" [6]	" (starved for 6 months)	F	36.2	1.8	0.34
[6]		M	32.2	1.8	0.29
<i>Urosalpinx perrugata</i> [6]	Florida, USA	F	23.3	1.5	0.23
(Conrad) [6]		M	18.5	1.1	0.19
<i>Urosalpinx tampiensis</i> [2]	Florida, USA	F	19.5	1.1	0.18
(Conrad) [1]		M	12.1	0.9	0.12

Note: Species names based in part on Keen (1971), Abbott (1974), and Diaz and Puyana (1994).

[ ] = number of specimens examined.

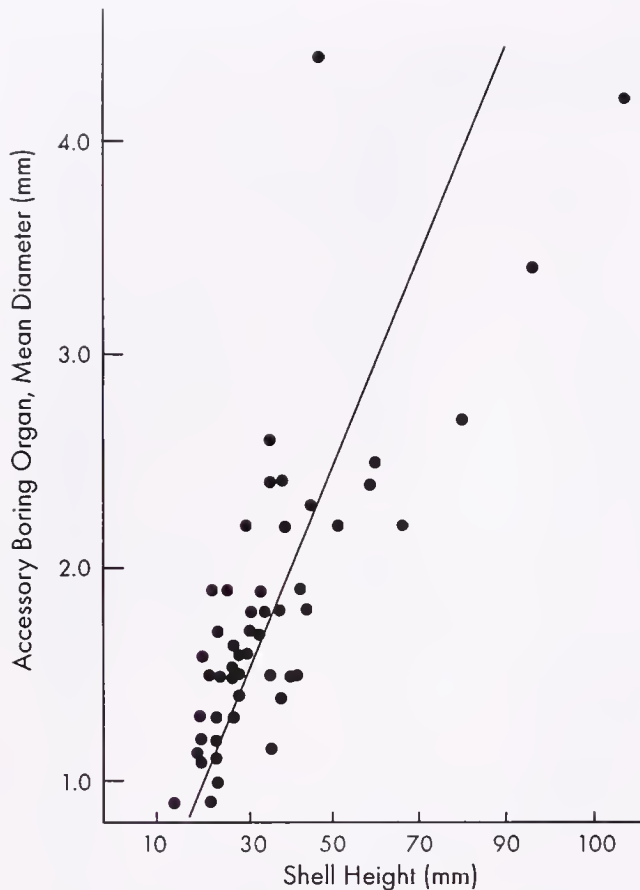


Figure 6. Relationship between shell height and diameter of the accessory boring organ in snails from widely different regions of the Earth.

zone, containing the nuclei, Golgi complexes, endoplasmic reticulum, ribosomes, autophagic vacuoles, secretion granules, and other vacuoles. The basal zone is highly active physiologically during shell boring.

The exterior surface of the ABO possesses a prominent brush border of long microvilli, with an occasional, longer tuft of cilia (possibly sensory) among the microvilli. A viscid secretion coats the brush border. Distally, each cell group is nearly circular in cross section. An interstitial "star-shaped" duct passes up the center of each cell group to the base of the microvilli. This is likely a passageway for secretion granules moving to the exterior of the ABO. The basal zone is extremely complex histologically, made so by the interdigitation of the secretory cells and their processes, and the maze of capillaries, muscle fibers, and nerve fibers that intertwine among them.

Mitochondria are mostly located close to the cell membranes bordering the hemocyanin-filled intergroup sinuses. Blood pigment is conspicuously more abundant in active than in inactive ABOs, the increased blood flow into the gland augmenting the supply of required oxygen and nutrients. The dense network of nerves throughout the secretory disc indicates an organ whose extension and withdrawal are closely synchronized with the movements of the proboscis, odontophore, and radula during hole boring, as well as, possibly, peristaltic pulsations of the muscular gland (such pulsations have not been observed) during discharge of secretion granules and other secretory products.

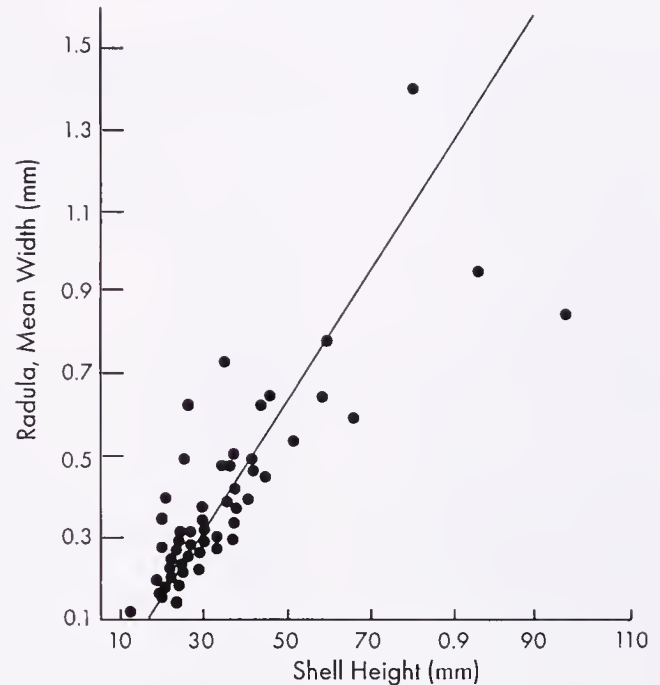


Figure 7. Relationship between shell height and width of radula in snails from widely different regions of the earth.

The ABO secretory epithelium possesses the cytological features characteristic of highly active cells: elaboration and extracellular discharge of secretory products, synthesis of glycogen in the inactive stage, and changes in the population of organelles relative to the physiological state of the organ. Golgi complexes in the basal zone produce the membrane-bound secretion granules and vesicles. How these are discharged onto the microvillar surface remains unclear. In electronmicrographs, granules are visible apparently fusing with plasma membranes at the base of the microvillar zone. This suggests that granules could be transported from the Golgi complexes at the base of the cells through the cell cytoplasm to the microvillar border, a distance of about 100  $\mu\text{m}$ , a discharge that would require considerable energy. A second, more probable course is the movement of granules into the interstitial ducts in the center of the cell groups, and extrusion by contractions of the musculature onto the microvillar border. However, electronmicrographs have not shown granules in the ducts (Nylen et al. 1969), possibly because the granules were expelled quickly to the ABO surface by the fixative. Also, Derer (1975) observed no interstitial ducts in the cell groups, possibly because of a different method of tissue preparation. However, Carriker (1973) confirmed the existence of interstitial ducts using scanning electron microscopy. Freshly excised active ABOs fixed slowly with agitation in increasing concentrations of glutaraldehyde and freeze dried by a special technique clearly showed openings on the surface of the ABO among the microvilli and discharged granule-like spheres (Figs. 10, 11). Each extremely dilated duct opening bore a conspicuous flange and was traceable into the interior of the ABO in fracture sections. The secretion-like granules appeared swollen and larger than those (0.2- $\mu\text{m}$  diameter) in Nylen et al.'s (1969) micrographs, probably a result of the preservation procedures.

Abundant immature secretion products in the Golgi complexes of active ABOs (in contrast to inactive ones) indicated that elabo-

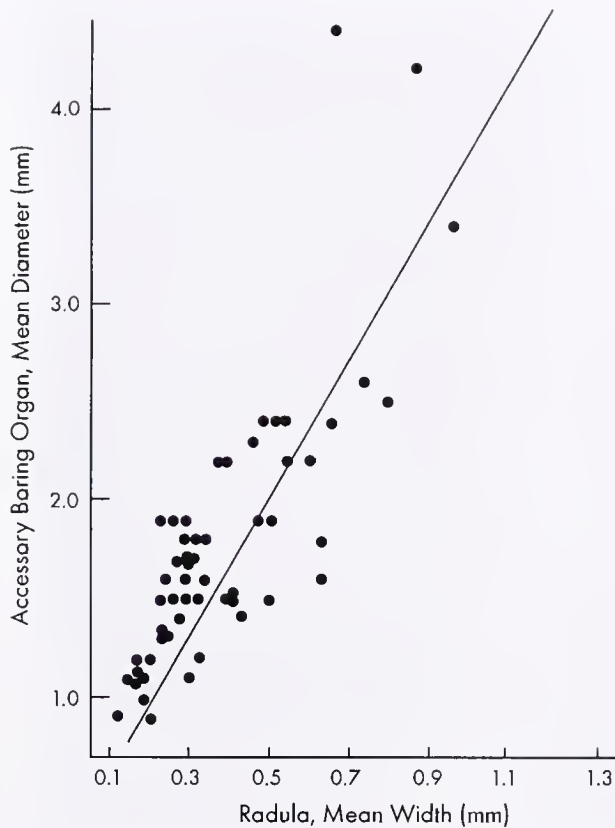


Figure 8. Relationship between width of radula and size of accessory boring organ in snails from widely different regions of the earth.

ration and discharge of secretion granules are highest during periods of active boring. The glycogen in the secretory disc of inactive ABOs provides a readily available source of energy for the change from the inactive to the active phase. Junctional complexes between the interstitial spaces and the free surface of the active ABO probably facilitate the passage of other secretion components (specific molecules, ions, water) from the cells onto the exterior brush border; the reverse movement is also likely.

#### PHYSIOLOGY OF THE ABO

Comprehensive studies on the physiology of the gastropod ABO have been reported for four species of Muricidae (Neogastropoda). These studies include:

- *Nucella lapillus* (Linnaeus), Chétail and Binot (1967), Rosenberg et al. (1968), Chétail et al. (1968), Chétail and Fournié (1969), Chétail and Fournié (1970), Webb and Saleuddin (1977), Chétail and Fournié (1980), Chétail et al. (1982);
- *Ocenebra erinacea* (Linnaeus), Humphrey (1990);
- *Ocenebrina edwardsi* (Payr.), Bolognani-Fantin et al. (1981);
- *Urosalpinx cinerea* (Say) and *U. c. follyensis* Baker Carriker et al. (1963), Provenza et al. (1966), Carriker et al. (1967), Person et al. (1967), Smarsh et al. (1969), Carriker (1969), Carriker and Chauncey (1973), Carriker (1978), Carriker and Williams (1978), Carriker et al. (1978a), Carriker et al. (1978b), Evans (1980), Carriker (1981).

There have been no comparable studies on the physiology of the naticoid ABO.

Because of its prominence and accessibility on the ventral tip of the proboscis, the naticid ABO attracted the attention of early

TABLE 4.  
Condition of the ABO in naticoid and muricid gastropods from tropical regions.

Species	Region	ABO
<i>Acanthina angelica</i> [3] I. Oldroyd	Guaymas, Mexico	Present
<i>Boreotrophon aculeatus</i> [2] (Watson)	Bahamas	Present
<i>Chicoreus brevifrons</i> [2] (Lamarck)	Santa Marta, Colombia	Present
<i>Coralliophila parva</i> [2] (E. A. Smith)	Guaymas, Mexico	Absent
<i>Drupa ricina</i> [4] (Linnaeus)	Oahu, Hawaii	Present
<i>Eupleura muriciformis</i> [4] (Broderip)	Guaymas, Mexico	Present
<i>Haustellum haustellum</i> [1] (Linnaeus)	Marinduque, The Philippines	Present
<i>Hexaplex erythrostomus</i> [2] (Swainson)	Guaymas, Mexico	Present
<i>Morula ferruginosa</i> [3] (Reeve)	Guaymas, Mexico	Present
<i>Murex troscheli</i> [2] <i>troscheli</i> Lischke	Marinduque, The Philippines	Present
<i>Muricanthus nigrinus</i> [2] (Philippi)	Guaymas, Mexico	Present
<i>Muricanthus princeps</i> [3] (Broderip)	Guaymas, Mexico	Present
<i>Muricopsis armatus</i> [5] (A. Adams)	Guaymas, Mexico	Present
<i>Natica chemnitzii</i> [2] Pfeiffer	Guaymas, Mexico	Present
<i>Neorapana tuberculata</i> [4] (Sowerby)	Guaymas, Mexico	Present
<i>Pteropurpura erinaceoides</i> [1] (Valenciennes)	Guaymas, Mexico	Present
<i>Purpura pansa</i> [3] Gould	Guaymas, Mexico	Present
<i>Purpura patula</i> [5] Linnaeus	Santa Marta, Colombia	Present
<i>Siratus plicifosoides</i> [1] (Kuroda)	Marinduque, The Philippines	Present
<i>Thais biserialis</i> [4] (Blainville)	Guaymas, Mexico	Present
<i>Thais coronata trini</i> [3] <i>tatensis</i> (Guppy)	Santa Marta, Colombia	Present
<i>Thais deltoidea</i> [3] (Lamarck)	Santa Marta, Colombia	Present
<i>Thais haemastoma</i> [1] <i>floridana</i> (Conrad)	Santa Marta, Colombia	Present
<i>Thais rustica</i> [3] (Lamarck)	Santa Marta, Colombia	Present
<i>Thais speciosa</i> [4] (Valenciennes)	Guaymas, Mexico	Present
<i>Thais triangularis</i> [2] (Blainville)	Guaymas, Mexico	Present

Note: Species names based in part on Keen (1971), Abbott (1974), and Diaz and Puyana (1994), and not updated.

[ ] = number of specimens examined.

naturalists long before the muricid ABO was known. Reaumur (1711), observing the smoothness of boreholes in molluscan prey, was the first to suggest that penetration was probably effected by chemical means. This report stimulated Schiemenz (1891) to study

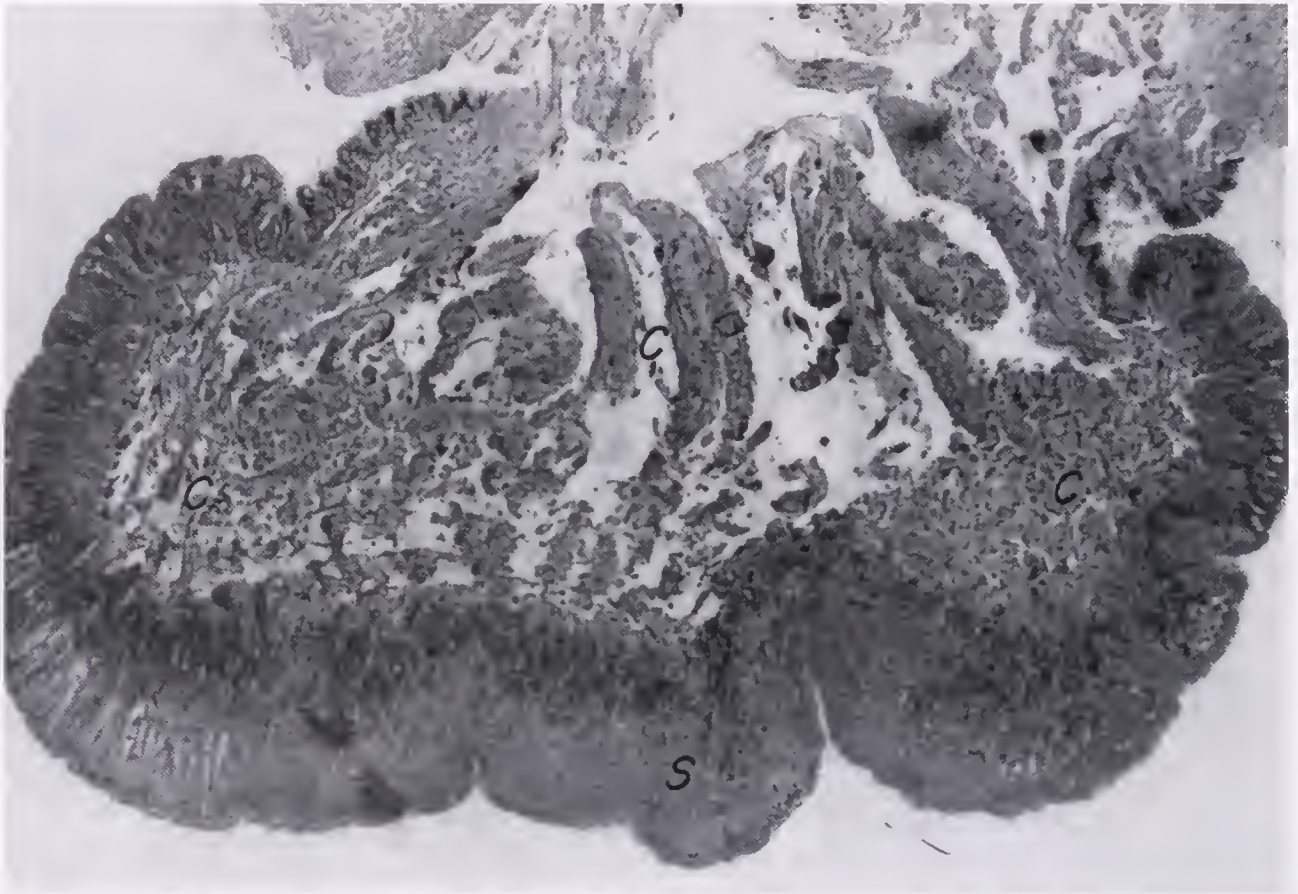


Figure 9. Histological sagittal section of accessory boring organ of *Urosalpinx cinerea follyensis* extended from foot. S, secretory epithelium. C, connective tissue in ABO sinus supporting retractor muscles, capillaries, and nerve fibers. ABO 1 mm in diameter. Light micrograph (from Carriker 1981).

naticids (at the Zoological Station in Naples) and to conclude that the ABO, which he named the "boring gland," secreted sulfuric acid. Blue litmus paper touched to the boring gland in three experiments was changed to red. This, he reported, supported his theory of shell penetration by chemical means. Hirsch (1915) refuted Schiemenz's results by using Congo red paper, which, he noted, is a true test of a free acid, and this acid did not change color when applied to the gland. He suggested that a specific enzyme, perhaps a calcase, could be involved in the shell boring. Fischer (1922) likewise tested the pH of the fluid on the ABO and the borehole with litmus paper and obtained neutral results; as did Ankel (1938) in later experiments. It would seem, then, that at least by these unreliable tests and conflicting results, the ABO secretion was neutral.

Carriker et al. (1963), questioning these determinations of hydrogen ion concentration, attempted more quantitative tests. Minute strips of short-range pH indicator papers, their color verified against standardized buffer solutions checked with a pH meter, were applied to normally extended ABOs, excised ABOs, and ABO homogenates of muricid snails. All tests proved neutral to slightly alkaline, confirming the observations of most earlier workers. Not satisfied with these results, Carriker et al. (1967) devised a glass-shell bivalve model that permitted observation of the boring process and access to the extended ABO through a pore in the glass plate. Using specific glass microelectrodes developed

by G. Charlton, they were able quantitatively to determine the pH of the viscid secretion on the surface of the ABO extended normally into an incomplete borehole, and to demonstrate that the secretion of the functioning ABO in the borehole is distinctly acid, ranging in pH from 3.8 to 4.1. The failure of earlier workers to detect convincing acid reactions with pH papers was because of the insensitivity of their methods and the basic fact that forcefully extruded or excised ABOs produce no, or minimal quantities of acid.

Subsequently, Carriker et al. (1978a), using a similar bivalve model, were able to monitor both hydrogen and chloride ions with microelectrodes touched to live, normally extended ABOs. They confirmed the acidity of the ABO secretion determined earlier (1967), and in addition observed that chloride ions were plentiful and increased stepwise in concentration from the time of extension of the ABO to its withdrawal. Qualitative analysis of dry ABO secretion by energy dispersive X-ray analysis confirmed the presence of chloride and of sodium. It is important to note that there had been no contact of the ABO with seawater during the tests.

Metabolically (relative to oxidase activity in mitochondrial rich particulate fractions), the boring ABO is much more active than the resting ABO (Person et al. 1967). Several investigators have identified a number of enzymes in the ABO: cytochrome oxidase, succinate dehydrogenase, and lactate dehydrogenase (Person et al. 1967); lipase and alkaline phosphatase (Chétail et al. 1968); car-

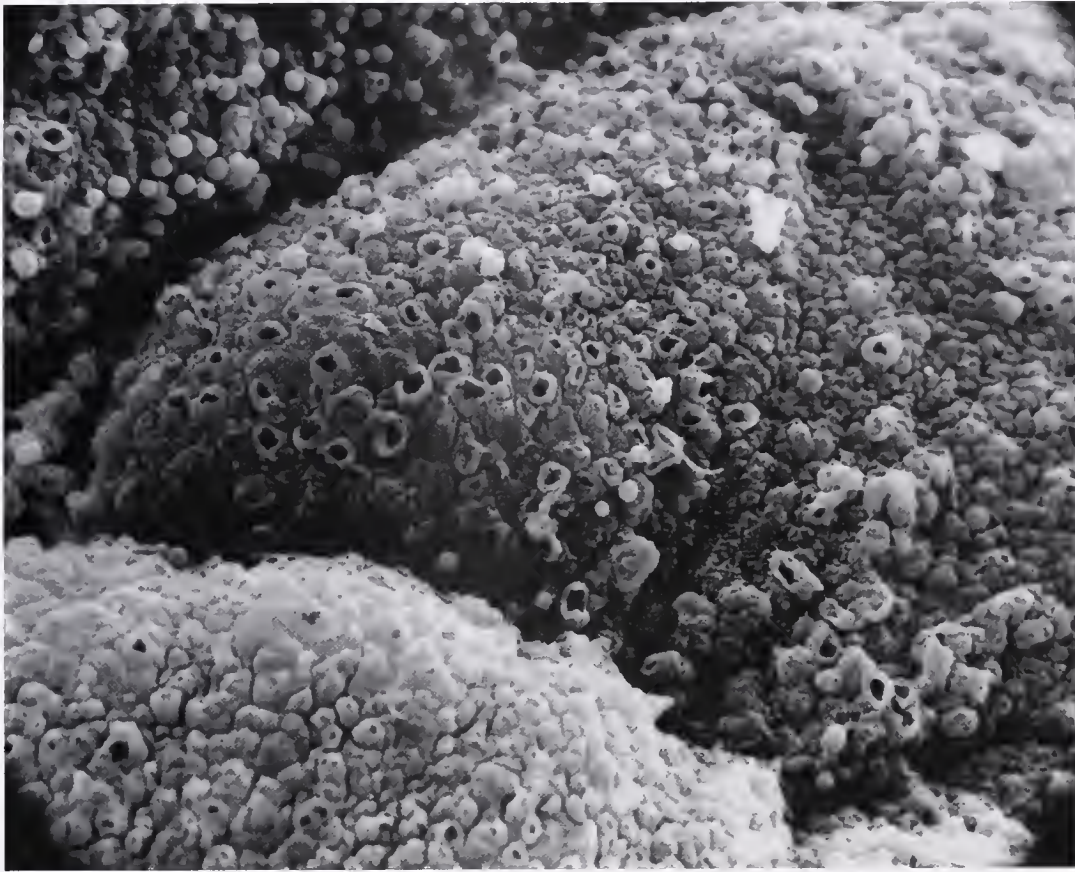


Figure 10. A part of the exterior surface of the secretory disc of the accessory boring organ of *U. c. follyensis* showing a field of dilated pore openings in the brush border. Outer diameter of largest pores 10  $\mu\text{m}$ . The light spheres could be secretion granules. Scanning electron micrograph.

bonic anhydrase, acid phosphatase, alkaline phosphatase, ATPase (Webb and Saleuddin 1977); carbonic anhydrase (Chétail and Binot 1967, Smarsh et al. 1969, Chétail and Fournié 1969, Carriker and Chauncey 1973); alkaline and acid phosphatase, carbonic anhydrase, Na-K-ATPase (Bolognani-Fantin et al. 1981); and Ca-ATPase, Na-K-ATPase, acid phosphatase, adenylate cyclase (Humphrey 1990). Whether the snails in these tests had been actively boring before their ABO was excised, was not indicated.

The most prominent enzyme in the secretory cells of the ABO is carbonic anhydrase (Carriker et al. 1968, Chétail et al. 1968, Webb and Saleuddin 1977). Furthermore, it is considerably more concentrated in the secretory epithelium than in the surrounding pedal tissues (Chétail and Binot 1967, Smarsh et al. 1969). Smarsh also found carbonic anhydrase in the ABO brush border; whereas, Webb and Saleuddin (1977) did not. Some researchers found the enzyme about equally abundant in both active (=boring) and inactive (=resting) ABOs (Chétail and Fournié 1969, Webb and Saleuddin 1977); whereas, others found it more abundant in active ABOs (Chétail et al. 1968, Bolognani-Fantin et al. 1981). This inconsistency could have resulted from different ways of excising and treating the ABOs.

Carbonic anhydrase is associated with secretory cells noted for active transport of hydrogen ions, bicarbonate ions, and carbon dioxide, and with some cells that transport sodium and chloride ions (Webb and Saleuddin 1977). Smarsh et al. (1969) and Carriker and Chauncey (1973) concluded that carbonic anhydrase

plays a vital role in shell dissolution during boring, but does not function as a direct demineralizing agent. Whether this is true in shell penetration by other invertebrates (Carriker and Smith 1969) remains to be determined. The required involvement of carbonic anhydrase in shell drilling was demonstrated by the significant inhibitory effect of Diamox, a specific inhibitor of the enzyme, on the incidence of boring by live snails in seawater to which the Diamox had been added (Chétail and Binot 1967, Rosenberg et al. 1968, Chétail and Fournié 1969, Carriker and Chauncey 1973). Chétail and Fournié (1969) and Webb and Saleuddin (1977) assumed that carbonic anhydrase is not released in the secretion during boring; yet Carriker and Chauncey (1973) identified the enzyme on blots of secretion taken from live, active ABOs. Again, handling of the ABO could explain the differing results.

As released on the surface of the active ABO, the secretion is highly viscid, granular, generally insoluble in seawater, hypertonic, and about 65% volatile, when dried it becomes highly hygroscopic. Its granular consistency is attributable to the secretory, membrane-bound granules and vesicles; its mucoid consistency prevents its dispersion during boring (Carriker 1981). Excised, live ABOs when placed on polished mollusk shell etch the surface; whereas, excised ABOs treated with heat and papain do not (Carriker and Van Zandt 1964), suggesting inactivation of enzymes.

Cytologically, there is a conspicuous difference between actively boring and inactive ABOs. Active glands possess a thicker secretory epithelium, longer microvilli, more secretory granules,



**Figure 11.** Another field of pores in the secretory disc of the ABO, twice the magnification of that in Figure 10. Outer diameter of largest pores 10  $\mu\text{m}$ . Scanning electron micrograph.

vesicles, endoplasmic reticulum, and lysosomes than inactive glands, and a higher concentration of hemocyanin molecules in the intergroup sinuses (Provenza et al. 1966). Active ABOs contain little glycogen; whereas, in inactive glands, it is abundant (Chétail et al. 1968).

The marked structural and microstructural correspondence of the ABOs of different species of boring gastropods suggest that their physiology and biochemistry are probably quite similar. Carriker and colleagues hypothesized that shell penetration is primarily a chemical process in which a combination of enzymes, an inorganic acid, and chelating agents solubilize the shell at the bottom of the borehole (Carriker et al. 1963, Carriker and Smith 1969, Carriker and Williams 1978, Carriker 1981; also reviewed by Bubel 1984). Borehole size and shape are, thus, a reflection of the external morphology of the extended ABO. Dissolution of the shell surface occurs first at the exterior, insoluble layers of the organic matrix of shell prisms and then proceeds into the interior organic-calcareous structure of individual prisms (Carriker 1996), weakening them for removal by the radula (Carriker 1981).

Membrane-bound secretion granules and vesicles in the discharged hypertonic ABO secretion (Carriker 1973, Carriker et al. 1978a, Nylén et al. 1969, Humphrey 1990) and solubilization by the secretion of the organic matrix of shell prisms provide circumstantial evidence for the presence of enzymes (possibly a concholinase). Inactivation of the shell-dissolving activity of the excised ABOs by heat and papain further suggest this view. That proteins are present in the ABO secretion of a muricid was demonstrated by

Evans (1980). Hydrogen, chloride, and sodium ion concentrations in the secretion indicate its acidic (probably HCl) and hypertonic (NaCl) characteristics (Carriker et al. 1978a). A chelating agent, although not chemically identified, and an acid mucopolysaccharide present in the secretory epithelium of the ABO (Bernard and Bagshaw 1969, Smarsh et al. 1969, Carriker and Chauncey 1973) could function in chelation.

The intricate distal zone of the secretory epithelium of the ABO is elaborately microstructured for active ionic transfer: long, dense microvilli, gap junctions, numerous microtubules, a rich capillary system, and a dense nerve net (Carriker et al. 1963, Nylén et al. 1969, Carriker 1973, Chétail et al. 1982, Webb and Saleuddin 1977, Humphrey 1990). The highly hydrophilic, negatively charged layer of acid mucopolysaccharides on the microvillar border of active ABOs facilitates the flow of ions and the function of secretion and absorption (Chétail and Fournié 1980).

Initial contact of the ABO epithelium with the borehole surface probably activates the carbonic anhydrase within the secretory cells. The enzyme catalyzes the hydration of metabolic carbon dioxide, producing hydrogen ions (from the dissociation of the carbonic acid), which are selectively transported into the borehole. These ions are involved in the release of calcium ions, water, and carbon dioxide from the shell calcium carbonate (and other shell carbonates, Carriker et al. 1978b). The carbon dioxide readily permeates the secretory cell membranes, and functions synergistically with cellular metabolic carbon dioxide and ATP to increase the rate of acid production (Webb and Saleuddin 1977). Simulta-



neously, an increase in oxygen and pedal blood raises the metabolic rate and the activity of the mitochondrial population in the secretory cells (Person et al. 1967). The sodium ions in the secretion (Carriker et al. 1978a) are active in exchange, reabsorption, and transport of solutes across the secretory cell membranes (Humphrey 1990). As sodium ions pass into the secretory cells along the chemical gradient, hydrogen ions transfer out, acidifying the narrow space between the ABO and the borehole wall. The activity coefficients of calcium and carbonate ions, thus, probably decrease because of the low pH and because of the ion pairing of carbonate and bicarbonate ions with sodium ions. Removal of the free calcium ions from the borehole by chelation and ion transport across the cells of the ABO further decreases the concentration of calcium ions and increases the solubility product of the borehole shell calcium carbonate. Calcium ions may also serve as counterions to hydrogen ion transport. The role of chloride ions (Carriker et al. 1978a) in ionic transport is less clear. Calcium ions resulting from the solubilization of the shell pass through the secretory epithelium into the ABO intergroup sinuses and thence into the snail's pedal blood stream (Chétail and Fournié 1970). When the borehole has been completed, the blood supply in the ABO is reduced, and metabolic rate and production of acid diminish (Webb and Saleuddin 1977). Glycogen in inactive ABOs is probably used to fuel ionic exchange pumps and for the synthesis and release of the secretory products in active ABOs.

#### BORING GASTROPODS LACKING AN ABO

The dorid nudibranch, *Okadaia elegans* Baba, bores holes in the calcareous tubes of spirorbid and serpulid polychaetes and feeds on them (Young 1969). Its holes are smooth, round, and beveled. However, the nudibranch does not possess a typical gastropod-type accessory boring organ; instead, a collar of conspicuous glandular cells surrounds the lumen of the proboscoidal stomodeum. During shell penetration, the stomodeum is everted fully, releasing a secretion, while simultaneously rasping with its radula.

*Cassia tuberosa* (Linnaeus) and *Cypraecassis testiculus* (Linnaeus) (tonnacean mesogastropods) drill round holes in the tests of echinoids to feed on them (Hughes and Hughes 1981). These snails possess two large salivary glands that deliver a secretion rich in sulfuric acid (pH 1) through long ducts passing through the nerve ring, along the proboscis, to the buccal cavity. Penetration of the prey test is achieved within about 10 minutes by the combined action of the acid and the radula.

The marginellid neogastropods, *Austroginella johnstoni* (Petterd) and *A. muscava* (Lamarck), which bore round holes in the shell of their molluscan prey, also lack an ABO. The anterior end of the proboscis is supplied with an abundance of subepithelial gland cells, which may secrete the solvent for dissolving the shell. The surface of the hole is highly corroded, lacks evidence of radular scraping, or wear of the radular teeth, and its inner diameter is proportionally very small. These observations suggest that penetration is dominantly solutional (Ponder and Taylor 1992). The marginellid holes are similar to those bored by octopods. The composition of the shell-dissolving secretion in the dorids and marginellids has not been determined.

#### EMBRYOLOGY OF THE ABO

The ABO develops early in the embryology of boring gastropods. In *Lunatia lewisii* larvae, for example, the ABO is first recognizable as a patch of slightly enlarged epidermal cells located

a short distance behind the ventral lip of the mouth on the proboscis tip. As development of the embryo progresses, the epithelial cells of the prospective ABO proliferate, grow taller and columnar in shape, and acquire darkly staining inclusions. In the laboratory within 3 to 5 days of metamorphosis, these larvae drill and ingest small bivalves and ostracods (Page and Pedersen 1998).

In *Nucella lapillus*, the ABO opening is soon visible in the foot of veligers (Ball et al. 1997). The early development of the ABO in muricids has not been reported.

It has been demonstrated by several investigators that naticid and muricid juveniles drill small prey and feed on them very soon after hatching: *Concholepas concholepas*, young barnacles and conspecifics (DiSalvo and Carriker 1994); *Nassarius festivus*, conspecifics (Morton and Chan 1997); *Natica gualtieriana*, *Bittium* sp. (Berg 1976); *Ocenebra erinacea*, small barnacles and conspecifics (Humphrey 1990); *Polinices duplicatus*, young *Gemma gemma* (Wiltse 1980); *Lunatia lewisii*, small bivalves and ostracods (Page and Pedersen 1998); *Urosalpinx cinerea*, small oysters, barnacles, clams (Carriker 1957).

Fretter (1946) noted that in newly hatched *Nucella lapillus*, the ABO is very large, its diameter being equal to nearly one-third of the width of the foot. Humphrey (1990) observed the same proportionately large ABO in the foot of young *Ocenebra erinacea*. It seems that cannibalism among these young snails occurs primarily when no other prey are present.

Morton and Chan (1997) were the first to report the evidence for shell boring by a species of the Nassariidae. The boring mechanism has not been described.

#### EVOLUTION OF THE ABO

The fossil record shows that shell boring by gastropods evolved late in the biological history of the Earth, probably in the Upper Cretaceous. After this, the frequency of bored shells increased dramatically, attesting the success of shell boring in obtaining food (Carriker and Yochelson 1968, Sohl 1969). The abundant, almost worldwide presence of gastropod borings in recent marine molluscan valves further demonstrates the ubiquity and dominance of boring gastropod species. Shell boring not only permits the predator to feed in relative safety, but allows it to consume prey many times larger than itself. Although the position of the muricoidean ABO in the foot and that of the Naticoidea on the proboscis tip differs, the function of the ABO in the two taxa is similar, and thus analogous. However, the glands in the two groups are not homologous; their development undoubtedly represents a case of convergent evolution (Wiley 1981, Kabat 1990).

Although possible modern precursors suggesting the line of evolution of the ABO from its late Cretaceous beginnings have not been found, embryology does suggest a possible course. In the larvae of *Polinices lewisii* (Page and Pedersen 1998), for example, the ABO is first recognized as a patch of slightly enlarged epidermal cells located a short distance behind the ventral lip of the mouth on the proboscis tip. As development progresses, the epidermal cells of the prospective ABO proliferates, become tall and columnar in shape and acquire darkly staining inclusions. From this sequence, we can hypothesize that in muricoideans mucus-secreting cells in the midventral pedal epithelium also evolved into unicellular glands that came to secrete a shell softener. For unknown reasons, when the specialized secretory pad invaginated, innervation and vascularization increased, and retracting muscles formed. Cilia from the original mucus cells, for the most part

disappeared and were replaced by a dense brush border of microvilli. The few remaining cilia assumed sensory functions. Because of the position of the organ in the sole of the foot, only evolution in the direction of invagination was practical. Other possible evolutionary experiments, such as evagination, which would have impeded locomotion, or remaining at the level of the pedal epithelium, which might have limited full development of the organ, must have failed. Evolvement of the ABO peduncle made possible lengthy eversion and the capacity to bore through thick-shelled prey.

In those species of female muricoideans in which the ABO lies atop the egg capsule gland utilizing a common duct, an origin of the ABO similar to that of males is difficult to envision. Kool (1993a) suggested that the condition of the ABO atop the capsule gland evolved first, and from this position, the two separate openings to the outside evolved. It is also likely that the two positions formed independently: that of the ABO in front of the capsule gland, in a way similar to that of the ABO in males; and that of the ABO atop the capsule gland, by specialization of a patch of epithelium in the roof of the capsule gland.

Table 1 shows that the ABO and ventral pedal gland in all species of Muricinae, Muricopsinae, Ergalataxinae, Trophinae, and Rapaninae (less *Concholepas concholepas* and *Cymia tecta*) possess a common duct, and that all species of the Ocenebrinae (less *Haustrum haustrum* and *Muricanthus fulvescens*) are characterized by separate openings. These data support Kool's (1993a) findings that ABOs with separate ducts are restricted primarily to the Ocenebrinae, and those with a common duct, to the Rapaninae. His report that *C. concholepas* possesses an ABO atop the ventral pedal gland may be in error (Gruber and Carriker 1990).

Evolution of the ABO under the end of the naticoidan proboscis, compared to the evolution of the muricoidean ABO, was probably a relatively simple one. It could have involved a transformation of mucus-secreting epithelium of the proboscis sheath into a pad of tall epithelium secreting a shell softener, and a consequent thickening of the pad in situ. Its formation as a slightly projecting, nonretractile gland was successful because of its position on the proboscis and the protection from mechanical injury afforded by a retractable proboscis.

The literature discloses little evidence of possible precursors of the ABO in modern gastropods. Fischer (1922) observed that the naticid *Sigaretus* sp. possesses under the end of its proboscis a bi-lobed organ that he suggested could be a homologue to the naticid ABO. This interesting possibility has not been pursued. In another case, Fischer searched for the ABO in *Natica catena* da Costa, but found it only in individuals "of great size." He considered this an example of tardy development in ontogeny.

In any event, presence of the long cephalic proboscis was undoubtedly necessary before initiation of the evolution of the ABO. Conversely, elongation of the cephalic snout into a proboscis did not necessarily serve as the catalyst for the evolution of the ABO, as demonstrated by the many species of gastropods that possess a lengthy proboscis, but lack an ABO.

What stimulated the original evolution of these specialized shell-dissolving epithelial pads in the first place, is a fascinating question for which there seems to be no answer. However, we do know that external molluscan epithelia are highly plastic physiologically, versatily capable of depositing shell or removing it as the state of development or other conditions require. During the ontogenetic development of the shell, for example, new shell is

deposited along the valve margins and inside the valve(s) accommodating the increase in size and reshaping the form of the soft parts. Several instances can be cited.

A striking case is that of the muricid gastropods *Chicoreus brevifrons* (Lamarck) and *Muricanthus fulvescens* (Sowerby), whose shells are ornamented with conspicuous spines that run the breadth of the whorls. In the process of the spiral growth of the shell, the snail must remove the older spines that come to lie along the inner lip of the aperture in order to make room for the new shell of the enlarging body whorl. If the spines were not removed, they would block the movement of the snail body in and out of the shell, entombing the animal in its own shell. The response of the snail is partially to dissolve the base of the offending spines with secretion from the mantle edge until the spines fall away. What part of the complex mantle margin secretes the dissolving substance and what part deposits new shell, or whether the same secretory epithelium functions alternately in shell dissolution and shell formation, is not yet known (Carriker 1972).

Another case is that of bivalves that employ a shell dissolving secretion to open burrows for themselves in molluscan shell. An unusual example is that of the pholad bivalve *Penitella conradi* Valenciennes that dissolves burrows in the shell of the abalone *Haliotis rufescens* Swainson. The burrowing process proceeds mainly by chemical dissolution of the calcareous substrate by a shell-dissolving secretion released by the mantle. This remains in close contact with the burrow wall as the excavation is enlarged to permit increase in the growth of the pholad (Smith 1969).

A final, unusual case is that in which minute, single-cell extensions of the mantle epithelium of such bivalves as *Corbicula fluminea* (Müller) dissolve microtubules that penetrate the shell to the outer periostracal covering. The function of the mantle extensions into the microtubules is unclear, but the tubules demonstrate the flexibility of the normally shell-depositing mantle epithelium in producing the microtubules (Aranjo et al. 1994).

It is clear that shell formation and shell dissolution, two sides of the physiological coin, are natural processes in the shelled members of the Phylum Mollusca. Consequently, it is not difficult to conceive of the evolution of special shell-dissolving organs like the ABO. The titillating question, however, is what stimulus initiated the development of such an organ in the first place, and then step-by-step carried the evolution to climax in the morphological-physiological complex that has successfully served many species of gastropods since at least the Cretaceous—seemingly with little, if any, change. A parallel question is why the ABO evolved in such different parts of the snail body in naticoideans and muricoideans. Bernard and Bagshaw (1969) liked to think of the variation as "one of the most interesting parallels in molluscan morphology." Indeed!

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In 1959, Dennis Crisp, John Blake, and the senior author col-

lected boring gastropods along the east and west shores of Florida. In 1966, during a brief expedition to Guaymas, Mexico, organized by the senior author's brother, Frederick R. Carriker, several of us collected intertidal and subtidal boring snails. And in 1981, the senior author, hosted by Director José A. Lozano, INVEMAR (Instituto de Investigaciones Marinas de Punto de Betin), searched for boring snails along shores in the vicinity of Santa Marta, Colombia. During these years, at different times, John Ballard, John Blake, Gregory Gruber, Alex Marsh, Mackie Willis, Langley Wood, and Dirk Van Zandt assisted in the ABO research.

In addition to the collections by us, live boring gastropods were airshipped to the senior author in Morehead City by friends from other parts of the world:

- Australia, Sydney Harbor: D. F. McMichael
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- Canada, St. Andrews: Neil Bourne
- England, Burnham-on-Crouch: D. A. Hancock, Duncan Waugh
- England, Plymouth: D. P. Wilson
- Hawaii, Oahu: E. Allison Kay
- Japan, Sendai: Akimitsu Koganezawa
- Korea, Seoul: Yongbok Cho

- Philippines, Marinduque (these were preserved snails from the DMNH, Smithsonian Institution): Jerry Harasewych
- Puerto Rico, Mayaguez: Juan Rivero, Jeff Rogers, Paul Shave
- United States, Massachusetts, Woods Hole, Gloucester: Langley Wood
- United States, Virginia, Chincoteague: Thomas Carver, Michael Castagna
- United States, Virginia, lower Chesapeake Bay: William Hargis
- United States, Washington, Quilcene: Lee Fosdick, Cedric Lindsey.

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## EFFECT OF FRESHWATER IMMERSION ON ATTACHMENT OF THE JAPANESE OYSTER DRILL, *CERATOSTOMA INORNATUM* (RECLUZ 1851)

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**ABSTRACT** The Japanese oyster drill, *Ceratostoma inornatum*, has plagued the northeast Pacific oyster industry for most of the 20th century. To prevent its spread in Washington state, the Washington Department of Fish and Wildlife regulates the intrastate transfer of shellfish between growing areas. Immediately after each transfer into or from an area infested with *C. inornatum*, all working surfaces (e.g., decks of vessels or beds of vehicles) must be rinsed or washed down, preferably with freshwater. The motivation for this rule was based on a previous study that showed that oyster drills detach under hyposaline conditions (7.2–18.0 ppt). The objectives of our study were to test the hypothesis that freshwater (0 ppt) causes *C. inornatum* to detach and to estimate the freshwater bath time,  $T_p$ , so that the probability of oyster drills remaining attached after  $T_p$  is less than  $P$  (range =  $1 \times 10^{-1}$  to  $1 \times 10^{-9}$ ). To determine the time to detach in freshwater  $T$ , individual *C. inornatum* ( $n = 373$ ) were placed on oyster valves at the bottom of a seawater (28.5 ppt) holding tank and allowed to attach themselves. Once attached, the oyster valves and oyster drills were removed and immersed in a bath of freshwater (0 ppt). Detachment was indicated by *C. inornatum* rolling off the oyster valves;  $T$  was measured using a digital stopwatch.  $T$  ranged from 0.7 to 190.5 s and varied considerably for all size classes. A linear regression of log-normal  $T$  on shell length revealed a significant and positive size correlation. Large *C. inornatum* took longer to detach than smaller conspecifics. Assuming a log-normal probability distribution for  $T$ ,  $T_p$  of oyster drills measuring  $\geq 40$  mm shell length ( $n = 53$ ) ranged from 82 to 1,213 s (1.4 to 20.2 min) for  $P = 10^{-1}$  to  $10^{-9}$  (i.e., 1 in 10 oyster drills is expected to remain attached after being immersed in freshwater for 82 s, and so on). Our results can be used by resource managers and shellfish growers alike to reduce the risk of *C. inornatum* infestation to both wild and cultured shellfish stocks.

**KEY WORDS:** *Ceratostoma inornatum*, oyster drill, shellfish pest control, shellfish transfer protocol

### INTRODUCTION

Washington state (USA) is a leading producer of cultured bivalves, including the Pacific oyster, *Crassostrea gigas* (Thunberg 1793) (Cheney and Mumford 1986). The Japanese oyster drill, *Ceratostoma inornatum*, is a predatory snail (Neogastropoda: Muricidae) that has plagued Washington's oyster industry ever since its accidental introduction to the state during the early 1920s with shipments of *C. gigas* from Japan (Galtsoff 1932, Quayle 1969, Chew 1990). Predation by *C. inornatum* can cause major losses to shellfish farmers in Washington. According to one study (Westley 1965) by the Washington Department of Fish and Wildlife (WDFW), formerly the Washington Department of Fisheries (WDF), in oyster drill-infested areas of the state, mortality in out-planted oyster seed increased by at least 25% because of predation during the first 6 months after planting, resulting in lower total yields. Furthermore, oyster drill control measures, such as transplanting oyster crops to uninfested areas, increased production costs by 17%. These combined with seed losses, Westley (1965) predicted, decreased net profits by 55% because of predation by *C. inornatum*.

By 1945, *C. inornatum* was detected in most of the commercial shellfish growing areas of Washington, the result of unchecked oyster transplanting activities during the previous decades. At the time, total eradication of the oyster drill was the favored solution to the problem. In response, the WDF explored a number of possibilities, including chemical molluscicides, barriers, and attractants. However, most methods failed or were considered impractical in the field because of logistic or environmental concerns

(Chambers et al. 1972). Therefore, to prevent the spread of oyster drills, the WDF began regulating the intrastate transplantation or transfer of oysters and oystering equipment between growing areas (Glud[Glude] 1947). Given the impact of *C. inornatum* on the shellfish industry, and the improbability of eradicating the pest, this practice is continued today by the WDFW.

The WDFW classifies all marine waters, tidelands, and shellfish handling facilities within the state as "restricted" with respect to the presence of aquatic diseases, including such harmful pests as *C. inornatum*. Those marine habitats and facilities lying outside restricted shellfish areas are designated "unrestricted." A WDFW pamphlet (Mueller et al. 1997) describes the guidelines and requirements for most types of shellfish transfers between these areas. According to the pamphlet, immediately after each shellfish transfer into or from a restricted area, all working surfaces (e.g., decks of vessels or beds of vehicles) must be rinsed or washed down, preferably with freshwater, at a location where the rinsed material or debris cannot reach tidelands. The purpose of the freshwater rinse or wash-down is to remove oyster drills from products and equipment used during a shellfish transfer.

The original motivation for this condition was based on the observations of Chapman and Banner (1949), who reported that oyster drills [*C. inornatum* as well as the native oyster drill, *Nucella lamellosa* (Gmelin 1791)] detach under hyposaline conditions ( $\leq 18.0$  ppt). In their study, Chapman and Banner monitored the activity of oyster drills exposed to different treatment salinities (7.2, 11.6, 15.2, 18.0, 22.4, 26.3, and 28.9 ppt) for 11 d. Each treatment consisted of a single holding jar containing 10 oyster drills immersed in water of prescribed salinity. After 15.5 h expo-

sure to salinities  $\leq 18.0$  ppt, the percentage of *C. inornatum* attached and crawling on the sides of holding jars declined with decreasing salinity. For example, in 18.0 ppt, 80% of the oyster drills remained attached; whereas, in 7.2 ppt, no oyster drills remained attached. One objective of our study was to extend the earlier study of Chapman and Banner by rigorously testing the hypothesis that freshwater (0 ppt) causes *C. inornatum* to detach. A second objective was to estimate the time to detach in freshwater, as a basis for improving methods of controlling a pest that affects both wild and cultured shellfish stocks alike.

## MATERIALS AND METHODS

The study was conducted at the WDFW's Point Whitney Shellfish Laboratory located on the Hood Canal, Puget Sound, Washington during 18 June to 14 August 1996 and 12 to 14 May 1997. Several hundred juvenile and adult *C. inornatum* were collected from the Point Whitney Lagoon (47°45'44"N. Lat; 122°51'07"W. Long.) and maintained in an aerated, seawater (28.5 ppt) holding tank (53 L) at ambient temperature (16–18 °C).

To determine the time to detach in freshwater  $T_i$ , we placed individual *C. inornatum* ( $n = 373$ ; size range = 5.7–50.5 mm shell length; Fig. 1) on single oyster valves at the bottom of the holding tank. Oyster drills were allowed to attach themselves, which was indicated by crawling movements across the oyster valves (elapsed time <2 min). The oyster valves and attached oyster drills were then removed from the holding tank by hand, tilted slightly (<30° from horizontal), and immersed in a plastic tub filled with freshwater (0 ppt) at ambient temperature. Detachment was indicated by *C. inornatum* rolling off the tilted oyster valves; time to detach was measured for each oyster drill using a digital stopwatch. To serve as a control, and to confirm that freshwater rather than handling caused *C. inornatum* to detach, we followed the same procedures described above, using an additional 50 oyster drills (size range = 8.5–44.6 mm shell length) with one difference: *C. inornatum* and the oyster valves were held in a bath of seawater instead of freshwater.

During preliminary testing, we observed longer times to detach for larger oyster drills. Based on the skewed shape of the histogram of times to detach in freshwater (Fig. 2), we log-transformed the data, then conducted a linear regression to confirm that size and time to detach were significantly and positively related. The linear regression model was

$$\ln(T_i) = \alpha + \beta(L_i) + \varepsilon_i$$

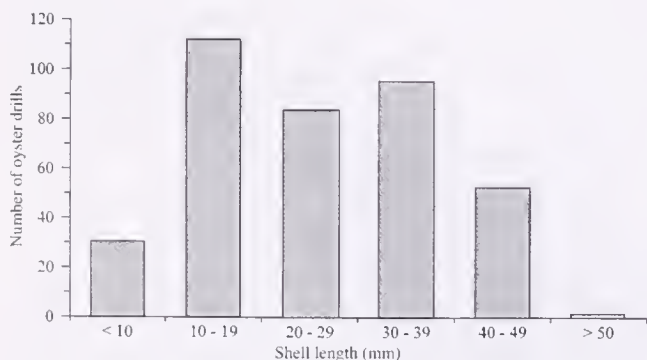


Figure 1. Length-frequency distribution of *C. inornatum* in treatment group ( $n = 373$ ).

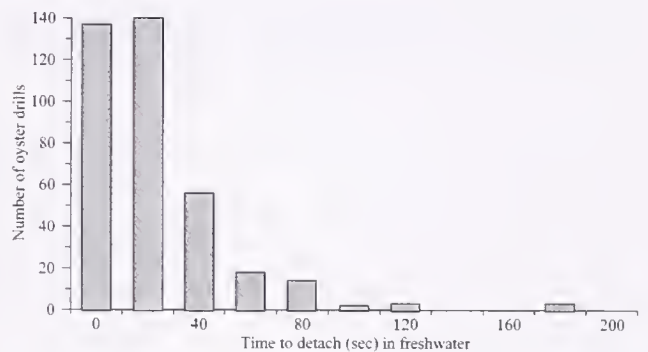


Figure 2. Frequency distribution of the time to detach in freshwater for all *C. inornatum* in treatment group ( $n = 373$ ; size range = 5.7–50.5 mm shell length).

where  $T_i$  was the time to detach of the  $i$ th oyster drill,  $L_i$  was the length of the  $i$ th oyster drill, and  $\varepsilon_i \sim \text{normal}(0, \sigma^2)$ . Because  $\beta$  was significantly positive, the oyster drills in the largest size class ( $\geq 40$  mm shell length; Fig. 1) were expected to have the longest times to detach. Consequently, we estimated freshwater bath time,  $T_p$ , so that the proportion of the largest oyster drills that remain attached after  $T_p$  is less than  $P$  (range =  $1 \times 10^{-1}$  to  $1 \times 10^{-9}$ ).

Because the regression was conducted on  $\ln(T)$ , we first estimated the required bath time,  $T_p$ , on a log scale and then exponentiated the result to obtain the required real time,  $T_p$ . To estimate  $T_p$ , we used the condition that the probability of  $\ln(T)$  being greater than  $T_p$  must be  $p$ , that is

$$P(\ln(T) > T_p) = p$$

This led to the solution

$$T_p = Z_p \sigma + \mu$$

where  $\ln(T)$  was normally distributed with mean  $\mu$  and standard deviation  $\sigma$ , and  $Z_p$  was the  $(1 - p)$ th quantile from a standard normal distribution (Zar 1984). We estimated  $\mu$  and  $\sigma$  from the  $\ln(T)$  of the oyster drills in the largest size class, so that

$$\hat{T}_p = Z_p \sigma + \mu$$

which resulted in an estimate of  $T_p$  as

$$\hat{T}_p = \exp(\hat{T}_p)$$

## RESULTS

Time to detach in freshwater ranged from 0.7 to 190.5 s, or roughly 3 min (Fig. 2). Although there was considerable variation in time to detach within size classes, a linear regression of the natural log of the time to detach on shell length revealed a slight, but positive significant relationship ( $P < 1 \times 10^{-15}$ ,  $n = 373$ ). On average, large *C. inornatum* took longer to detach in freshwater than smaller conspecifics (Fig. 3). In the control group, all but one of the oyster drills ( $n = 50$ ) remained attached to the oyster valve after 210 s. This individual (size = 23.8 mm shell length) detached after 178 s while crawling along the thin lip of the oyster valve (i.e., lost its foothold and fell).

The estimated mean and standard deviations for  $\ln(T)$  of the largest oyster drills ( $\geq 40$  mm shell length,  $n = 53$ ) were  $\hat{\mu} = 3.68$  and  $\hat{\sigma} = 0.57$ . The estimated freshwater bath times,  $T_p$ , so that the proportion of the largest oyster drills remaining attached after  $T_p$  was less than  $p$  are given in Table 1. For example, after being



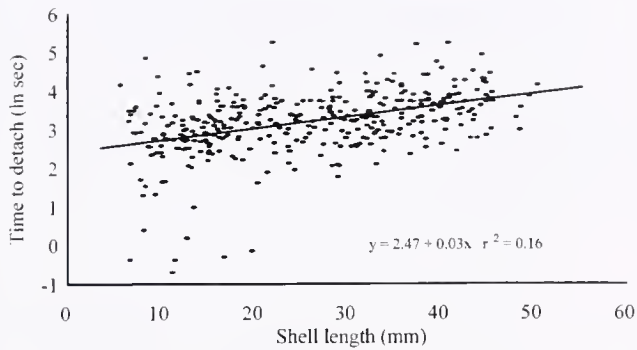


Figure 3. Relationship of the natural log of the time to detach (s) on shell length (mm) of *C. inornatum* immersed in freshwater ( $P < 1 \times 10^{-15}$ ;  $n = 373$ ).

soaked in freshwater for 82 s, one in ten of all *C. inornatum*  $\geq 40$  mm shell length are expected to remain attached.

#### DISCUSSION

The use of fresh- and brackish water has long been declared effective in controlling oyster drills. For example, Federighi (1930) described a procedure called "floating," wherein oysters infested with the Atlantic oyster drill, *Urosalpinx cinerea* (Say) 1822, were immersed in large containers of brackish water (12–14 ppt) for 10 days. Evidently, this treatment was sufficient to kill *U. cinerea* without harming the oysters. Butler (1953) concluded that, although the southern oyster drill, *Stramonita haemastoma* Linnaeus, 1758, was capable of surviving prolonged exposure to freshwater, the only way to prevent the spread of *S. haemastoma* was through sustained hyposaline conditions ( $<15$  ppt). Accordingly, Pollard (1973) proposed the diversion of freshwater from the Mississippi River to control populations of *S. haemastoma* by flooding Louisiana state oyster grounds with brackish water. A follow-up study by Breithaupt and Dugas (1979) indicated that Pollard's proposal was plausible and, since 1991, *S. haemastoma* has been eliminated from some oyster grounds because of a successful freshwater diversion program (Greg Laiche, Louisiana Wildlife and Fisheries Commission, pers. comm.).

Our study showed unequivocally that freshwater causes *C. inornatum* to detach from its foothold. We also showed that time to detach was positively correlated to size and that the bath times required to remove the largest oyster drills could be over 1,200 s or 20 min. Because the regression was significantly positive, the largest oyster drills represented the "worst case scenario," requir-

TABLE 1.

Estimated freshwater bath times  $T_p$ , so that the proportion of the largest *C. inornatum* ( $\geq 40$  mm shell length) remaining attached after  $T_p$  is less than  $P$  (range =  $1 \times 10^{-1}$  to  $1 \times 10^{-9}$ ). Values are based on the log-normal distribution of bath times,  $T$  ( $n = 53$ ), with  $\hat{\mu} = 3.68$ ,  $\hat{\sigma} = 0.57$ , and  $Z_p = (1 - p)$ th quantile from a standard normal distribution (Zar 1984).

$P$	$Z_p$	$\hat{T}_p = \exp\{(0.57 \times Z_p) + 3.68\}$
$1 \times 10^{-9}$ (1 in 1 billion)	6.00	1,213 s (20.2 min)
$1 \times 10^{-6}$ (1 in 1 million)	4.75	596 s (9.9 min)
$1 \times 10^{-3}$ (1 in 1 thousand)	3.09	231 s (3.9 min)
$1 \times 10^{-1}$ (1 in ten)	1.28	82 s (1.4 min)

ing the longest bath times. Because the motivation for this study was removal of most, if not all, oyster drills, estimating the freshwater bath times for the largest individuals was the most conservative approach, precluding the need to estimate bath times for smaller oyster drills.

We confirmed the observations made by Chapman and Banner (1949) that hyposaline conditions cause oyster drills to detach and support the recommendation to use freshwater, if available, when rinsing or washing down working surfaces, products, or equipment after each shellfish transfer into or from areas infested by *C. inornatum*. Our results strongly suggest that a short, haphazard freshwater rinse or wash-down will not be sufficient to remove all oyster drills, because *C. inornatum* is capable of maintaining its foothold for potentially 1,213 s or 20.2 min when completely immersed in freshwater (Table 1). Still, we have shown that longer exposures to freshwater substantially decrease the probability of *C. inornatum* maintaining its foothold. Our results can be used by resource managers and shellfish growers alike when developing shellfish transfer protocol to reduce the risk of *C. inornatum* infestation to both wild and cultured shellfish stocks (*sensu* Elston 1992).

#### ACKNOWLEDGMENTS

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## FATTY ACIDS AND STEROLS OF *RAPANA VENOSA* (VALENCIENNES, 1846)

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**ABSTRACT** The composition of fatty acids and sterols was investigated in different organs of *Rapana venosa*. The isolated lipids were saponified then esterified, and fatty acid esters were analyzed by gas chromatography (GC). The total fatty acid content in the hepatopancreas was > right massive gland salivary > gonad > flesh. In the flesh, the ratio of saturated to unsaturated fatty acids was 0.50. The highest amounts of fatty acids in the flesh were stearic acid in the saturated fatty acids, and n-3 and n-6 acids in the polyunsaturated fatty acids at 0.30, 0.32, and 0.38 µg/mg, respectively. The sterols isolated from *R. venosa* were analyzed by gas chromatography/mass spectrometry (GC/MS). Two sterols were identified in the flesh and nine sterols in the whole organs.

**KEY WORDS:** *Rapana venosa* organs, fatty acids, sterols

### INTRODUCTION

Lipids and sterols of marine invertebrates have been intensively studied, but relatively little is known for shellfish. *Rapana venosa* (Valenciennes 1846) (formerly *R. thomasiensis thomasiensis*), Mollusca, Gastropoda. In shellfish, lipids percentage (Rosoiu and Serban 1981, Rosoiu and Panait 1992) and whole body fatty acid content of lipids (Christie et al. 1988) were investigated.

Cholesterol is the only sterol reported in *R. venosa* (Tsujimoto and Koyanagi 1934). Other contents of *R. venosa*, such as heparin (Güven et al. 1991), insulin, and some enzymes (Akinci et al. 1998a, Akinci et al. 1998b, Akinci et al. 1998c) were also investigated.

Fatty acids (FAs) composition in marine organisms includes highly polyunsaturated fatty acids (PUFAs). The medically important PUFAs are 20:5n-3, eicosapentaenoic acid (EPA) and 22:6n-3 docosahexaenoic acid (DHA). These PUFAs can reduce the risk of cardiovascular diseases and reduced platelet aggregation (Seidelin et al. 1992; Eritsland et al. 1995; Daviglus et al. 1997). Shellfish as a food source in such countries as Japan entails the need to investigate fatty acids as nutritional components. In this paper, the composition of fatty acids and sterols in different organs of *R. venosa* is reported.

### MATERIALS AND METHODS

*Rapana venosa* was collected between July to September 1997 from the Black Sea near the entrance of the Bosphorus at a depth of 20–30 m and stored at –30 °C. Flesh, hepatopancreas, right massive gland salivary, and gonad were separated according to Lupu (1977) and stored at –70 °C until analysis. The standard fatty acid methyl esters were obtained from Sigma and authentic cholesterol from Merck.

#### Total Lipid Extraction

The frozen organs were thawed, carefully weighed (200–300 mg), and homogenized in cold 154 mM NaCl. The total lipids were extracted according to the method of Folch et al. (1957) modified by Yazici et al. (1994). 0.1 ml internal standard (200 µg heptadecanoic acid in chloroform), 0.1 ml of methanol containing 20 g/l butylated hydroxytoluene, as antioxidant, 2 ml methanol, and 4 ml chloroform added to the homogenated tissue. The sample was mixed vigorously using a vortex for 2 min, followed by cen-

trifugation at 2,000 g for 10 min at 4 °C. The chloroform phase was separated and evaporated to dryness at 37 °C under a stream of nitrogen.

#### Saponification of Lipids, Methylation and Analysis of Fatty Acid Esters

The total lipid extract was hydrolyzed in 8 ml of KOH/ methanol (2% v/w) under reflux in a water bath for 30 min. After cooling, 1 ml of 14 % BF<sub>3</sub>-methanol was added and heated for 2 min at 100 °C then cooled, 5 ml distilled water and 2 ml hexane were added, vortex-mixed for 2 min, and centrifuged (2,000 g; 10 min at 4 °C). The hexane phase was separated, evaporated to dryness under a stream of nitrogen, and the residue was dissolved in hexane in a volumetric flask and applied to gas chromatography (GC).

#### Isolation of Sterols

Sterols were extracted from the flesh and in whole organs of *R. venosa* with dichloromethane (DCM) in Soxhlet apparatus for 4 h. The extract was distilled and hydrolyzed with 5 % KOH in methanol for 30 min under reflux in water bath. After hydrolysis, two volumes of water were added and extracted with DCM, and the organic phase was separated then distilled. The residue was collected with hexane and applied to gas chromatography/mass spectrometry (GC/MS) analysis.

#### GC Analysis

The FA methyl esters were analyzed by capillary gas chromatography (Perkin-Elmer 8420 Capillary Gas Chromatography, Gouda, The Netherlands). Column: 25 × 0.25 mm ID, QC2/BP × 70, 0.25 µm film; flame ionization detector temperature 300 °C; split injector temperature 300 °C; oven temperature program from 150 to 230 °C at 2 °C min; carrier gas N<sub>2</sub>.

FAs were identified by their retention time and compared to those of the standards. Their amounts were estimated by calculating the corresponding areas.

#### GC/MS Analysis

The analysis of sterols was run on an HP6890 capillary gas chromatograph connected to an HP MSD and controlled by an HP

TABLE 1.  
Fatty acid profiles of lipids of *Rapana venosa* organs.

Fatty Acid	Flesh	Hepatopancreas	Right Massive Gland		P
			Salivary	Gonad	
14:0	0.07 ± 0.00 B	2.21 ± 0.27 C	1.28 ± 0.17 A	0.21 ± 0.03 B	0.0000
15:0	0.04 ± 0.01 B	0.62 ± 0.03 A	0.42 ± 0.11 A	0.42 ± 0.07 A	0.0016
15:0 iso 14-methyl	Trace	0.20 ± 0.02	0.14 ± 0.06	Trace	
16:0	0.20 ± 0.01 B	5.63 ± 0.52 C	3.35 ± 0.73 A	0.65 ± 0.09 B	0.0001
16:1n-7	0.09 ± 0.00 B	1.73 ± 0.17 C	0.64 ± 0.05 A	0.23 ± 0.04 B	0.0000
16:1n-9	trace	0.49 ± 0.21 B	0.24 ± 0.09 A	0.06 ± 0.01 A	0.0084
16:0 iso 14-methyl	trace	0.45 ± 0.06	0.27 ± 0.06	trace	
16:0 iso 15-methyl	0.02 ± 0.01 B	0.33 ± 0.02 C	0.24 ± 0.03 A	0.09 ± 0.02 B	0.0006
17:0 iso 16-methyl	0.09 ± 0.02 D	0.84 ± 0.04 C	0.61 ± 0.04 A	0.31 ± 0.04 B	0.0001
17:0 iso 15-methyl	0.07 ± 0.02 B	0.53 ± 0.09 A	0.31 ± 0.10 AB	0.23 ± 0.06 B	0.0137
18:0	0.28 ± 0.01 D	2.02 ± 0.01 C	1.44 ± 0.28 A	0.89 ± 0.03 B	0.0001
18:1n-13	0.07 ± 0.01 A	0.45 ± 0.03 B	0.27 ± 0.08 A	0.19 ± 0.04 A	0.0090
18:1n-9	trace	0.68 ± 0.090	0.34 ± 0.18	0.06 ± 0.01	
18:1n-11	trace	0.51 ± 0.05 B	0.22 ± 0.08 A	0.03 ± 0.01 A	0.0090
18:1n-7	trace	0.34 ± 0.02 B	0.16 ± 0.03 A	trace	0.0115
18:2n-6	0.09 ± 0.07 B	0.40 ± 0.03 A	0.29 ± 0.07 A	0.32 ± 0.05 A	0.0043
18:3n-3	0.01 ± 0.00 B	0.35 ± 0.04 A	0.21 ± 0.06 A	0.09 ± 0.00 B	0.0140
20:0	trace	0.36 ± 0.05	0.18 ± 0.09	trace	
20:1n-9	0.12 ± 0.02 B	4.78 ± 0.47 A	2.94 ± 1.31 A	0.50 ± 0.03 B	0.0047
20:1	0.02 ± 0.00 B	1.20 ± 0.01 C	0.78 ± 0.12 A	0.08 ± 0.01 B	0.0001
20:1	0.02 ± 0.00 B	1.17 ± 0.13 C	0.67 ± 0.12 A	0.10 ± 0.02 B	0.0012
20:2n-9	0.03 ± 0.01 B	1.01 ± 0.29 A	0.55 ± 0.28 AB	0.22 ± 0.06 AB	0.0401
20:2n-7	0.05 ± 0.01	0.36 ± 0.24	0.30 ± 0.05	0.35 ± 0.04	
20:3n-6	0.03 ± 0.00 D	0.33 ± 0.05 C	0.23 ± 0.01 A	0.12 ± 0.01 B	0.0002
20:4n-6	0.19 ± 0.00	1.49 ± 0.06	1.24 ± 0.19	1.59 ± 0.59	0.0456
20:5n-3	0.08 ± 0.01 B	3.24 ± 0.17 C	1.91 ± 0.57 A	0.64 ± 0.09 B	0.0003
22:2n-9	0.15 ± 0.10 C	4.57 ± 1.17 A	3.10 ± 0.80 B	1.09 ± 0.09 BC	0.0093
22:2n-7	0.13 ± 0.01 B	2.14 ± 0.14 A	1.64 ± 0.27 A	0.53 ± 0.07 B	0.0001
22:3n-6	0.03 ± 0.00 B	0.51 ± 0.06 A	0.38 ± .012 A	0.15 ± 0.01 B	0.0031
22:4n-6	0.05 ± 0.01 C	0.26 ± 0.03 A	0.24 ± 0.05 A	0.39 ± 0.03 B	0.0007
22:5n-3	0.14 ± 0.01 B	1.34 ± 0.21 A	1.21 ± 0.18 A	0.52 ± 0.06 B	0.0009
22:6n-3	0.10 ± 0.02 B	3.85 ± 0.41 A	2.49 ± 0.93 A	0.52 ± 0.06 B	0.0026
n-3	0.32 ± 0.04 B	8.78 ± 0.77 A	5.83 ± 1.72 A	1.77 ± 0.20 B	0.0009
n-6	0.38 ± 0.01 B	3.00 ± 0.23 A	2.38 ± 0.40 AB	2.24 ± 0.94 AB	0.0360
n-9	0.40 ± 0.01 B	12.74 ± 2.03 A	7.92 ± 2.24 A	1.91 ± 0.50 B	0.0016
Saturated	0.77 ± 0.04 B	13.19 ± 0.95 C	8.24 ± 1.69 A	2.99 ± 0.31 B	0.0001
Unsaturated	1.4 ± 0.10 B	30.52 ± 3.40 C	20.05 ± 5.34 A	7.78 ± 1.10 B	0.0007
saturated/unsaturated	0.55 ± 0.50	0.43 ± 0.16	0.41 ± 0.03	0.384 ± 0.19	
Total	2.17 ± 0.14 B	43.71 ± 3.33 C	28.29 ± 7.02 A	10.77 ± 1.34 B	0.0003

One-way analysis of variance. The means are compared by the Student–Newman–Keuls test; values with no common capital letter differ at  $P < .05$ . Mean values are from three replicate ( $\mu\text{g}/\text{mg}$  wet weight).

ChemStation. Capillary column; Column 50 m × 200  $\mu\text{m}$  ID. fused HP PONA (methyl siloxane). Column temperature program was from 110 °C to 290 °C at 6 °C  $\text{min}^{-1}$  and 290 °C at 10  $\text{min}^{-1}$ ; split injector temperature 250 °C; carrier gas helium, 44.7 psi.

The cholesterol was identified using a cholesterol standard, and the other sterols were identified by comparing the spectrum of each peak with its corresponding spectrum from HP memory.

## RESULTS AND DISCUSSION

Fatty acid content of lipids isolated from different organs of *R. venosa* are shown in Table 1. The main saturated FAs were palmitic and stearic acid in all organs studied. Palmitic (16:0) and stearic (18:0) acid contents were lower in flesh as compared to the other organs tested. The important PUFAs, were EPA and DHA

with percentages of the total FAs in the flesh of 3.7 EPA, 4.6 DHA, in the hepatopancreas 7.4 EPA, 8.8 DHA, in the right massive gland salivary 6.8 EPA, 8.8 DHA, and in the gonad 5.9 EPA and 4.8 DHA. EPA level was lower in the flesh than in the hepatopancreas and the right massive gland salivary. The amount of EPA

TABLE 2.  
Pentaenoic and hexaenoic acids level in *R. venosa* and fish flesh (%).

Acids	Animals			
	<i>R. venosa</i>	Salmon <sup>a</sup>	Herring <sup>a</sup>	Mackerel <sup>a</sup>
Pentaenoic	10.1	8	7	7
Hexaenoic	4.6	16	5	5.5

<sup>a</sup> Nøtveit and Cyvin (1962).

TABLE 3.  
Sterols identified in *R. venosa* whole organs (1), and flesh (2).

Sterols	GC (RT)	MS Peaks
22 Dehydrocholesterol (1)	33.002	55, 69, 95, 111, 145, 159, 213, 255, 271, 300, 351, 366, 384 (M+)
27-Norengosta-5, 22-dien-3, ol. (3. beta. 22 Z) (1)	33.512	55, 69, 95, 111, 145, 161, 213, 255, 273, 300, 351, 366, 384 (M+)
Cholesterol (1), (2)	34.450	55, 95, 145, 161, 213, 231, 255, 275, 301, 353, 368 (M+)
7-Dehydrocholesterol (1)	35.130	55, 95, 119, 145, 159, 211, 325, 351, 366, 384 (M+)
Crimosterol (1)	35.473	69, 95, 133, 159, 255, 271, 300, 337, 355, 380, 398 (M+)
24-Methyl-24-dehydrocholesterol (1)	36.814	55, 81, 105, 121, 145, 161, 185, 213, 229, 271, 299, 314, 365, 383, 398 (M+)
24. beta Methyl cholesterol (1)	37.058	55, 81, 95, 119, 145, 161, 213, 273, 289, 315, 382, 400 (M+)
Cholest-4 en-3 one (1)	37.501	55, 95, 124, 147, 229, 245, 261, 342, 384 (M+)
Cholesterilene (1), (2)	31.584	55, 81, 95, 107, 120, 133, 147, 159, 213, 247, 260, 326, 353 (M+)

(0.08 µg/mg) and DHA (0.10 µg/mg) was similar to that of arachidonic acid (0.19 µg/mg). EPA and DHA competitively inhibits the utilization of arachidonic acid (Dyerberg et al. 1978).

Notevarp and Cyvin (1962), studied pentaenoic and hexaenoic acid levels in fish flesh. Their levels were high in fish but low in animal lipids. Table 2 shows the comparison of the pentaenoic (EPA, C20:5n-3; Docosapentaenoic acid, C22:5n-3) and hexaenoic (DHA, C22:6n-3) acids level in flesh of *R. venosa* with fish. The pentaenoic acids level was higher in *R. venosa* than in salmon, herring, or mackerel (Table 2).

The amount of saturated FAs in flesh was 0.77 µg/mg in comparison to 1.40 µg/mg unsaturated FAs. So far, little is known about the exact FAs composition of different organs of *R. venosa*. Total organ lipid content (Rosoiu and Serban 1981; Rosoiu and Panait 1992) and total body fatty acids content (Christie et al. 1988) were investigated earlier. In the present investigation, quantitative and qualitative studies were carried out on *R. venosa* FAs. Comparison of our findings to those of Christie et al. (1988) as calculated from their tables on saturated and unsaturated fatty acid levels shows similarity (the percentage of saturated 29.20, 29.66, and unsaturated 70.70, 70.34, respectively).

The ratio of saturated to unsaturated fatty acids were 0.55 in flesh, 0.43 in hepatopancreas, 0.41 in right massive gland salivary,

and 0.38 in gonad, thus unsaturated FAs were twice the level of the saturated FAs. The total fatty acid (saturated and unsaturated FAs) contents were ranked as hepatopancreas greater than gland salivary greater than gonad greater than flesh.

When the lipid levels of *Mytilus galloprovincialis* (Christie et al. 1988) are compared with the flesh, which is the consumed part of *R. venosa*, the lipid content of the latter was lower than the total mussel consumed.

In *R. venosa*, cholesterol was the only sterol that was reported by Tsujimoto and Koyanagi, (1934). In this work, nine sterols were identified in whole organs and two in the flesh of *R. venosa* (Table 2). Sterols were identified by using standard cholesterol and the others from the HP memory. GC/MS spectral data are given in Table 3. The mass peaks were compared in the spectra of the lipid extract obtained from *R. venosa* organs with the spectra taken from the HP memory. The similarities of both spectra (quality 96–99) were noted. In contrast, more sterol compounds were isolated from *M. galloprovincialis*, and differed from those of *R. venosa*. Considering the lipid and sterol contents of *R. venosa*, its flesh has an advantage over mussels.

In conclusion, the flesh of *R. venosa* can be considered as suitable for human consumption based on its content of FAs and sterols.

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## ANALYSIS OF KARYOTYPE, CHROMOSOME BANDING, AND NUCLEOLUS ORGANIZER REGION OF PACIFIC ABALONE, *HALIOTIS DISCUS HANNAI* (ARCHAEOGASTROPODA: HALIOTIDAE)

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**ABSTRACT** Chromosome preparations of *Haliotis discus hannai* larvae were subjected to karyotype analysis using a scanning electron microscope (SEM), banding analysis using a salt solution treatment, and nucleolus organizer region (NOR) analysis by silver staining. Standard values of the relative length and arm ratio of each chromosome pair in this species were determined by SEM measurements of the chromosome arm length. The arm ratios indicated that this species possessed 11 pairs of metacentric and seven pairs of submetacentric chromosomes ( $2n = 36$ ). This is the first study of Haliotidae in which banding is attempted (i.e., not partial banding, such as C-banding) as well as NOR analyses. The characteristic banding pattern served as a useful marker for the identification of homologous chromosomes that were difficult to identify using only morphological analysis. The NORs were located terminally on the long arms of two chromosome pairs with variations in the NOR-bearing chromosome. The techniques of karyotype identification, banding, and NORs employed in this study may serve as useful indexes in future research on cytogenetics in Haliotidae.

**KEY WORDS:** chromosome, karyotype, banding, NOR, abalone, *Haliotis discus hannai*, mollusca

### INTRODUCTION

Abalone (Haliotidae) is an economically important marine product in Japan. Therefore, wide-ranging studies on Haliotidae from various perspectives, including seed production, taxonomy, genetics, and ecology have been intensively conducted. Attention has focused on cytogenetics, because this field furnishes useful information on chromosome manipulation and taxonomy in Haliotidae. To conduct cytogenetic studies, it is necessary to establish chromosome identification techniques for reliable comparisons of intra- and interspecific karyotypes.

Chromosomal studies on Haliotidae have been performed using *Haliotis aquatilis* (Nishikawa 1962, Nakamura 1986), *H. cracherodii* (Minkler 1977), *H. discus hannai* (Arai et al. 1982), *H. d. discus* (Arai et al. 1982, Miyaki et al. 1997), *H. lamellosa* (Colombera and Tagliaferri 1983), *H. tuberculata* (Colombera and Tagliaferri 1983, Arai and Wilkins 1986), *H. diversicolor aquatilis* (Nakamura 1985), *H. varia* (Nakamura 1986, Arai et al. 1988, Jarayabhand et al. 1998), *H. gigantea* (Nakamura 1986, Miyaki et al. 1997), *H. planata*, *H. diversicolor diversicolor* (Arai et al. 1988), *H. asinina*, *H. ovina* (Jarayabhand et al. 1998), and *H. madaka* (Miyaki et al. 1999). These studies have revealed the chromosome number ( $2n = 28 \sim 36$ ) in 14 species of the above-mentioned Haliotidae. In most of these abalone species, morphological classification of the chromosome (metacentric, submetacentric, or others) has been established. However, none of these researchers has described the concrete numerical values of relative length, arm ratio, or centromeric index of the chromosomes, or reported the chromosome banding. The only chromosome banding study on Haliotidae was performed with C-banding in *H. d. hannai* (Okumura et al. 1995). Therefore, at this time the systematic approaches to Haliotidae cytogenetics based on reliable measurement of chromosome size and morphology (relative length and arm ratio) and banding patterns are difficult. Accordingly, the establishment of reliable indexes of karyotypes based on accurate measurements of chromosome arm length and the application of band-

ing or nucleolus organizer region (NOR) analysis in Haliotidae are required.

In mollusca, some species of Bivalvia and Gastropoda have been analyzed with NORs and chromosome banding. Chromosomal NORs of *Cerastoderma glaucu* (Thiriot-Quievreux and Wolowicz 1996), *Mytilus galloprovincialis* (Martinez-Exposito et al. 1997), *Ostrea angasi* (Li and Havenhand 1997), and *Nucella lapillus* (Pascoe and Dixon 1994, Pascoe et al. 1996) have been analyzed by the silver staining method. In addition, C-banding has previously been employed in investigations of *Ostrea denselamellosa* (Insua and Thiriot-Quievreux 1991) and *M. galloprovincialis* (Martinez-Exposito et al. 1997). Furthermore, a banding technique has been established for *M. galloprovincialis* using  $2 \times$  SSC/Giemsa-stain treatment (Mendez et al. 1990). Based on these studies, banding and NOR patterns have been shown to be useful markers not only for the identification of homologous chromosomes, but also to serve as effective indexes for discriminating intra- and interspecific genetic differences. Because these approaches have been successfully performed in the mollusca species noted above, banding and NOR analyses on Haliotidae may provide an avenue for differentiating chromosomal construction in Haliotidae.

In the present study, we demonstrated a reliable chromosome index from accurate measurements of the chromosome length using a scanning electron microscope (SEM) and applied the banding and Ag-NOR-staining techniques to *H. d. hannai* chromosomes.

### MATERIALS AND METHODS

*H. d. hannai* larvae were obtained at 15–20 h after fertilization by artificial hatching in a hatchery (Marine Development Company, Iwate, Japan). The larvae were treated with 0.1% colchicine, 0.075 M KCl and fixed in Carnoy solution according to the method of Arai et al. (1984). The chromosome preparations used for conducting SEM determinations of chromosome arm lengths, banding, and NOR analyses were made by the chopping method

TABLE 1.  
Chromosome measurements derived from 12 metaphase plates in  
*Haliotis discus hannai*.

Chromosome Pair No.	Relative Length		Arm Ratio		Classification <sup>b</sup>
	Mean	SD <sup>a</sup>	Mean	SD <sup>a</sup>	
1	6.88	0.20	1.21	0.08	m
2	6.69	0.33	2.09	0.19	sm
3	6.49	0.32	1.36	0.11	m
4	6.48	0.25	1.59	0.14	m
5	6.10	0.32	2.59	0.22	sm
6	5.78	0.32	1.28	0.15	m
7	5.75	0.35	2.00	0.12	sm
8	5.74	0.35	2.24	0.17	sm
9	5.71	0.41	1.63	0.08	m
10	5.47	0.24	1.84	0.10	sm
11	5.32	0.35	1.45	0.14	m
12	5.20	0.15	1.15	0.07	m
13	4.95	0.41	2.13	0.13	sm
14	4.74	0.37	1.31	0.14	m
15	4.73	0.24	1.48	0.07	m
16	4.70	0.27	1.91	0.13	sm
17	4.66	0.24	1.61	0.09	m
18	4.64	0.22	1.10	0.05	m

<sup>a</sup> Standard deviation.

<sup>b</sup> m, metacentric; sm, submetacentric.

(Yamazaki et al. 1981) using 30 to 50 fixed larvae per slide. Slides were either dried at room temperature for SEM and NOR analyses, or incubated at 50 °C before drying for banding analysis, as described below.

For observations using the SEM, slides were stained with Giemsa solution according to the method of Okumura et al. (1995), and slides with well-spread metaphases were monitored by light microscopy (LM; Nikon Optiphot). The slides were cut with a glass cutter into 15 × 15 mm<sup>2</sup> pieces bearing well-spread metaphases before attachment to a brass disk (specific for the SEM, 26-mm diameter, 7-mm thick) using adhesive carbon tape. The disk-mounted pieces were lightly coated with gold for 3 ~ 5 min using an ion coater (Jeol JFC-1100), and the metaphase plates were photographically analyzed using SEM (Jeol JSM-6400) at 5,000 ~ 10,000 magnification. The short and long arm lengths of chromosomes in 12 well-spread metaphase plates were displayed in SEM photographs and measured with a digital map meter (Koi-zumi Sokki Mfg. Co.). Relative lengths were calculated from the total chromosome length (Thiriot-Quievreux 1984), and arm ratios were derived (Levan et al. 1964) accordingly. The arm ratios and relative lengths were used for the morphological classification (Levan et al. 1964) and the numbering of chromosomes, respectively.

For banding analysis, cell suspensions from the chopped larvae expanded with Carnoy solution on the slides were incubated at 50 °C until dry. The slides were treated with a salt solution (Ohnuki 1968) at room temperature for 150 min before rinsing with tap water. The slides were then stained with Giemsa solution by the above method. Nine well-banded metaphase plates were photographed using LM or SEM, and chromosomes were measured and paired on the basis of their relative lengths, arm ratios, and banding patterns.

Ag-NOR staining was performed according to the method of Howell and Black (1980) with slight modifications. After Giemsa staining for 40 min, the slides were briefly treated with a mixture

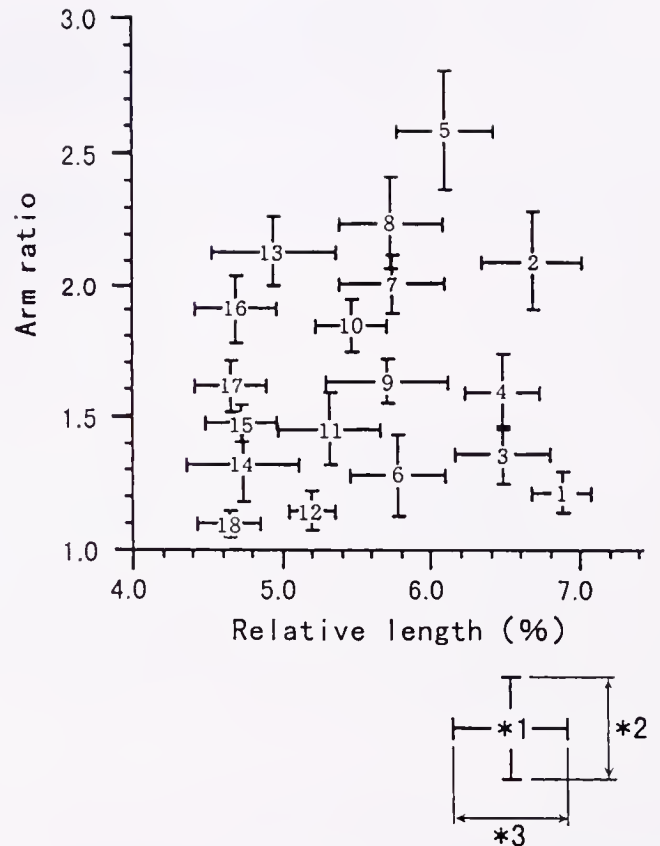


Figure 1. A two-dimensional reference plot of the relative chromosome length and arm ratio distributions in *H. d. hannai* derived from Table 1. \*1: Chromosome number and position of mean value. \*2: Mean  $\pm$  standard deviation of arm ratio. \*3: Mean  $\pm$  standard deviation of relative length.

of silver nitrate, gelatin, and formic acid (Howell and Black 1980) for 5 min at 70 °C. Four-well NOR metaphase plates were photographed before measuring chromosome arm lengths.

## RESULTS

The mean values of the relative lengths and arm ratios of 18 chromosome pairs were estimated from arm length measurements in 12 well-spread metaphase plates from the larvae using an SEM (Table 1). Chromosome pairs were numbered in the order of their relative lengths. The arm ratios indicated that this species possessed 11 pairs of metacentric and seven pairs of submetacentric chromosomes. The distribution of relative chromosome lengths and arm ratios (Fig. 1) was derived from the values in Table 1. This two-dimensional graph served as a reference plot for the identification of karyotypes in subsequent individual *H. d. hannai* analyses, and can be used for comparisons with members of other Haliotidae species in future research. Chromosome pairs 7 and 8, 7 and 10, and 14 and 15 were difficult to identify using only such morphological characteristics as the relative length and arm ratio, because of their mutually overlapping standard deviations (Fig. 1).

Banding was accomplished in *H. d. hannai* by heat and salt solution treatment using nine well-spread metaphase plates. Some intermetaphase variations in the banding pattern, such as shading or numbers of bands, were found. Nevertheless, the banding patterns indicated the same characteristics within each homologous chromosome pair in the same metaphase plate. A typical banded karyotype of *H. d. hannai* is shown in Figure 2. In Fig. 3, a



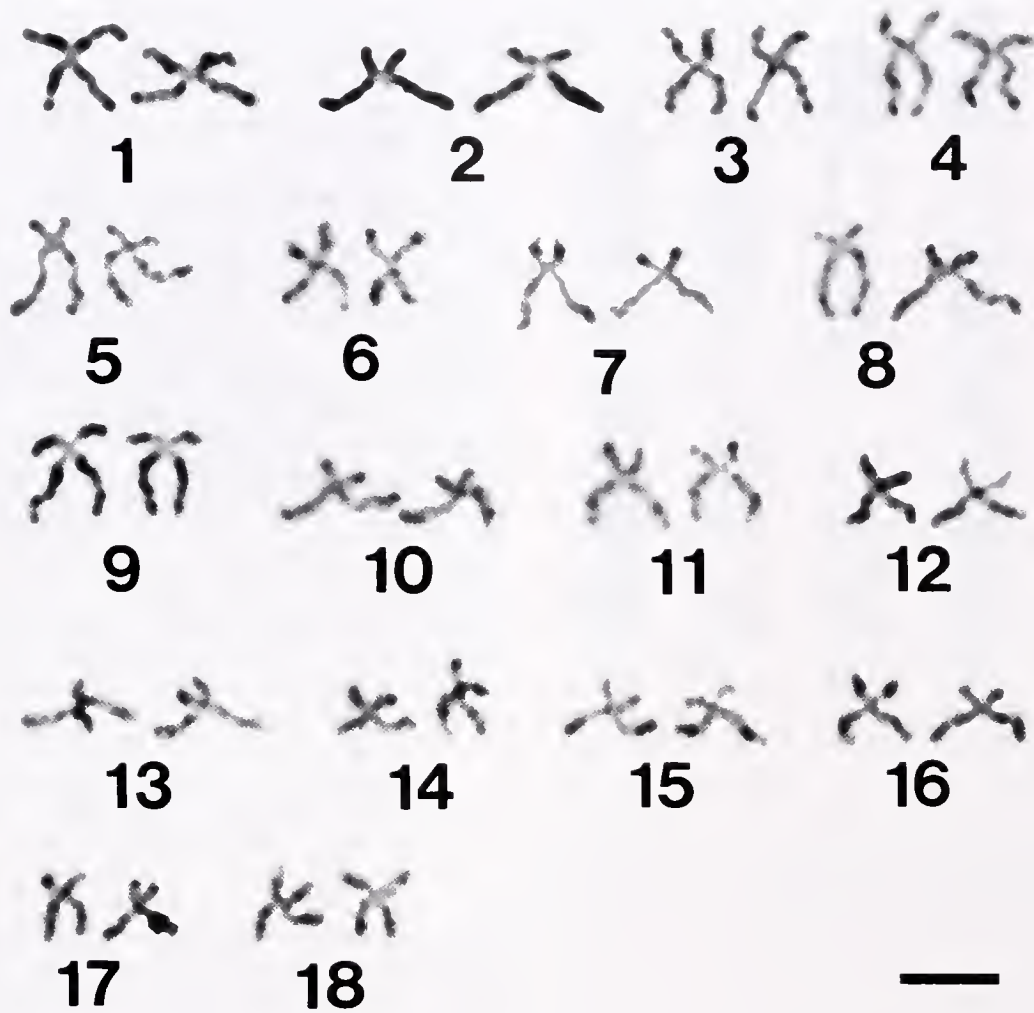


Figure 2. Banded karyotype of *H. d. hannai* after treatment with heat and salt solution (scale bar = 5  $\mu$ m).

diagram of the banding patterns of chromosome pairs no. 7, 8, 10, 14, and 15 from Figure 2 is shown. These chromosomes were among those in the combinations mentioned above for which it was difficult to identify homologous chromosomes using only morphological characteristics. Chromosomes 7 and 15 had unique bands deeply stained on the short arm (7) or the long arm (15) (Figs. 2,3). These staining characteristics simplified the differentiation between chromosome pairs 7 and 8, 7 and 10, and 14 and 15.

Ag-NOR staining was performed on four well-metaphase plates of *H. d. hannai*. The NORs were located terminally on the long arms of two chromosome pairs in all metaphases. The chromosome numbers of each pair bearing the NOR in those four metaphases were identified by referring to the relative chromosome lengths and arm ratios illustrated in Figure 1. In the metaphase shown in Figure 4, the two chromosome pairs bearing the NOR were identified as nos. 5 and 6. In a similar manner, NOR-bearing chromosome pairs in the other three metaphases were identified as nos. 8 and 11, 5 and 11, and 5 and 6, respectively.

#### DISCUSSION

Based on the accurate measurement of chromosome arm lengths using an SEM, we determined the mean values of the

relative lengths and arm ratios of 18 chromosome pairs in this study. This is the first report of concrete numerical values described for the Haliotidae species. These values serve as reliable indexes of morphological characteristics in *H. d. hannai* chromosomes, and our novel approach may furnish information on the *H. d. hannai* karyotype useful for future research. Although previous findings using LM (Arai et al. 1982; Okumura et al. 1995) indicated the presence of 10 pairs of metacentric and eight pairs of submetacentric chromosomes, the present study using SEM measurements indicated that 11 pairs of metacentric and seven pairs of submetacentric chromosomes exist in this species. This difference may be attributable to the existence of chromosomes 4, 9, or 17 showing borderline values of the arm ratio in the metacentric/submetacentric classification (Fig. 1). Comparisons of chromosome classifications based on observations using LM between *H. d. hannai* and *H. d. discus* (Arai et al. 1982), as well as between *H. d. discus* and *H. gigantea* (Miyaki et al. 1997), suggest that there is no difference in chromosome number ( $2n = 36$ ) or morphology among these species. However, if comparisons using the mean values of SEM-determined chromosome arm lengths and arm ratios are performed, some morphological differences might be found, despite their being within the same chromosome classification.

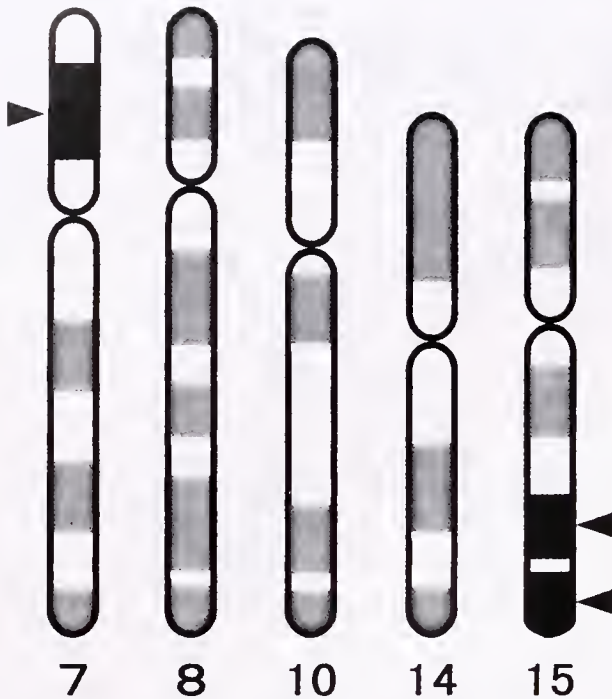


Figure 3. Diagram of banding patterns of chromosome numbers 7, 8, 10, 14, and 15 from Figure 2. Arrowheads indicate the deeply stained bands.

This is the first study in which chromosome banding analysis (i.e., not partial banding, such as C-banding) of Haliotidae by heat and salt solution treatment is attempted. This solution was originally used for the observation of chromosome spiral structures in humans (Ohnuki 1968); however, in the present study, when the metaphase plates of *H. d. hannai* were treated with this solution after heat treatment, clear chromosome bands appeared. These findings may well be applicable to mollusca species that have not been successfully subjected to banding analyses using the general methods of G-banding. The banding patterns may be similar to patterns of G-banding; however, we cannot compare our patterns to G-banding patterns, because although some general methods of G-banding (Sumner et al. 1971, Seabright 1971) have been previously attempted on this species (Okumura unpublished), the G-bands were not convincingly clear as compared to our present banding patterns. The characteristic banding patterns serve as reliable and useful markers for discriminating homologous chromosomes in the same metaphase plates, which had hitherto been difficult to identify using only morphological analysis. Nevertheless, the banding patterns showed some intermetaphase variations. Mendez et al. (1990) described variations in the quality of banding attributable to differing extents of chromosomal condensation in mollusca. We speculate that the variation observed in the present study was caused by the same factors.

NOR analysis was performed here for the first time in Haliotidae, and variations in NOR-bearing chromosomes were observed. Some species of shellfish and finfish have been reported to manifest intraspecific variations in NOR patterns: *Ostrea angasi* shows variations in three chromosome pairs (Li and Havenhand 1997), and differences in NOR number and NOR-bearing chromosome types have been observed in Erythrinidae fish (Antonio and Bertollo 1996). In those studies, the NOR variations have been used in comparative studies of intra- or interspecific genetic features. Variations in NOR chromosomes may confuse the identifi-

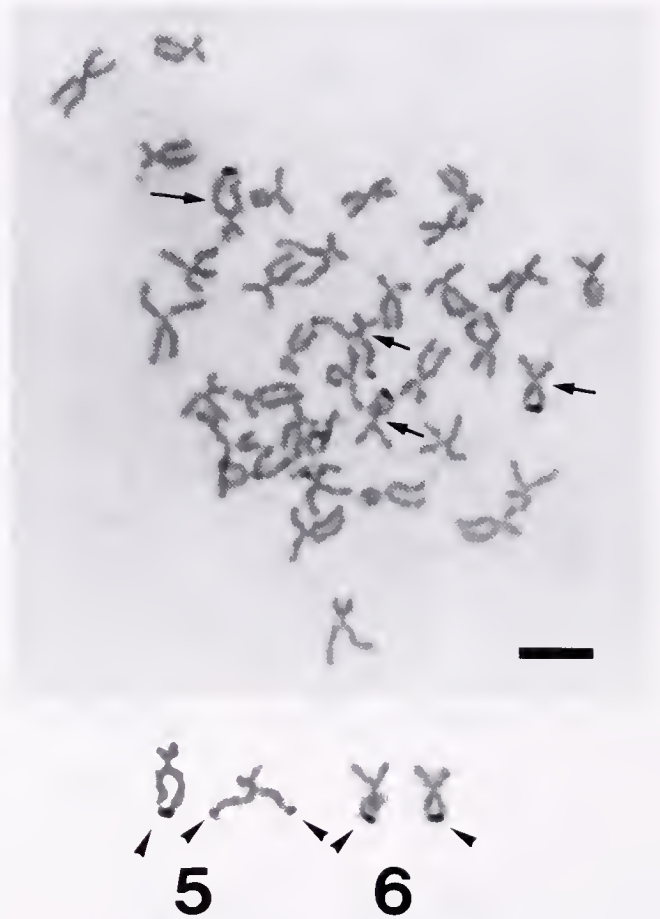


Figure 4. Ag-NOR-stained metaphase plate from *H. d. hannai*. Arrows indicate the NOR-bearing chromosomes. Arrowheads indicate NOR spots on the two chromosome pairs cut out from the upper photograph of the metaphased plate (5 and 6 represent the chromosome pair numbers. Scale bar = 5  $\mu$ m).

cation of chromosome karyotypes when NOR markers are used. However, the variation may serve as a useful index for monitoring genetics and taxonomy in Haliotidae.

In the present study, we developed a method of reliable identification of karyotype, chromosome banding, and NOR analyses in *H. discus hannai*. In fact, we showed the morphological values and banding patterns in this species. Because the present study was successfully performed for this species, similar analyses in other species of Haliotidae are highly likely to succeed. In the future, comparative studies of Haliotidae chromosomes based on the results for *H. d. hannai* in this study are warranted. Recently, studies on fluorescent in situ hybridization of mollusca chromosomes have been undertaken (Pascoe et al. 1996, Guo and Allen 1997, Martinez-Exposito et al. 1997, Insua et al. 1998). This technique is useful for chromosome and gene locus identification, and it may well be applicable for the Haliotidae species.

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## EFFECT OF pH ON GROWTH RATE, OXYGEN CONSUMPTION RATE, AND HISTOPATHOLOGY OF GILL AND KIDNEY TISSUE FOR JUVENILE GREENLIP ABALONE, *HALIOTIS LAEVIGATA* DONOVAN AND BLACKLIP ABALONE, *HALIOTIS RUBRA* LEACH

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**ABSTRACT** Juvenile greenlip abalone, *Haliotis laevigata*, (mean whole mass  $2.30 \pm 0.73$  g, mean  $\pm$  SD,  $n = 561$ ) and juvenile blacklip abalone, *Haliotis rubra*, (mean whole mass  $1.56 \pm 0.64$  g, mean  $\pm$  SD,  $n = 559$ ) were grown for 50–68 days in bioassay tanks at a range of pH levels adjusted using hydrochloric acid (HCl) or sodium hydroxide (NaOH). For greenlip abalone, specific growth rate (SGR) was significantly affected by pH, whether measured on a length or whole mass basis ( $P < .001$ ). For blacklip abalone, SGR was significantly affected by pH whether SGR was measured on a length or whole mass basis. For growth, expressed on a whole mass basis, the EC<sub>5</sub> values (5% growth reductions) were at pH 7.78 and 8.77 for greenlip abalone, and at 7.93 and 8.46 for blacklip abalone. The EC<sub>50</sub> values (50% growth reductions) were at pH 7.39 for greenlip abalone, and 7.37 and 9.02 for blacklip abalone. Survival of both species was significantly reduced at pH 6.79, and survival of blacklip abalone was also significantly reduced at pH 7.76. At the end of the bioassay, groups of abalone were transferred to respiratory chambers. A significant reduction in respiratory activity was observed at both high and low pH values for greenlip abalone ( $P < .001$ ). Greenlip abalone exposed to pH 7.16 showed alterations in kidney definition, tubule and lumen size, and an increase in gill hyperplasia and abnormalities. Blacklip abalone exposed to pH 7.16 demonstrated alterations to kidney and gill definition, and lumen size was increased.

**KEY WORDS:** abalone, *Haliotis laevigata*, *Haliotis rubra*, growth, mollusks, pH, histology

### INTRODUCTION

With the demand for premium abalone products rising steadily (Oakes and Ponte 1996), abalone culture is expanding in both land- and sea-based culture systems (Fleming and Hone 1996). With land-based culture, some recirculation of water is often employed to reduce costs, and a variety of conditions can be experienced with sea-based culture, depending upon site (Hindrums et al. 1996). In both cases, abalone may be exposed to levels of ammonia, nitrite, pH, and dissolved oxygen that may, at least, vary from their optima. Biochemical, physiological, and/or morphological changes can occur as a response to water quality levels that are in excess of those tolerated in aquatic animals (Meyers and Hendricks 1985). Often the gills are among the organs most affected by waterborne pollutants (Mallat 1985), because the respiratory surface provides an extensive interface with the aquatic environment. In many fish, the kidney often forms a site of histological changes in response to toxicants (Russo 1985). In a previous study, both gill and kidney tissue of greenlip abalone provided some indicators of environmental stress (Harris et al. 1998a).

The current emphasis toward water reuse for land-based abalone culture systems is likely to have an impact on pH levels. The process of nitrification, central to biofilter operations in recirculating water systems, causes pH levels to decline (Wickins 1983). In abalone culture systems, diatom surfaces are often used for juvenile rearing (Fleming and Hone 1996). Both respiration by the abalone and nitrification in a biofilter will depress the water pH; however, photosynthetic activity by diatoms will cause pH to increase. In a previous experiment with abalone, pH values ranged from 7.96–8.06. pH levels outside a range of 5–9 are lethal to many aquatic animals (Randall 1991); whereas, pH variation can also have such secondary effects as altering ammonia toxicity (Thurston and Russo 1981). However, limited information is currently available regarding the effects of pH on mollusks and is mostly concerned with bivalves (Calabrese and Davis 1966, Bamber 1987, Bamber 1990).

Oxygen uptake has been widely used to help indicate the health of animals and their over-all energy expenditure or activity levels (Innes and Houlihan 1985) and is also a critical factor in assessments of stress in aquatic organisms (Beitinger and McAuley

1990, Willows 1994), including abalone (Harris et al. 1997, Harris et al. 1999). Wells et al. (1998) demonstrated the New Zealand abalone, *Haliotis iris* and *Haliotis australis*, to have reversed Bohr and Root effects at low pH, leading to an increase in the binding affinity of the respiratory pigment, haemocyanin, and oxygen, and subsequent decrease in released oxygen.

Previous bioassays on Australian greenlip abalone have determined the effects of some aspects of water quality to abalone, such as the chronic toxicity of nitrite (Harris et al. 1997), ammonia (Harris et al. 1998b) and dissolved oxygen (Harris et al. 1999). The aim of this research is to determine the effects of chronic exposure to a variety of pH levels on growth, survival, food consumption, oxygen consumption, and histopathology of gill and kidney tissue of the greenlip abalone, *Haliotis laevigata*, and the blacklip abalone, *Haliotis rubra*, because both species are of increasing importance to abalone aquaculture in Australia.

#### MATERIALS AND METHODS

The juvenile greenlip abalone used in these experiments were approximately 2 years old and were obtained from a commercial hatchery at Bicheno, Tasmania, Australia, where the research was conducted (E148'18", S41'53"). The juvenile blacklip abalone were approximately 12 months old and were obtained from a commercial farm at Swansea, Tasmania, Australia. The initial mean length and mass of the greenlip and blacklip abalone were 26.49 ± 2.83 mm and 2.30 ± 0.73 g and 22.92 ± 2.92 mm and 1.56 ± 0.64 g, respectively (mean ± SD; n = 561 and 559). For 2–3 months before experimentation, the greenlip abalone were maintained on a mixture of formulated abalone feed and benthic diatoms, and the blacklip abalone had been maintained on a formulated abalone feed (Adam & Amos). Blacklip abalone were initially removed using a spatula before transport to the experimental site, and acclimatized for 3 days in flowing, aerated seawater before further handling. All abalone were anesthetized (0.1% benzocaine) until they could be easily removed from the tank surfaces. Subsequently, they were weighed to the nearest 0.01 g, measured with callipers to 0.1 mm, tagged (Hallprint, Adelaide, Australia), and randomly distributed into 18 bioassay units to give 30 of each species within each tank. Mortalities from pH 7.76 resulted in the stocking of this treatment 15 days after the trial commenced, using more from the initial group of blacklip abalone.

#### Bioassay System

Seawater from an exposed coastline, free from freshwater runoff, was filtered through a commercial sand filter and delivered to six 1,100 L reservoirs. pH was adjusted using AR grade NaOH or HCl, thoroughly dissolved in each reservoir. Each reservoir was connected to a constant head chamber (150-mm diameter, vertical PVC pipe, operating volume 30 L) that supplied constant flow to three bioassay chambers via standard lengths of black 4-mm polypropylene tubing that entered the bioassay tanks. These tanks were cylindrical with a conical base to concentrate solid wastes. In each 70-L bioassay tank, there were two cages (100-mm × 35-cm PVC tube with 6-mm mesh floor and 8-mm mesh wall sections) suspended vertically, containing greenlip and blacklip abalone. Daily flow rates averaged 193 ± 1.4 mL/min<sup>-1</sup> (n = 108; 18 tanks on six occasions) giving an effective replacement rate of 90% of bioassay tank volume in 10–12 h. This was within the recommended flow rates for aquatic toxicological studies by Sprague (1969) of 90% replacement in 8–12 h. Identical 5 W submersible pumps were placed in each tank to stimulate similar current flow (8.7 L/min<sup>-1</sup> output at zero head). The experiment was conducted using 200 and 300 W aquarium heaters in the bioassay tanks and constant head chambers, respectively, to maintain relatively uniform daily temperature at 19.0 ± 1.0 °C (mean ± SD) (n = 71 days) (range 16.5–21.7 °C) (Table 1).

#### Water Quality Analysis

The pH, temperature, salinity, and DO in all tanks were measured on all days (Table 2). A pH meter and combination glass electrode (TPS) were calibrated with phosphate (pH = 7.00) and borate (pH = 9.28) buffers daily before use (Bruno and Svoronos 1989). A TPS oxygen electrode, used for daily measurements, was calibrated before use in "air-saturated" seawater. The efficiency of this calibration was validated occasionally using Winkler's titration. Water samples were collected in acid-washed glassware, and ammonia was measured using the indophenol blue spectrophotometric method (Solórzano 1969, as modified by Dal Pont et al. 1974). The concentration of ammonia was measured as total ammonia-nitrogen (TAN), and free ammonia-nitrogen (FAN) was calculated from appropriate temperature, pH, and salinity tables (Bower and Bidwell 1978) (Table 1). Nitrite was measured occasionally, using the diazotisation method (Grasshoff 1989).

TABLE 1.  
Food consumption and survival of greenlip abalone, *Haliotis laevigata* and blacklip abalone.

mean ± SE	pH		Food Consumption (g/g <sup>-1</sup> /day <sup>-1</sup> )		% Survival	
	Min	Max	Greenlip	Blacklip	Greenlip	Blacklip
9.01 ± 0.01	8.34	9.40	0.087 ± 0.011 <sup>ab</sup>	0.139 ± 0.024 <sup>a</sup>	81.9 ± 10.3 <sup>a</sup>	55.0 ± 15.1 <sup>ab</sup>
8.27 ± 0.00	7.86	8.77	0.070 ± 0.003 <sup>ab</sup>	0.088 ± 0.007 <sup>ab</sup>	95.8 ± 2.1 <sup>a</sup>	78.8 ± 8.7 <sup>a</sup>
7.76 ± 0.01	6.71	8.27	0.102 ± 0.010 <sup>a</sup>	0.428 ± 0.294 <sup>†</sup>	94.4 ± 1.1 <sup>a</sup>	20.0 ± 18.4 <sup>b</sup>
7.46 ± 0.02	6.97	8.17	0.061 ± 0.011 <sup>ab</sup>	0.074 ± 0.015 <sup>ab</sup>	98.9 ± 1.1 <sup>a</sup>	55.2 ± 13.4 <sup>ab</sup>
7.16 ± 0.01	6.45	7.93	0.036 ± 0.006 <sup>b</sup>	0.034 ± 0.005 <sup>b</sup>	70.4 ± 14.8 <sup>a</sup>	75.8 ± 8.6 <sup>a</sup>
6.79 ± 0.03	6.04	7.62	0.052 ± 0.007 <sup>ab</sup>	0.078 ± 0.015 <sup>2</sup>	3.1 ± 3.1 <sup>b</sup>	0 <sup>b</sup>

*Haliotis rubra* exposed to a range of pH conditions (mean ± SE) (means sharing a common superscript are not significantly different [ $P > .05$ ]).

<sup>a</sup> Water quality: ammonia concentrations ranged from 0–0.026 mg FAN/l<sup>-1</sup>, nitrite concentrations ranged from 0.003–0.005 mg NO<sub>2</sub>-N/l<sup>-1</sup>, temperatures ranged from 18.6–19.3 °C, flow rates ranged from 184.2–202.9 mL/min<sup>-1</sup>, salinity ranged from 33.8–34.6 ppt, and oxygen levels ranged from 6.96–7.19 mg DO.l<sup>-1</sup>.

<sup>†</sup> Data for pH 7.76 and pH 6.79 were not included in statistical analyses because of lack of replicates.

TABLE 2.

Water quality parameters and biomass for respiratory experiments on greenlip abalone, *Haliotis laevigata*, and blacklip abalone, *Haliotis rubra*.

Greenlip Abalone				Blacklip Abalone			
pH	Biomass (g)	Temperature (°C)	Salinity (ppt)	pH	Biomass (g)	Temperature (°C)	Salinity (ppt)
9.25 ± 0.07	76.31 ± 7.16	18.4 ± 0.2	34.4 ± 0.3	9.27 ± 0.00	29.08 ± 17.05	18.5 ± 0.5	—
8.45 ± 0.15	105.57 ± 12.56	18.5 ± 0.2	34.7 ± 0.1	8.56 ± 0.01	50.07 ± 1.80	18.8 ± 0.3	—
7.95 ± 0.12	68.65 ± 7.71	16.7 ± 0.2	33.7 ± 0.1	8.02 ± 0.19	26.05 <sup>a</sup>	19.5 <sup>a</sup>	34.2 <sup>a</sup>
7.30 ± 0.03	68.22 ± 2.66	16.9 ± 0.2	34.1 ± 0.1	7.11 ± 0.31	44.48 ± 14.98	20.4 ± 1.1	34.8 <sup>a</sup>
6.72 ± 0.06	51.12 ± 0.75	19.0 ± 0.3	34.2 ± 0.3	6.88 ± 0.02	37.76 ± 5.47	18.9 ± 0.2	35.0 ± 0.1
6.08 ± 0.05	5.77 <sup>a</sup>	18.8 ± 0.3	34.4 ± 0.0	—	—	—	—

All measurements expressed as mean ± SE.

<sup>a</sup> n = 1.

**Experiment 1: Chronic pH exposure**

Six experimental treatments were established (Table 1); average pH ranged from 9.01–6.79. The abalone were acclimatized to the bioassay system for 4–6 days before pH adjustment commenced. pH adjustment occurred over several days, with a gradual increase in chemical levels (HCl or NaOH) each day, until the desired level was attained. All cages were checked daily for mortality.

All tanks were fed a proprietary, formulated abalone diet (AB-CHOW) every 2 to 3 days. The feeding ration was adjusted in response to food consumption data as the trial progressed. Food consumption was estimated on four occasions from uneaten food removed from the base of the cages after 2 days and drying it for 24–48 hours at 55–60 °C. Residual food mass was not corrected for soluble and particulate nutrient losses over the 2 days. Apparent food consumption (amount of food supplied minus residual food as g dry mass) was divided by the initial tank biomass, less the mass of any mortalities to that point, and expressed as g dry mass food remaining per g whole wet body mass per day.

A valve in the base of each bioassay tank was opened daily to remove organic wastes. Tanks were also cleaned more thoroughly, on average, every 9 days. Cleaning involved lowering the water level, siphoning enough water from the bioassay tank into a 20-L bucket to cover the cages, removing cages to the bucket, draining the tank, scrubbing the tanks and cages, refilling the tanks directly from the reservoirs, and returning the cages to the tanks. This took under 10 minutes for any tank.

Abalone remained in the bioassay system for up to 68 days and were removed in staggered groups for respirometry over 14 days. This is unlikely to be sufficient time for significant differences in growth because of stocking density to arise. All abalone were weighed and measured for the final growth data. Specific growth rate data were calculated for mass and length of each abalone as  $SGR = [\ln(\text{final}) - \ln(\text{initial})] 100 \text{ days}^{-1}$ .

**Experiment 2: Oxygen Consumption Rates at End of the Chronic Bioassay**

The respirometer system included five elliptical perspex chambers (of 2.3 l) normally set up with two replicate chambers for each treatment and one chamber as a control (no animals), as described in Harris et al. (1997).

Commencing on day 57, abalone from the bioassay system were transferred to respirometer chambers for a series of 3-day experiments. All abalone remaining in two of the three replicate bioassay tanks for each treatment level were transferred to the respirometer system so that data could be obtained for duplicate tanks of each species at each nominal treatment level. These animals had been fed before removal. Abalone that did not attach to transferable plastic strips in the cages within the bioassay units were removed manually, either by sliding them directly from the substrate or by inserting a thin, plastic card underneath each abalone's foot. Temperature and pH levels were measured within the constant head chambers (Table 3).

TABLE 3.

Scoring schedule for histological sections of abalone.

		1	2	3
Gill	Definition	Well-defined brush border	Some filaments showing irregular brush borders	Poorly defined filaments
	Hypertrophy	No cells	Isolated cells	widespread
	Hyperplasia	No evidence	Isolated cells	widespread
	Abnormalities	No evidence	Isolated incidences	several per gill
Right kidney	Definition	Well-defined cells	Some difficulty in defining tubules	Tubules very difficult to define
	Tubule size	Small, plenty of tubule contents	Some enlargement of tubules	large tubules
	Lumen size	Small, plenty of lumen contents	Some enlargement of lumen	large lumen space
	Cytoplasm	Small % of each cell	>half cell volume	< half cell volume
	vacuoles	Small % of each cell	>half cell volume	< half cell volume

### Histological Sample Preparation

Five abalone were sampled from two of the triplicate bioassay tanks for each treatment. These abalone were dissected to remove the posterior portion of the viscera containing the gills and kidney. This tissue was fixed in phosphate-buffered formalin at room temperature (15–18 °C) then dehydrated through a graded ethanol series to xylene in a Tissue-Tek II tissue processor. Dehydrated tissue samples were embedded in paraffin resin on a Shandon Histocentre 2 and sectioned on a Microm HM 340 microtome at 4  $\mu$ m. Routine Harris' Hematoxylin and Eosin (H & E) staining were carried out on all tissues processed using a Shandon Linistain GLX automatic tissue stainer. All sections were mounted in DPX and examined under a light microscope.

Insufficient animals remained in pH 6.79 for histological analysis, so abalone from pH 7.16 were the most extreme treatment considered. The tissue sample from each abalone was examined and scored regarding several aspects of gill and kidney structure (Table 3).

### Statistical Analysis

Data were subjected to one factor analysis of variance (ANOVA) after meeting assumptions of normality using the Shapiro–Wilk test (Zar 1996) and homogeneity of variance using Cochran's test (Underwood 1981). Replicates were considered to be independent, and pH concentration was analyzed as a fixed factor. Survival data (as percentage) and whole wet body mass (WWBM): shell length (SL) ratio were transformed ( $\arcsin \sqrt{\%} \times 0.01$  and log, respectively) to satisfy assumptions of normality and homogeneity of variance before analysis. Results for each pH level were compared using Tukey's HSD (Sokal and Rohlf 1995). Preliminary analysis indicated that initial abalone size did not affect growth rate. All analyses included assessment of FAN, nitrite-N, DO and temperature as covariates (Sokal and Rohlf) and were conducted using JMP 3.0 software (SAS Institute, Cary, North Carolina). The effect of each toxicant on gill and kidney structure was examined using Chi-squared ( $\chi^2$ ) analysis to compare two proportions (one-tailed) (Sokal and Rohlf).

## RESULTS

### Experiment 1: Chronic pH Exposure

For greenlip abalone, SGR was significantly affected by pH ( $P < .001$ ), whether measured on a SL or WWBM basis. SL growth (Fig. 1) and WWBM gain (Fig. 2) were highest at pH 8.27–7.76, however, significant growth rate reductions occurred at pH 9.01 and 7.46–6.79 ( $P < 0.05$ ). Second order regression of the SGR length data indicated that there was no shell growth below pH 6.90 (Fig. 1). The EC values (effective concentration where reductions of  $x\%$  occur) from the modeled mass data were pH 8.77 and 7.78 ( $EC_5$ ), and pH 7.39 ( $EC_{50}$ ) (Fig. 2). Highest WWBM and SL growth rates were observed for greenlip abalone at pH 8.27.

For blacklip abalone, SGR was significantly affected by pH whether measured on a length ( $P < .001$ ) or whole mass ( $P < .01$ ) basis. SL growth rates were highest at pH 8.27–7.76, with further depression of SL growth rates at pH 9.01 and at pH 7.46–6.79 ( $P < .05$ ). Second-order regression of the SGR length data indicated that there was no shell growth below pH 6.99 (Fig. 3). For WWBM gain, significant growth rate reductions occurred at pH 7.16 (Fig. 4) ( $P < .05$ ). The EC values from the mass data were pH 8.46 and 7.93 ( $EC_5$ ), and pH 9.02 and 7.37 ( $EC_{50}$ ). Highest WWBM and SL growth rates were observed for blacklip abalone at pH 7.76 al-

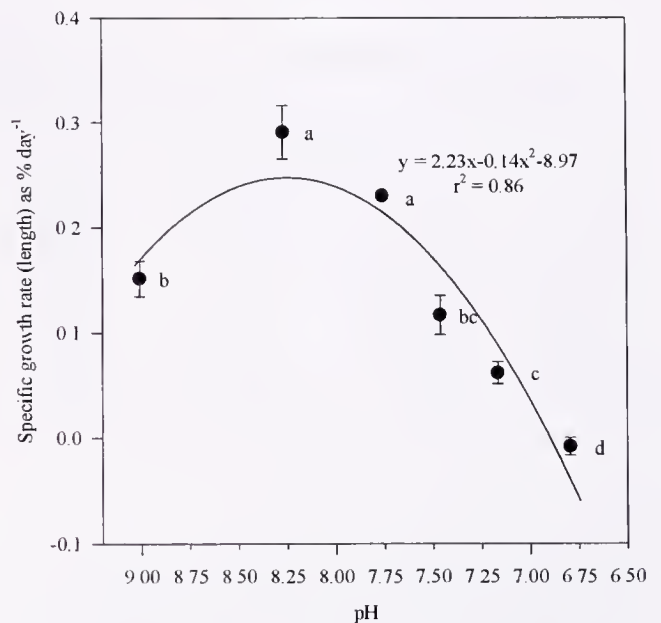


Figure 1. Specific growth rate (length) of juvenile greenlip abalone, *Haliotis laevigata*, subjected to chronic pH conditions in Experiment I (mean  $\pm$  SE,  $n=3$ ). Regressions based on data for each replicate rather than treatment means.

though WWBM growth rate data at this pH were highly variable (Fig. 4).

Survival of greenlip abalone was significantly affected by pH exposure ( $P < .0001$ ). Survival was high in all but pH 6.79, where significant mortalities occurred ( $P < .05$ ) (Table 1). Survival of blacklip abalone was also significantly affected by pH exposure ( $P < .01$ ). Survival was high in all but pH 7.76 and 6.79, where

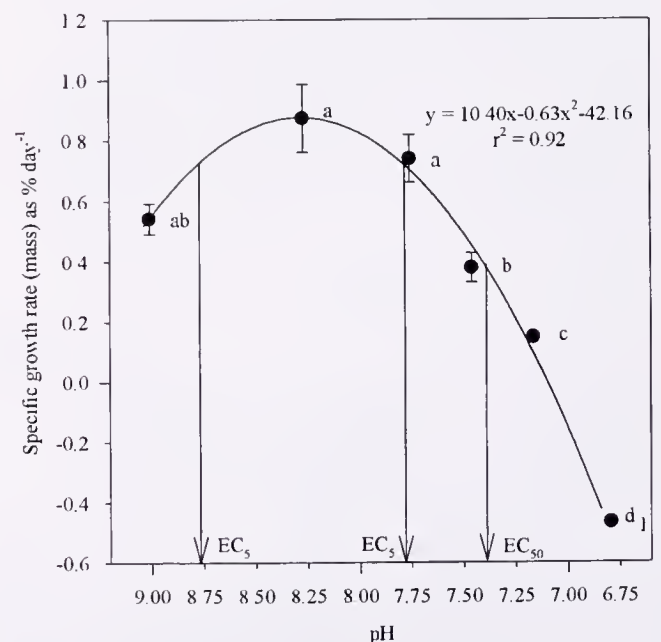


Figure 2. Specific growth rate (mass) of juvenile greenlip abalone, *Haliotis laevigata*, subjected to chronic pH conditions in Experiment I (mean  $\pm$  SE,  $n=3$ ). Regressions based on data for each replicate rather than treatment means ( $n=1$ ).



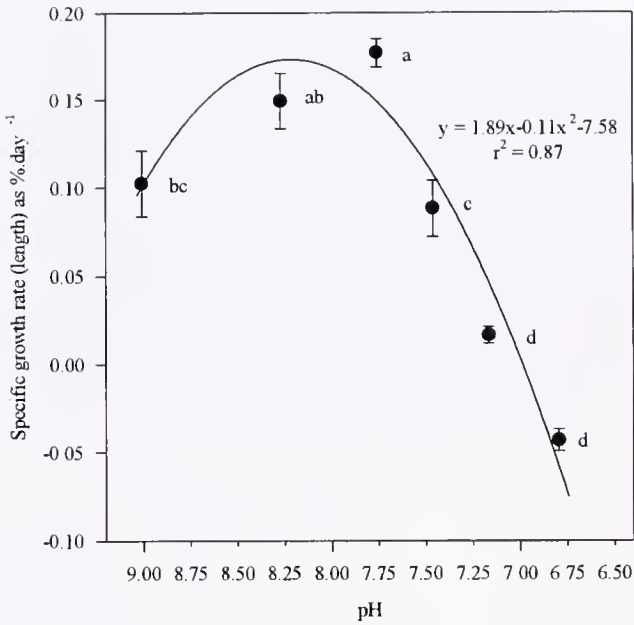


Figure 3. Specific growth rate (length) of juvenile blacklip abalone, *Haliotis rubra*, subjected to chronic pH conditions in Experiment 1 (mean ± SE, n = 3). Regressions based on data for each replicate rather than treatment means.

significant mortalities occurred ( $P < .05$ ) (Table 1). During the experiment, if the pH fell below 6.2, significant mortalities for both species followed for up to 7 days. Abalone exposed to these conditions lost attachment and collected toward the bottom of the cages. At the end of the experiment, all blacklip abalone at pH 6.79, all greenlip abalone in two replicates at pH 6.79, and all blacklip abalone of one replicate at pH 7.76 had died, so only

length measurements until their day of removal were calculated, not mass data.

pH had a significant effect on WWBM:SL for both greenlip abalone (Fig. 5) ( $P < .001$ ) and blacklip abalone ( $P < 0.05$ ) (Fig. 6). Greenlip abalone from pH 6.79 had significantly lower ratios than abalone from pH 8.27 ( $P < .05$ ). For blacklip abalone, pH 7.16 produced a significantly ( $P < .05$ ) lower ratio than pH 7.76.

There was a significant effect of pH on food consumption by greenlip abalone ( $P < .01$ ), where abalone from pH 7.76 had significantly higher food consumption than abalone from pH 7.16 ( $P > .05$ ) (Table 2). The variability of the data for abalone in treatments that also had significant mortality rates prevented the conditions of homogeneity of variance being satisfied for statistical analysis, so these treatments (pH 7.76 and pH 6.79) were omitted from analysis. A significant effect of pH on food consumption by blacklip abalone was observed ( $P < .05$ ), with abalone held at pH 7.16 demonstrating significantly lower rates than abalone from pH 9.01 (Table 1).

Significantly lower temperatures were recorded at pH 9.01 and 7.46 than other treatments ( $P < .001$ ). DO levels were also significantly different between treatments ( $P < .001$ ), although average treatment oxygen saturation was  $96.7 \pm 0.2\%$ . Salinity was also significantly affected by pH ( $P < .001$ ). The salinity at pH 9.01 was significantly lower than the controls (Table 1) ( $P < .05$ ). Statistical analysis of log-transformed ammonia levels determined all treatments to be significantly different to the control ( $P < .05$ ). Nitrite levels were at or below  $0.005 \text{ mg NO}_2\text{-N.l}$ .

Experiment 2: Oxygen Consumption Rates at End of Chronic Bioassay

Oxygen consumption rate of juvenile greenlip abalone was significantly affected by pH ( $P < .001$ ), with greenlip abalone of pH 9.25 and 6.72–6.08 recording significantly lower ( $P < .05$ ) oxygen consumption rates than the controls (pH 8.45) (Fig. 7). Mortality

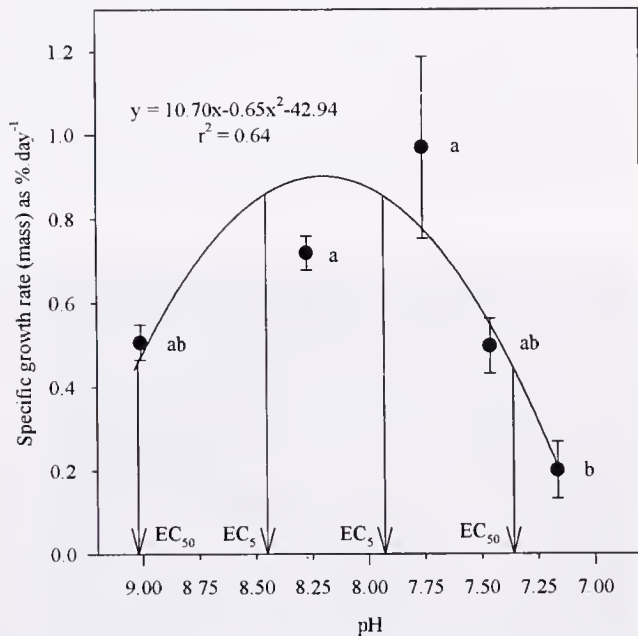


Figure 4. Specific growth rate (mass) of juvenile blacklip abalone, *Haliotis rubra*, subjected to chronic pH conditions in Experiment 1 (mean ± SE, n=3). Regressions based on data for each replicate rather than treatment means.

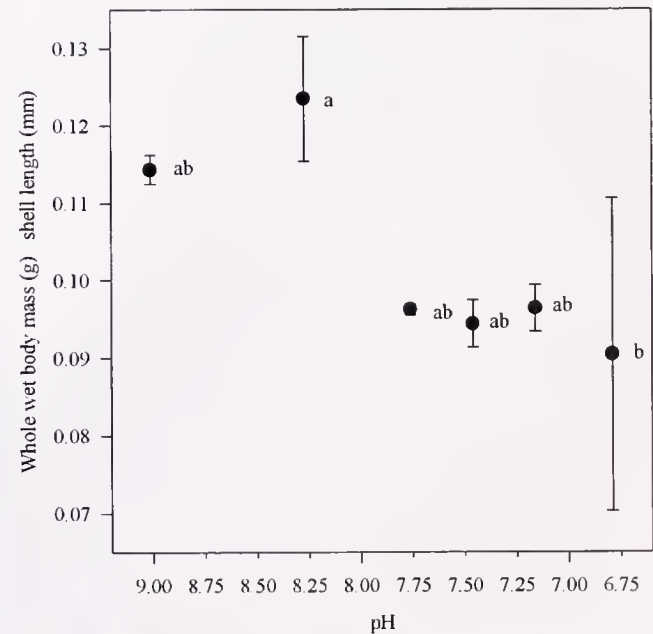


Figure 5. Whole wet body mass: shell length of juvenile greenlip abalone, *Haliotis laevis*, subjected to chronic pH conditions in Experiment 1 (mean ± SE, n = 3). Regressions based on data for each replicate rather than treatment means.

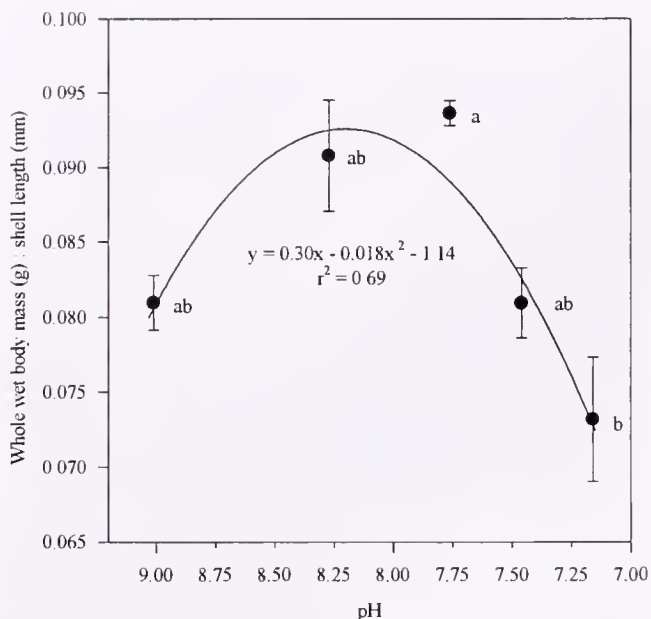


Figure 6. Whole wet body mass: shell length of juvenile blacklip abalone, *Haliotis rubra*, subjected to chronic pH conditions in Experiment 1 (mean  $\pm$  SE, n = 3). Regressions based on data for each replicate rather than treatment means.

among blacklip abalone before and during respirometry prevented statistical analysis of this species.

#### Histological Sample Examination

Chi-squared analysis of histological observations from greenlip abalone exposed to slightly acidified seawater (pH 7.16) demonstrated significantly different kidney definition ( $\chi^2$  calc. = 8.57; v

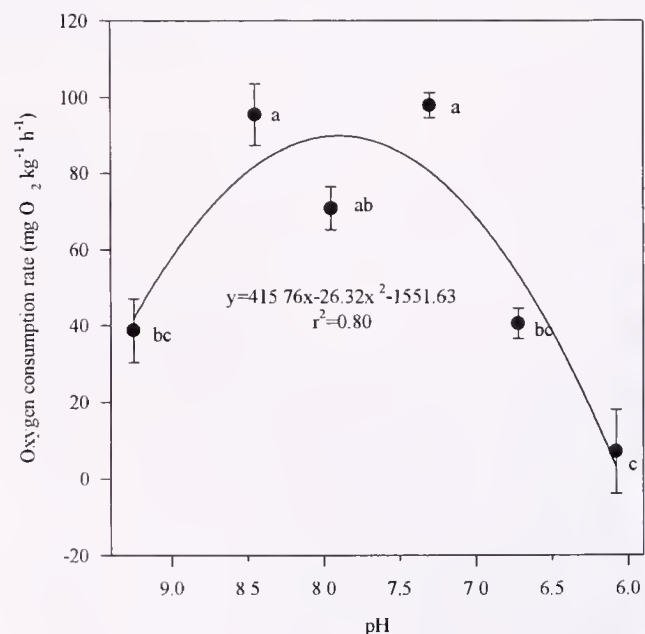


Figure 7. Oxygen consumption rate of juvenile greenlip abalone, *Haliotis laevigata*, subjected to a range of pH conditions (mean  $\pm$  SE, n = 2). Regressions based on data for each replicate rather than treatment means.

= 1,  $P < .005$ ) and tubule enlargement ( $\chi^2$  calc. = 8.57;  $P < .005$ , v = 1) (Fig. 8). Kidney lumen size was also significantly larger in greenlip abalone from pH 6.79 ( $\chi^2$  calc. = 15; v = 1,  $P < .001$ ). Analysis of gill structure in greenlip abalone revealed significant increases in hyperplasia ( $\chi^2$  calc. = 4.29; v = 1,  $P < .05$ ), and abnormalities ( $\chi^2$  calc. = 6.2; v = 1,  $P < .005$ ), with exposure to pH approaching acidity (pH 7.16) (Fig. 9) Blacklip abalone exposed to pH 7.16 demonstrated similar significant differences in kidney definition ( $\chi^2$  calc. = 4.8; v = 1,  $P < .05$ ), kidney lumen size ( $\chi^2$  calc. = 4.8; v = 1,  $P < .05$ ) (Fig. 10), and gill definition ( $\chi^2$  calc. = 8.24; v = 1,  $P < .005$ ) (Fig. 11). High pH did not induce any detectable alterations to the structure of the gill or kidney tissue of the abalone examined.

#### DISCUSSION

The fastest growth rates of greenlip abalone (SGR mass =  $0.87 \pm 0.11$  %/day<sup>-1</sup>; SGR length =  $0.29 \pm 0.03$  %/day<sup>-1</sup>) and blacklip abalone (SGR mass =  $0.97 \pm 0.22$  %/day<sup>-1</sup>; SGR length =  $0.18 \pm 0.01$  %/day<sup>-1</sup>) in this experiment were much higher in comparison to a previous bioassay conducted at a similar temperature in the same experimental system with greenlip abalone only (Harris et al. 1997) (SGR mass =  $0.48 \pm 0.04$  %/day<sup>-1</sup>; SGR length =  $0.12 \pm 0.01$  %/day<sup>-1</sup>). One difference in the system design involved the incorporation of small submersible pumps into the tanks to improve water flow, as increased water movement stimulates feeding for Australian abalone (Shepherd 1973, Higham et al. 1998).

The abalone in this study seem to be less tolerant to alterations in pH than other species. From the EC<sub>5</sub> estimations, greenlip abalone have a wider range of pH over which whole body growth is not inhibited, although outside this pH range, inhibition seems more severe than for blacklip abalone. In comparison, bivalves exhibited slightly higher levels of tolerance to pH than the abalone in this study. *Ostrea edulis* and *Crassostrea gigas* grown for 30–60 days lost shell at pH 6.0 and 7.0, respectively (Bamber 1990) and young *Venerupis decussata* grown for up to 30 days also lost shell at pH 7.0 (Bamber 1987). The flatfish *Paralichthys orbignyanus* demonstrated no adverse effects at pH 6.0 (Wasielesky et al. 1997), and the pH level where a 5% growth reduction occurred for the marine shrimp *Penaeus monodon* was pH 5.9 (Allan and Maguire 1992), both substantially lower than for abalone.

The greenlip abalone also demonstrated a different pattern of

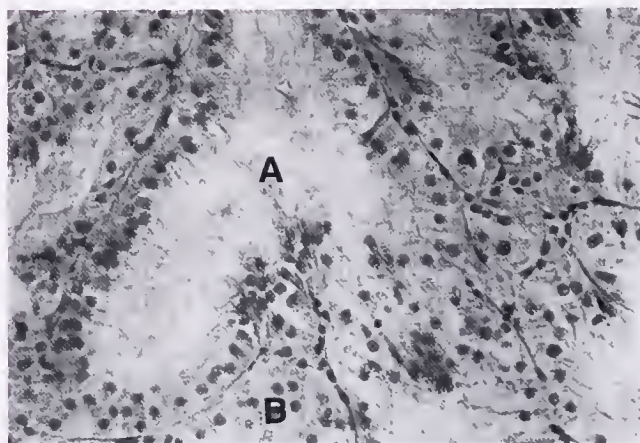


Figure 8. Right kidney of *Haliotis laevigata* exposed to pH 7.16. Magnification 400X. A: enlarged lumen; B: enlarged tubule.

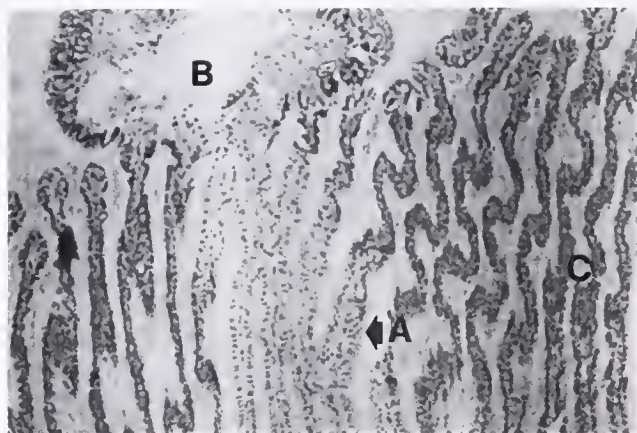


Figure 9. Gill lamellae of *Haliotis laevigata* exposed to pH 7.16. Magnification 100X. A: fused lamellae; B: expanded distal gill lamellar tip with dilated haemolymph channel (aneurysm); C: hyperplasia.

shell growth inhibition than blacklip abalone. The drop in WWBM:SL for greenlip abalone to a plateau is in contrast with the data for blacklip abalone, which demonstrated a pattern more similar to previous studies for greenlip abalone (Harris et al. 1998b). Shell and body mass growth rates of greenlip abalone both seem affected in similar patterns by pH, as indicated by levels where significant growth reduction occurs, yet WWBM:SL data indicate that shell growth rate is less affected than WWBM growth rate at pH > 7.76. The decline in WWBM:SL of blacklip abalone outside the EC<sub>5</sub> range indicates a decline in body growth, as opposed to shell growth, outside this range.

The data for WWBM:SL suggest that pH can affect whole animal growth (mass) and shell growth (length) differently. In a previous bioassay, we argued that ammonia affected shell growth more than whole body growth (mass) at low ammonia concentrations but that this pattern was reversed at high concentrations (Harris et al. 1998b). The low ratio at more extreme pH may reflect a limitation on whole body growth imposed by depressed shell growth rates in gastropods (Palmer 1981, Preston et al. 1996), in addition to the effect of the toxicant on body growth. In another bioassay on chronic nitrite toxicity, a more complex pattern of WWBM:SL growth appeared (Harris et al. 1997). Further work is required to examine the relationship of body mass growth to shell growth.

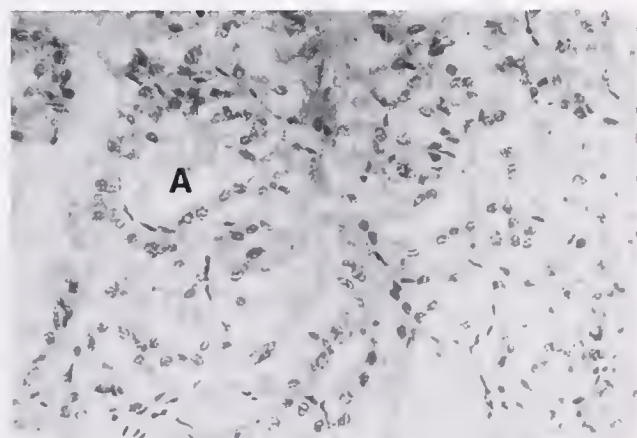


Figure 10. Right kidney of *Haliotis rubra* exposed to pH 7.16. Magnification 400X. A: enlarged lumen of kidney tubule.

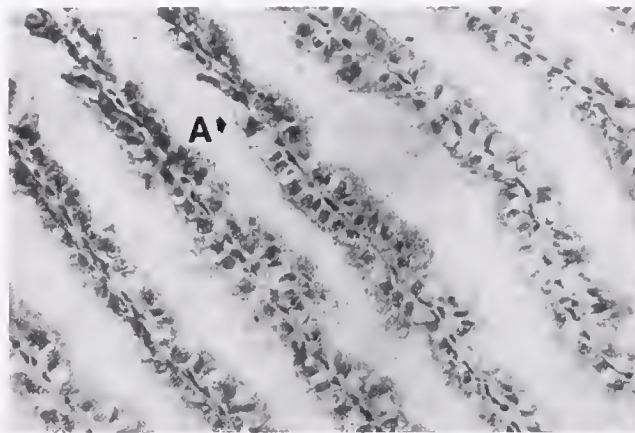


Figure 11. Gill lamellae of *Haliotis rubra* exposed to pH 7.16. Magnification 400X. A: poorly defined brush border.

The low pH level at which reductions in survival occurred for greenlip abalone and blacklip abalone is comparable to bivalves, because significant mortalities were observed for abalone at pH 6.79. Small *O. edulis*, *C. gigas*, and *Mytilus edulis* demonstrated reduced survival at pH 6.6, 6.0, and 6.6, respectively (Bamber 1990) and for *V. decussata* at pH 6.1–6.4. However, other species have much higher tolerance to acid stress, including *P. monodon* (96 h LC 50 = pH 3.7) (Allan and Maguire 1992) and *Paralichthys orbignyanus*, which had 100% survival after 96 h in pH 5.2 (Wasielky et al. 1997).

No growth reduction occurred for blacklip abalone at pH 7.76, a level where survival was affected, compared with the lower level, where both growth and survival were affected. The decrease in survival for blacklip abalone at pH 7.76 does not seem related to treatment levels and is more likely caused by stress on the abalone from handling. This treatment was restocked on day 15, when all blacklips from the most acidic treatment (pH 6.64, n = 3; 12 days) were replaced because of total mortality, hence the possibility of differences to handling for these abalone.

In this study, greenlip abalone demonstrated a similar pattern of oxygen consumption in response to pH as had been observed previously for the prosobranch gastropod *Viviparus contectoides* (Buckingham and Freed 1976). In their study, *V. contectoides* demonstrated two peaks in oxygen consumption at pH values 7.1 and 8.9, with an intervening trough. They suggested that, although energetically possible for *V. contectoides* to exist at pH 7.1 and 8.9, it involved substantial energy cost. In rainbow trout, this decline in oxygen consumption rate is a response to the reduced scope for activity at pH levels beyond those at which the organism can easily metabolise (Ye et al. 1991). In the case of greenlip abalone, the low pH experienced by the abalone depressed oxygen consumption rates. According to Wells et al. (1998), low internal pH should produce conditions where oxygen–hemocyanin affinity is highest. However, abalone rely heavily on anaerobic metabolism during exercise or environmental hypoxia, and the subsequent metabolic acidosis will conserve this oxygen further (Wells et al. 1998). It is likely that the abalone from this experiment have altered oxygen–hemocyanin affinity caused by the experimental conditions, which is further exacerbated by the products of anaerobic metabolism, thus causing the decline in oxygen consumption.

The alterations to gill epithelium that were observed in this study for both greenlip and blacklip abalone are of note, because similar changes are known to occur in fish, as the cells become

damaged through accumulation of bicarbonate in the mucus layer (Randall 1991). The decrease in kidney definition and increased lumen size noted for both species of abalone indicate that pH can alter kidney structure. A decrease in nuclear size and staining intensity was observed in kidney cells of the brook trout, *Salvelinus fontinalis* at pH 4.0 (Mudge et al. 1977).

The variations in water quality experienced during this experiment are not believed to have influenced the results. Although a significant reduction in salinity was observed at pH 9.01, the reduction was in the order of 0.5 ppt from the over-all mean of all other treatments (1.5% reduction). Short-term survival of greenlip abalone is known to be affected at 23 ppt, and at 28 ppt, if inappropriately fed (Boarder 1997). The daily temperature average of 19.0 °C is little different to the preferred temperature of greenlip abalone (18.3 °C) and blacklip abalone (17.0 °C) (Edwards 1996). FAN levels were higher at increased pH because of the influence of pH on ammonia ionisation (Bower and Bidwell 1978), though they were below 0.041 mg FAN l<sup>-1</sup>, the EC<sub>5</sub> for greenlip abalone (Harris et al. 1998b).

Within recirculation systems, there is greater likelihood of a

combination of adverse water quality factors occurring, rather than just one factor deteriorating. Because nitrification is a complex of processes by which ammonia is converted first to nitrite then nitrate, with concomitant acidification (Collins et al. 1975, Wickins 1983), then studying the effects of all these parameters in combination would provide much greater understanding of the tolerances of abalone to recirculating systems. Presently, the effect of chronic exposure to ammonia (Harris et al. 1998b), nitrite (Harris et al. 1997), dissolved oxygen (Harris et al. 1999) and pH (this study) are known, although this knowledge would be enhanced though subsequent combination studies.

#### ACKNOWLEDGMENTS

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## GENETIC VARIATION FOR SURVIVAL AND SHELL LENGTH OF CULTURED RED ABALONE (*HALIOTIS RUFESCENS*) IN ICELAND

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**ABSTRACT** A research program was started in 1996 to study genetic variation in survival and size of imported red abalone cultured in Iceland. A total of 100 families was produced from a hierarchical mating design using 29 males and 88 females. Larvae from each family were settled and grown in separate tanks until tagged at the age of 10 months, at which time individuals from all families were reared in one common environment, but at two farms, until the age of 24 months. After tagging, the abalone were fed with the macroalgae species *Palmaria palmata* and *Laminaria digitata*. The mean survival rate of the population was estimated at 9.6% at the age of 4 months, with large variation among families where the highest survival was 31.5% but the lowest 0.02%. The average shell length at the age of 8, 10, 18, and 24 months old was 13.10, 15.88, 34.99, and 50.90 mm, respectively. Heritability for survival at 4 months of age was estimated to 0.11 (0.33 on the liability scale) and for shell length at the age of 8, 10, 18, and 24 months to 0.08, 0.06, 0.27, and 0.34, respectively. A low, but negative, genetic correlation between the survival rate to 4 months and shell length was observed. Genotype by environment interaction was negligible after rearing individuals from the same families in two different farms for 14 months. Based on the heritability estimates observed, it was concluded, that, theoretically, it is possible to double the growth rate of cultured red abalone in just four generations by selection at the end of 2 years of rearing.

### INTRODUCTION

Selection of quantitative traits has been one of the most important tools in the improvement of production from farm animals and plants over the past few decades. The red abalone (*Haliotis rufescens*) is one of over 100 abalone species belonging to the genus *Haliotis* (Howorth 1978). The red abalone is the largest in the world often reaching shell lengths greater than 27.5 cm and weighing over 1.7 kg (McAlister 1976). A population of 2,300 individuals was imported to Iceland from California in 1988 to establish an aquaculture industry (Steinarsson 1993). Abalone culture is a rapidly growing industry throughout the world, because abalone is one of the most valuable shellfish on the international market (Gudnason pers. comm.). Despite the great interest in aquacultural production of abalone, no study for estimating genetic parameters in abalone has yet been reported.

Results from breeding experiments with other shellfish species suggest that additive genetic variance leads to successful selection programs for growth rate. Hara and Kikuchi (1989) demonstrated an increase in daily growth rate in the Japanese abalone, *Haliotis discus hannai* of 21% in animals of shell size 20–30 mm and a 65% increase in those of 30–70 mm after three generations of selection, when compared to the maximum growth of control commercial animals. Toro and Newkirk (1991) demonstrated response to selection for shell height in the Chilean oyster (*Ostrea chilensis*) with a realized heritability estimated to be  $0.34 \pm 0.12$ . Jarayabhand and Thavornnyutikarn (1995) also demonstrated a response to selection for growth rate of the oyster *Saccostrea cucullata* with a realized heritability to  $0.28 \pm 0.006$ .

The importance of estimating phenotypic and genetic param-

eters of economically important life history traits, the genetic correlation between them, and predicting responses to selection and estimating breeding values are crucial for planning a breeding program. The magnitude of additive and nonadditive genetic variance in relation to the total variance of each trait will determine which breeding method (purebreeding and/or crossbreeding) and selection method (individual and/or family selection) should be applied.

A research project was initiated in 1996 to study the economically important life history traits of survival, growth rate, meat yield, and age at maturity in farmed red abalone in Iceland. The aim of the project was to produce a breeding plan that would reduce production costs. In this paper, we report on estimates of the genetic parameters (heritabilities and genetic correlation) for survival at 4 months posthatching and shell length at 8, 10, 18, and 24 months of age.

### MATERIALS AND METHODS

The broodstock used in this study were 9 years old from the original population imported to Iceland in 1988. The average shell length of the 88 females used for spawning was 14.6 cm (SD 2.01), body weight 517.5 g (SD 185.7); the average shell length of the 29 males was 14.6 cm (SD 2.40) and body weight 576.3 grams (SD 245.7).

#### *Spawning and Settlement of Larvae*

Spawning at the Saebyli Ltd.'s farm (Farm 1, Table 1) was induced by increasing the water temperature and by exposure to UV light-treated seawater. The temperature was increased from 13 to 17 °C overnight before introduction of UV-treated seawater for

TABLE 1.

Shell length and number of animals (*n*), and coefficient of variation during the experiment, reared in two farms after the age of 10 months.

Trait	Age Months	Farm	<i>n</i>	Mean	C.V.
Length 1	8	1	9,648	13.10	27.9
Length 2	10	1	3,468	15.91	24.3
Length 2	10	2	2,391	15.83	23.6
Length 3	18	1	2,438	37.45	16.0
Length 3	18	2	2,098	32.14	19.9
Length 4	24	1	1,701	51.30	16.8
Length 4	24	2	1,354	50.40	17.3

a few hours. Altogether, 29 males and 88 females were used to produce 100 full- and half-sib families over a period of 1 month (September 1996). A hierarchical mating design was used where each male (sire) fertilized eggs of 2–6 females (dams). After fertilization and hatching, the larvae were held in separate tanks for 6 days at which time, the number of larvae per three 5-mL samples were counted in each tank. Thereafter, a sample of 5,000 larvae from each family were put into a 20-L tank in a greenhouse with feeder plates covered with microalgae. The feeder plates had been pregrazed by older abalone to induce settlement, these were removed before input of the larvae (Seki and Kan-no 1981). Each family was put in one tank, and the families were randomly distributed over two stands and three shelves (Fig. 1) to reduce possible variation caused by different environmental conditions within the greenhouse. Growing of microalgae was induced by direct sunlight, but at later stages, additional electrical lighting was used.

#### Feeding with Macroalgae

At the age of 6 months, fresh algae *Palmaria palmata* was added to the diet, and at the same time, the greenhouse lights were turned off for 5 hours during the night. At the age of 8 months, the greenhouse lights were turned off and fresh *Laminaria digitata* was added to the diet.

#### Recording Traits

Survival was estimated from the age of 6 days posthatching, at the time larvae were settled into 20-L tanks, until the age of 4 months, when all surviving individuals within each tank were counted. Into each tank, a random 250 survivors were returned for

continued culturing. For families with lower survival, 5% of the remaining individuals were counted and returned. The mean shell length at 4 months of age was 3.30 mm (SD 0.91). At the age of 4 months, some of the animals in each family were too small to be measured. Therefore, the first growth measurement was postponed until the age of 8 months, when approximately 100 individuals were measured per family. The widest diameter of the shell was measured to the nearest 0.01 mm. At the age of 10 months, a random sample of approximately 60 individuals from each family were tagged and measured; one family was not tagged because of high mortality. At tagging, each family was split up in two groups; one for each of two rearing sites. Two weeks after tagging, one group of all families was transported to the Marine Institutes research farm (Farm 2, Table 1) 20 km from the Saebyli's farm. All tagged individuals were placed together in one big common tank at each farm. Two weeks later, the animals were randomly placed into 40-L tanks with about 170 animals in each tank. At the age of 18 and 24 months, all tagged individuals were measured again. Tag-loss was observed; therefore, fewer than 30 individuals were measured per family at each site.

#### Temperature

The average water temperature was 14.2 °C during the first 4 months of growth, 15.8 °C for the next 4, and increased to 17.1 °C for the next 2 (to age 10 months). At Saebyli's farm the average temperature from the age of 10 to 18 months was 17.3 °C and 16.9 °C from the age 18 months to 24. At the Marine Institute's farm, the average temperature from age 10 to 18 was 16.0 °C, and from age 18 to 24 months it was 16.8 °C.

#### Statistical Analysis

Variance components for each of the four shell length measurements were estimated according to an animal model. The animal model allows both the recorded individuals and the parents without records to be included in the analysis in order to account for all known additive genetic relationships between animals; also, the model includes both fixed and random effects. In matrix notation, the model can be written.

$$y = Xb + Za + Zf + e$$

where *y* is the observation vector; *b* is the vector of fixed (environmental) effects (for length, these were farm, number of animals in tanks, and age from hatching; and for survival, they were shelf and stand) *a* is the vector of random animal effects; *f* is the vector of random effects of full-sib group caused by factors other than additive genetics; *e* is the vector of individual random error effects. *X* and *Z* are the corresponding design matrices.

The additive effects, the common full-sib effects attributable to other factors than additive gene effects, and the residual effects were assumed to have independent normal distributions with zero means and variances of  $\sigma_a^2$ ,  $\sigma_f^2$ , and  $\sigma_e^2$ , respectively. The variance components were estimated from a derivative-free restricted maximum likelihood (df-REML) algorithm (Meyer 1989) based on software MTDFREML written by Boldman et al. (1995).

Survival was calculated as percentage survival. The observations for survival were coded as 0 (dead individuals) and 1 (live individuals). The heritability estimate for survival was estimated on the observed scale. Therefore, the estimate was transformed to the underlying liability scale (Robertson and Lerner 1949).

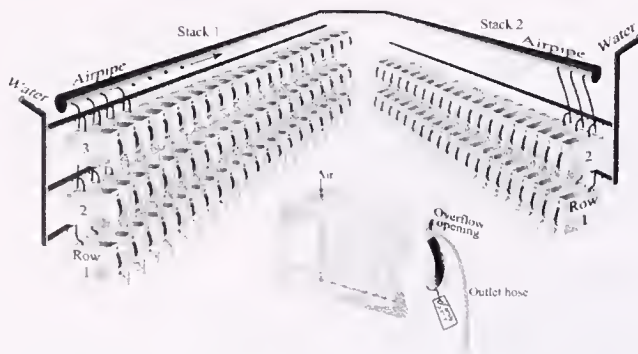


Figure 1. Location of the shelves and stands and the type of tank used from larval stages until tagging.



TABLE 2.

Survival (%) in stands and shelves during the first 4 months.

Stand Shelf	1	2
3	7.2	
2	7.8	10.7
1	8.1	14.2

Genetic Correlation Between Survival and Shell Length

For estimation of the genetic correlation between survival and shell lengths 1 (8 months) and 4 (24 months), a bivariate analysis was run in which survival and shell length were regarded as two different traits. A genetic correlation between survival and length 2 and length 3 was not computed. The error covariance between the traits was set to zero, because they are not measured on the same animal when running a multitrait analysis for the two traits. The same models as above for survival and shell lengths were used, but excluding the common full-sib effect. When estimating the genetic correlations between the four shell lengths 2 (10 months), 3 (18 months), and 4 (24 months), the error covariance was computed, because the measurements are made on the same individuals at different ages. The genetic correlation between length 1 and lengths 2, 3, and 4 was not computed, because lengths 1 and 2 were measured with only a 2-month interval.

Genotype by Environmental Interaction

For estimation of the genetic correlation between shell length at the age of 18 (length 3) and 24 (length 4) months in the two different farms, a univariate analysis was run as described above but excluding fixed effect of farm. Subsequently, a bivariate analysis was run with shell length at each farm for both age classes, regarded as two different traits. The error covariance between the traits was set to zero, because they are not measured on the same animal. The common full-sib effect was not included.

RESULTS

The mean rate of survival for the 100 families at the age of 4 months was 9.6%, with large variation among families where the highest survival was 31.5% but the lowest 0.02%. Significant effect (99.5% level) was found for the position of the family tank on the two stands and shelves (Table 2). Higher survival was in ob-

TABLE 3.

Shell length (standard deviation) at the age of 8 months in stands and shelves.

Stand/Shelf	1		2	
	n	Mean (SD)	n	Mean (SD)
3	1964	13.39 (3.6)		
2	1907	12.49 (3.1)	2117	12.72 (3.3)
1	1770	13.36 (3.8)	1889	13.42 (4.2)
Total	5641	13.08 (3.5)	4006	13.05 (3.8)

TABLE 4.

Proportion of partial sum of square for shell length, effect of each fixed (environment) effect, as R<sup>2</sup> in % on shell length during the experiment.

Effect	Length 1	Length 2	Length 3	Length 4
Shelf	0.01	1.11	0.2	0.19 <sup>NS</sup>
Stand	0.0	0.16	0.97	1.13
Animals (n) in tank	18.22	11.6	2.74	0.01
Animals (n <sup>2</sup> ) in tank	0.56	1.28	0.62	0.92
Age from hatching	—	3.53 <sup>NS</sup>	2.45	3.40
Age <sup>2</sup> from hatching	—	0.01 <sup>NS</sup>	2.01	2.46
Farm	—	0.0 <sup>NS</sup>	14.96	0.33
Total	18.8	17.7	24.0	8.4

<sup>NS</sup> Nonsignificant; all other effects significant at 99.5% level.

served on stand 2 and particularly on shelf 1, at 14.2%. No significant effect of tank position was observed for shell length at 8 months of age (Table 3).

The importance, expressed as percentage of partial sums of squares (R<sup>2</sup>), of each environmental effect included in the model for the shell length measurements are given in Table 4. The difference in number of animals in a tank had the greatest effect on shell length at 8 and 10 months of age, with minimum effect evident by 24 months of age (length 4). The difference between farms was most evident at 18 months (length 3), with the main effect at 24 months being age-at-hatching. The effect of stand and shelves in the first 10 months of growth had minor effect on all the models although the effect was significant on the 99.5% level.

The mean shell lengths observed were 13.10 mm at 8 months (length 1), 15.88 mm at 10 months (length 2), 34.99 mm at 18 months (length 3), and 50.90 mm at 24 months (length 4) (Table 1). A substantial variation between families was observed in shell length at the age of 24 months (Fig. 2). The largest mean shell length for a family was 66.9 mm and the smallest was 42.2 mm.

Heritabilities for survival (0.11) and shell lengths 1 and 2 (0.08 and 0.06) were low (Table 5), but when survival was transformed to the underlying liability scale, the heritability was 0.33. A higher growth heritability estimate was observed after 18 months (0.27) and even higher after 24 months (0.34). Effect other than additive effect (f<sup>2</sup>) was lower than the heritability for all traits (Table 5).

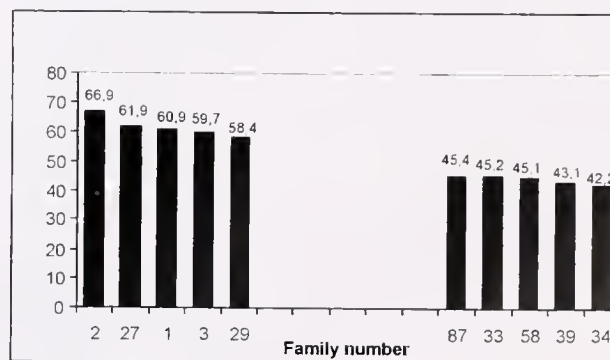


Figure 2. Mean shell length of family groups for the five largest and the five smallest families at the age of 24 months from hatching. Mean shell length for the 100 families tested was 50.9 mm.

TABLE 5.

Heritability estimates (diagonal), with then full-sib group effect caused by factors other than additive genetics (in parenthesis) and genetic correlations for the 5 traits recorded.

	Survival	Length 1	Length 2	Length 3	Length 4
Survival	0.11 (0.0)				
Length 1	-0.18	0.08 (0.06)			
Length 2	—	—	0.06 (0.04)		
Length 3	—	—	0.28	0.27 (0.0)	
Length 4	-0.12	—	0.09	0.09	0.34 (0.0)

The estimated genetic correlation between survival and shell lengths 1 and 4 were low and negative at  $-0.18$  and  $-0.12$ , respectively. As shown in Table 5, the genetic correlation between shell lengths 2 and 3 was low and even lower between shell lengths 2 and 4. Genetic correlation between shell lengths 3 and 4 was close to unity (0.9). The genetic correlations between shell length at the age of 18 months and 24 months in the two farms was estimated to 0.94 and 0.95, respectively.

#### DISCUSSIONS

A low survival rate of 10% during the first 4 months is quite common in red abalone culture (Fallu 1991). The mean growth rate during the experimental period of 24 months was 2.12 mm/month. Such a growth rate was similar to the 2.4–2.5 cm/year growth rate reported by Leighton (1974), Ebert and Houk (1984). An effect of density on growth during the first 8 months of rearing was observed in our families. A large variation was observed between full-sib families in survival and shell length. Limited literature is available on the subject of growth variation in abalone. Hara (1990) observed significantly lower growth in one of three families over a 180-day growing period.

The heritability for survival during the first 4 months was low at 0.11 but does suggest that it may be possible to improve the trait by selection. However, in general, survival is not of great economic importance, because of the high fecundity of females (Ault 1985); a single female can easily produce over 3 million eggs allowing sufficient spat production even at a survival rate of only 10%.

Heritability for shell length was low for lengths 1 and 2 but higher for lengths 3 and 4. An increase in heritability with age has also been reported for other shellfish species; for example, oysters (0.24–0.5; Longwell 1976, Newkirk et al. 1977, Losee 1978) and blue mussel (0.12–0.43; Innes and Haley 1977, Newkirk 1980). It must be emphasized that all the estimates presented for oysters and blue mussels were estimated from data from few families. It is not uncommon that heritability estimates at early life stages are lower than when estimated nearer to market size. This is also the case for Atlantic salmon and rainbow trout. (Jónasson 1993, Jónasson et al. 1997, Refstie 1980, Gjerde and Schaeffer 1989).

The statistical model used allowed estimates of effects other than additive genetics ( $f^2$ ). The observed low estimates for shell lengths 1 and 2 are most likely a transitory common environmental effect introduced when the groups were reared in separate tanks during the first 10 months of the experiment. This is demonstrated in Table 4, where it is shown that the effect of shelf, stand, and number of animals in tanks in the first 10 months is greatly reduced as the animals get older and are reared together in a common

environment. However, nonadditive genetic effects cannot be ruled out.

Genetic correlation between survival and shell lengths 1 and length 4 was negative but low. Worth keeping in mind is the categorical nature of the survival observation. The estimated genetic correlations are, therefore, systematically underestimated (Kendall and Stuart 1961). The present results indicate an unfavorable genetic correlation between survival in the first 4 months and shell length until 2 years of age. This is not in agreement with studies in oyster (Jarayabhand and Thavorniyutikarn 1995), where survival of groups selected for fast growth was relatively higher than groups selected from the medium and slow growing part of the population. In studies on Atlantic salmon ranching, Jónasson (1992) estimated genetic correlation between survival and body length of Atlantic salmon fingerlings to  $0.39 \pm 0.26$ , and Jónasson et al. (1997) estimated the genetic correlation between body weight and survival of grilse at sea in Atlantic salmon ranching to  $0.16 \pm 0.16$ . More data for abalone are needed; furthermore, survival during the first 4 months will most likely be correlated to other life history traits.

The low genetic correlation between shell length at the age of 10 months and at the age of 2 years indicates that shell length at young age is a poor estimate of the individual shell length at older stages. Market size is between 50–100 mm (Gudnason pers. comm.), and the results show that, in a breeding program for abalone, selection for growth should be taken as close to market size as possible.

Genetic correlation between the two farms for shell length is close to unity. This indicates that genotype by environmental interaction is negligible in this experiment for the first 2 years of rearing. It may, therefore, be concluded that selective breeding program for red abalone in Iceland can be based on one breeding population only and shell lengths of full- and half-sib groups can be recorded based on rearing in one farm.

The heritability for shell length at age 24 months was estimated at 0.34, indicating that prospects for improving growth rate are good. By using the standard formula for genetic gain for individual selection ( $\Delta G = i^* \sigma_p^* h^2$ , Falconer 1989), assuming that 5% of the largest animals will be selected as broodstock and using the phenotypic standard deviation (8.8 as in Table 1) obtained after 24 months of age, the genetic gain will be 6.0 mm per generation. Such a gain represents a shortening in production time of 2.83 months to the mean size of 56.90 mm. Should this predicted gain be real, then it would take just over four generations to double the growth rate in red abalone in Iceland by selection. By applying a combined individual and family selection for growth, the genetic gain during the first 2 years will be higher, because combined individual and family selection is more effective for traits with low heritabilities (Falconer 1989).

At this stage, it is too early to suggest a breeding plan for red abalone culture, because market size is usually reached in 2–3 years of age. Genetic parameters for such other life history traits as age at market size, meat yield, and age at maturity will be estimated on these same families, and economic evaluation of all traits will be performed. However, our results do suggest that significant production gains can be made in the culture of abalone through a selective breeding program.

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## GROWTH OF TAIWAN ABALONE *HALIOTIS DIVERSICOLOR SUPERTEXTA* FED ON *GRACILARIA TENUISTIPITATA* AND ARTIFICIAL DIET IN A MULTIPLE-TIER BASKET SYSTEM

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**ABSTRACT** Taiwan abalone, *Haliotis diversicolor supertexta*, juveniles ( $26.64 \pm 2.30$  mm) were placed in 4 sets of 7-basket tiers, 35 abalone in each basket, and reared indoors with running seawater (31–35‰) for 395 days. Food was generally given every other day at a rate of 30% and 3% of total body weight for *Gracilaria tenuistipitata* and artificial diet, respectively. Survival was 64.3–71.4% and 61.4–78.6% in the *Gracilaria* and artificial diet, respectively. Growth of abalone placed on the top tier was inferior to those placed on the middle and bottom tiers. In the first 33 days, the growth rate of *H. diversicolor supertexta* fed *Gracilaria* and the artificial diet was 61–101 and 105–163  $\mu\text{m}/\text{day}$ , respectively. The abalone fed an artificial diet gained twice as much total body weight as those fed *Gracilaria* after 96 days. The overall growth rate of abalone fed an artificial diet was 24.4, 47.7, and 47.9 mg/day, or 1.8, 2.2, and 2.2 times higher than abalone fed *Gracilaria*, on the top, middle, and bottom tiers, respectively. The FCR (feed conversion ratio) in dry weight of food was 3.10, 2.68, and 2.76 for the abalone fed an artificial diet, and 4.43, 3.03, and 3.04 for those fed *Gracilaria*. It is concluded that *H. diversicolor supertexta* placed in a multiple-tier basket system and fed an artificial diet could grow to market size (> 40 mm shell length) in less than half the time in comparison to those fed on *Gracilaria*.

**KEY WORDS:** *Haliotis diversicolor supertexta*, *Gracilaria tenuistipitata*, artificial diet, growth

### INTRODUCTION

Taiwan abalone (also known as small abalone) *Haliotis diversicolor supertexta* Lischke, which live in the littoral zone of rocky shores along the south coast of Japan and northeast coast of Taiwan, grow to 100 mm shell length in the wild (Nie 1992). Culture of *H. diversicolor supertexta* has expanded greatly since 1986 due to development of successful propagation and larval rearing techniques (Chen and Yang 1979, Lin 1986, Yang and Ting 1986). The farmed production of *H. diversicolor supertexta* in Taiwan was 502 tonne in 1986, doubled in 1987, and reached 2,213 tonne in 1997 (Taiwan Fisheries Bureau 1998).

Culture of *H. diversicolor supertexta* in Taiwan is commercially divided into 3 phases. The first stage is the culture of newly settled spat on corrugated plastic plates until a shell length of 2–3 mm is attained. The second stage is the culture of 2–3 mm larvae on cement plates to 20–30 mm juveniles. The third stage is the culture of 20–30 mm juveniles on cement plates or baskets to reach a market size shell length of more than 40 mm.

*Gracilaria* has been cultured commercially since 1962 on land farms. Most *Gracilaria* produced is used as food for the Taiwan abalone. According to the Taiwan Fisheries Bureau (1998), farmed *Gracilaria* totaled 9,232 and 12,576 ton in 1986 and 1997, respectively. This amount is not sufficient to meet the needs of the abalone produced, and recently a number of artificial diets have been developed for abalone culture.

Growth of abalone feeding on macroalgae and artificial diets has been studied for *H. discus* (Ogino and Ohta 1963), *H. discus hannai* (Uki et al. 1985, Nie et al. 1986, Uki et al. 1986a, 1986b), *H. fulgens* (Viana et al. 1993), *H. asinina* (Capinpin and Core 1996), *H. iris* (Stuart and Brown 1994), *H. tuberculata* (Kolke et al. 1979, Mgaya and Mercer 1995), *H. laevigata* (Morrison and Whittington 1991) and *H. midae* (Britz 1996a, 1996b, Knauer et al. 1996). However, little is known about the growth of *H. diversicolor supertexta* (Chen 1984).

A multiple-tier basket system for the culture of abalone has

recently been developed in Taiwan. In this system, abalone juveniles are placed inside the baskets and the baskets are stacked in 4 to 14 tiers in indoor cement ponds. This system is the best way to culture *H. diversicolor supertexta* in Taiwan where land is limited. Most farmers install a mechanical device to lift the basket for feeding and cleaning. However, we do not know how much variation there is in the harvest when using a multiple-tier system. The objectives of this research were to quantify the growth rate of *H. diversicolor supertexta* fed on *Gracilaria* and artificial diet, and compare the growth of abalone placed on the top, middle, and bottom tiers in a multiple-tier basket system. This is the first report of a comparatively long experimental trial to show the effect of diet on growth performance for *H. diversicolor supertexta*.

### MATERIALS AND METHODS

#### Test abalone

*H. diversicolor supertexta*, which were hatched and reared for about 3 months at a private farm in Kaohsiung, Taiwan, were shipped to our University on October 10, 1996. They were reared on cement plates in a concrete pond and fed red macroalga *Gracilaria tenuistipitata* Var. *lini* Zhang et Xia prior to shipping. They were placed inside the greenhouse and acclimated to running seawater at 35‰ for 20 days before experimentation. During the acclimation period, the juveniles were divided into 2 groups. One group of abalone juveniles was fed *Gracilaria*, and the other group was fed artificial diet (see Experiment Diet for details). For the experiment, 420 abalone juveniles were used, mean ( $\pm$  SD) body weight  $2.137 \pm 0.52$  g and shell length  $26.64 \pm 2.30$  mm. No significant difference in weight and shell length was observed among the treatments.

#### Experimental System

Perforated plastic baskets (39-cm long, 31-cm wide, 12-cm high; with perforations  $9.2 \times 9.6$  mm) were employed in the study.

Each basket was stocked with 35 juvenile abalone. Seven baskets were stacked into a tier and connected to a cement block to the bottom of the tier. In all, 4 sets of the 7-basket tiers were used: 2 for feeding with *Gracilaria* and 2 for feeding with artificial diet. The 4 sets of baskets were placed in an oval fiberglass tank, 2.0-m diameter and 1.2-m deep. The bottom of the tank was covered with gravel and sand 12-cm deep, and aerated with airlift to create a biological filter (Fig. 1). Seawater pumped from the coast adjacent to the University passed through a sand filter into the tank continuously at a flow rate of  $479 \pm 6$  L/h. The abalone placed in the 1st, 4th, and 7th basket of each tier served as the top, middle, and bottom, respectively. There were 6 treatments (2 diets  $\times$  3 tiers – top, middle, and bottom), and each treatment was conducted in 2 replicates.

#### Experiment Diet

Two diets were used in the study. The red macroalgae, *Gracilaria tenuistipitata* var. *liue* Zhang et Xia, was harvested from farms in Tainan, Taiwan, and shipped to the University. The percentage composition ( $\pm$  SE) of *Gracilaria* was moisture  $89.03 \pm 0.85$ , crude protein  $26.08 \pm 2.64$ , crude lipid  $1.52 \pm 0.70$ , crude fiber  $6.58 \pm 0.15$ , ash  $35.29 \pm 0.88$ , and nitrogen-free extract  $30.53 \pm 3.03$  in dry base. The artificial diet was manufactured by Tonlee Feed Company (Pingtung, Taiwan) based on seaweed powder, soy

bean powder, and wheat powder as main ingredients. The artificial diet was prepared in a row of 3 cylinder-shaped pellets (4-mm diameter and 10–30-mm long). The percentage composition of artificial diet was moisture 12.99, crude protein 30.02, crude lipid 4.41, crude fiber 3.14, ash 10.93, nitrogen-free extract 51.50 in dry base.

#### Feeding experiment

Food was generally given every other day at a rate of 30 and 3% of body weight of abalone for *Gracilaria* and artificial diet, respectively, based on preliminary observation and the experience of abalone growers. However, feeding amount was reduced to 20 and 2% of body weight for *Gracilaria* and artificial diet, respectively, from March to April 1997, the period considered by farmers to be dangerous for the animals due to the south wind. In general, feeding was 15 times a month, but was reduced to 7 times in August 1997 by power failure caused by 2 typhoons; feed was not given during this period to avoid accumulation of toxic ammonia. Fresh *Gracilaria* was collected every 4–5 days, blotted on filter paper, weighed, and fed to the abalone. The uneaten food was removed every other day, blotted on filter paper, and weighed. The artificial diet kept its shape for 48 h, but its stability in water over 48 h is 73.4%. Consumption of artificial diet corrected for leaching was calculated using the formula of Britz (1996b).

The experiment started November 1, 1996, and lasted for 395 days. The baskets were checked every other day for abalone survival, and dead abalone were removed. Growth was generally measured every month as body weight and shell length. An electronic balance was used to register the weight in mg, and a vernier caliper was used to measure the shell length in mm. Percentage weight gain, percentage length increase, growth rate in weight (mg/day) and shell length ( $\mu\text{m}/\text{day}$ ), and FCR (feed conversion ratio) were calculated. FCR was calculated based on the wet weight feed consumed (g) per wet weight gain (g), based on the dry weight feed consumed (g) per wet weight gain (g), and expressed as FCR (Wet) and FCR (Dry), respectively.

During the experimental period, the temperature range was 14.5–31.0 °C (Fig. 2) (measured with thermometer model HOBO HTEA, Onset Computer Corp., Pocasset, MA). The illuminance in the top, middle, and bottom tier was measured with an illuminometer (model HOBO HLI OK-P) and averaged 0.3, -2.1, and -2.5 log  $\text{lm}/\text{ft}^2$ , respectively (Fig. 3). Salinity, monitored with a refractometer, ranged from 31 to 35‰, and the dissolved oxygen, measured with a YSI Model 58 DO meter (YSI, USA), ranged from 5.8 to

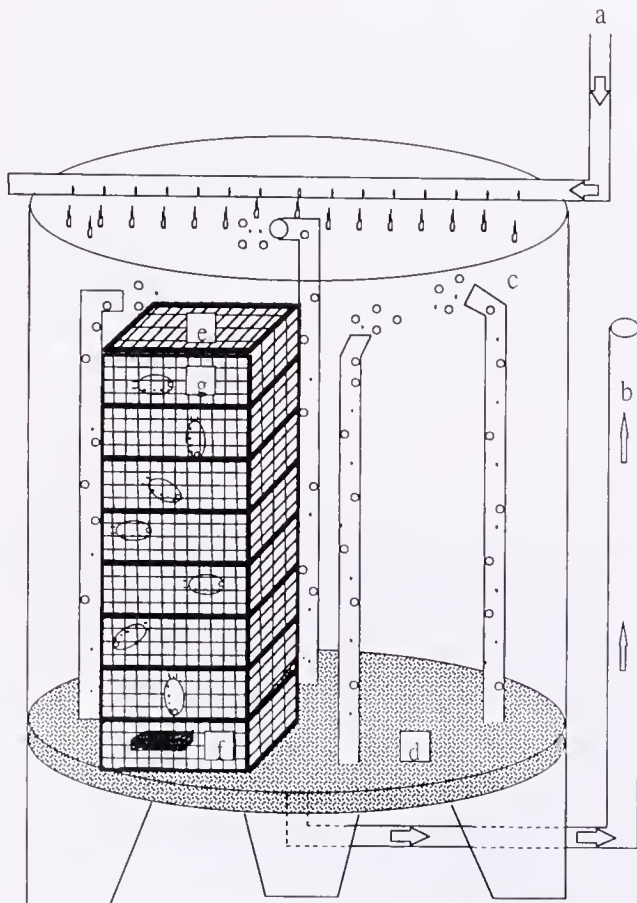


Figure 1. A schematic diagram of the 7-tier basket system. a, water inlet; b, water outlet; c, airlift; d, gravel; e, basket; f, cement block; g, abalone.

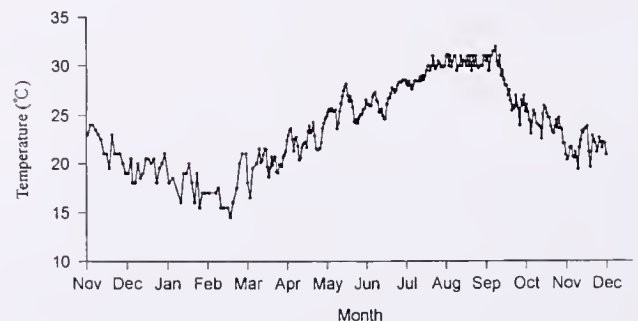


Figure 2. Water temperature in the culture system from November 1996 to November 1997.

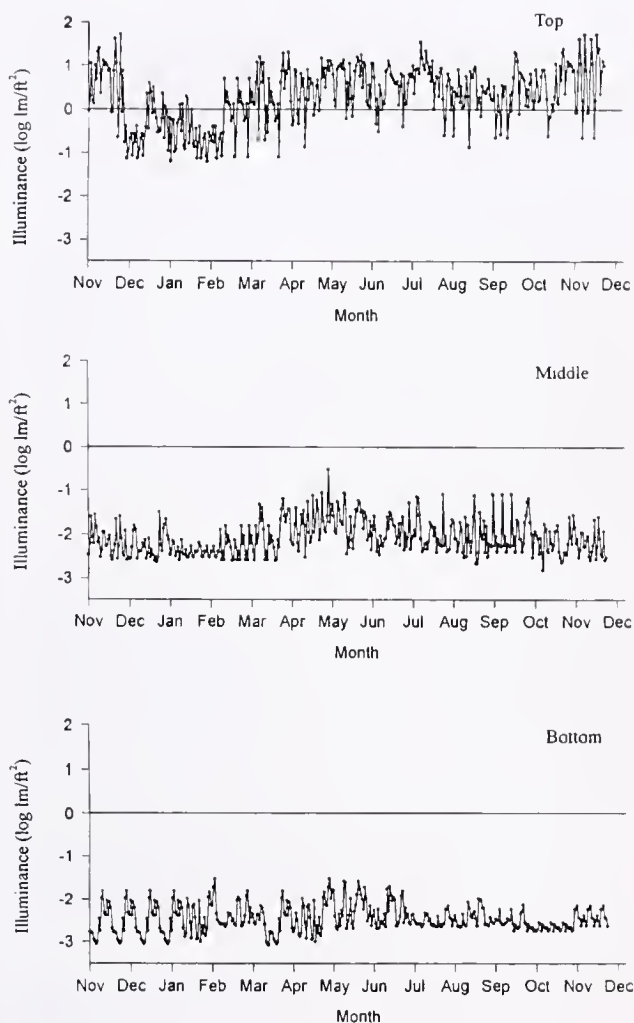


Figure 3. The illuminance in the top, middle, and bottom tier of culture system from November 1996 to November 1997.

8.1 mg/L. During the experimental period, concentrations of ammonia-N and nitrite-N, measured using the methods described by Solorzano (1969) and Bendschneider and Robinson (1952), were less than 0.044 mg/L and 0.018 mg/L, respectively.

Statistical analysis

All data were subjected to one way analysis of variance (ANOVA) followed by a Tukey's Studentized Range Test (SAS 1988) with a significance level of  $P < .05$ .

RESULTS

In the artificial diet group, the survival rate of abalone placed on the top, middle, and bottom tier was 78.6%, 67.1%, and 61.4%, respectively. In the *Gracilaria* group, the survival rate of abalone placed on the top, middle, and bottom tier was 65.7%, 71.4%, and 64.3%, respectively, after 395 days. No significant difference of survival was observed among 6 treatments.

A significant difference in body weight and shell length was observed between the abalone fed on artificial diet and *Gracilaria* over the time course of the experiment. In the artificial diet group,

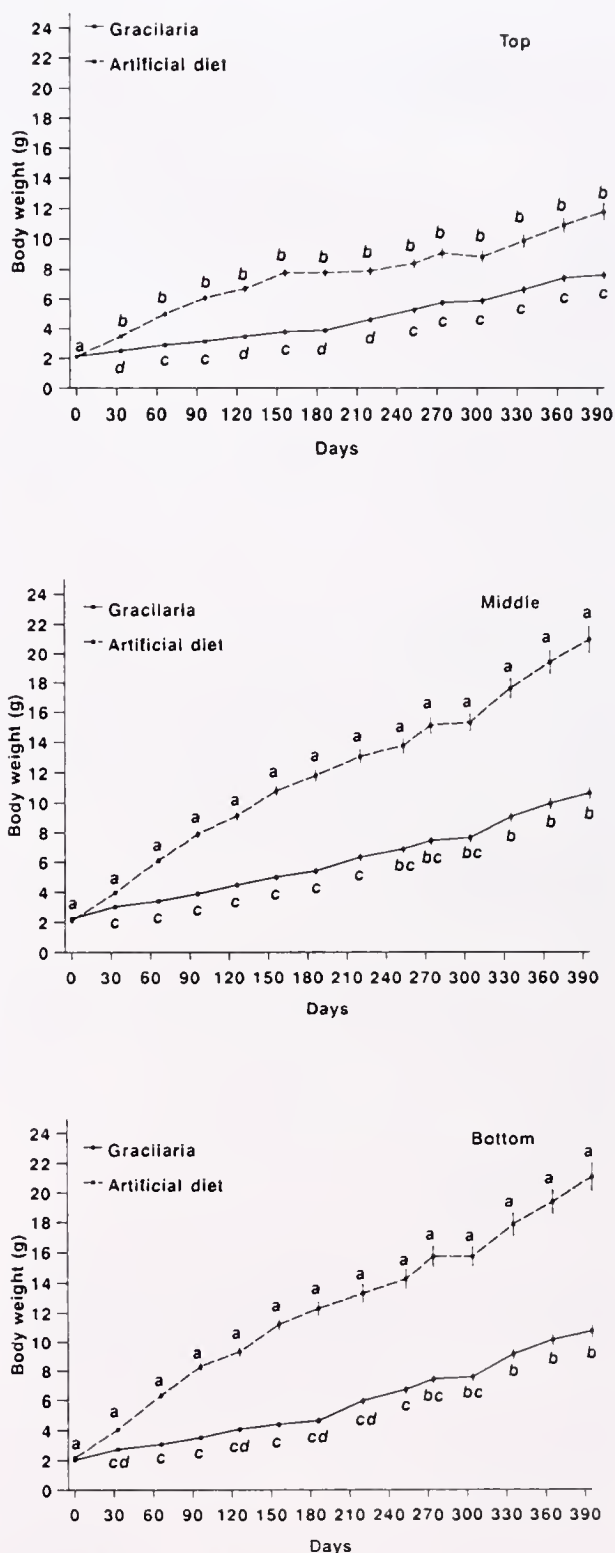


Figure 4. Mean body weight with standard error of *Haliotis diversicolor supertexta* placed on the top (T), middle (M), and bottom (B) tier, and fed on *Gracilaria* and artificial diet, after 395 days. Data in the same time period having different letters are significantly different ( $P < .05$ ) among 6 treatments.

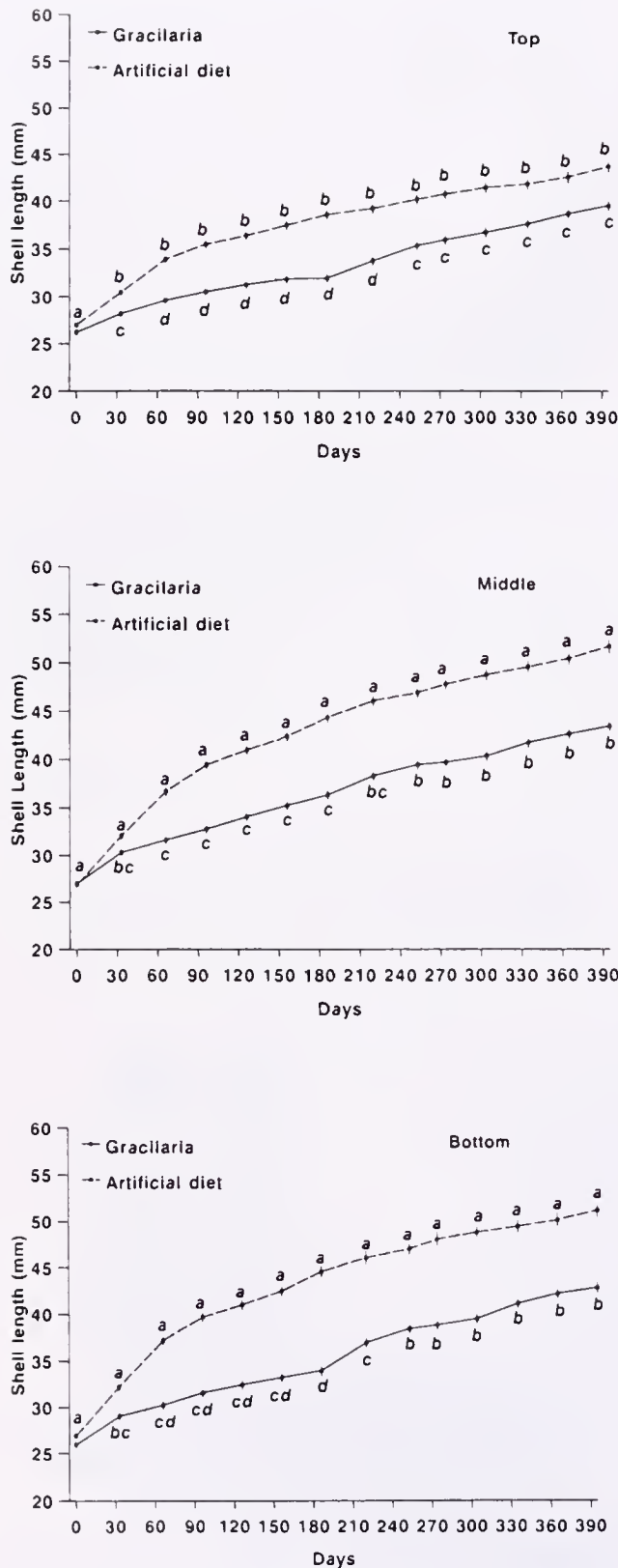


Figure 5. Mean shell length with standard error of *Haliotis diversicolor supertexta* placed on the top (T), middle (M), and bottom (B) tier, and fed on *Gracilaria* and artificial diet, after 395 days. See Figure. 4 for statistical information.

body weight and shell length of abalone placed on the top tier were significantly less ( $P < 0.05$ ) than those placed on the middle and bottom tiers at every observation time (Figs. 4 and 5).

In terms of total body weight, in the *Gracilaria* group, the abalone placed on the bottom tier grew to 10.76 g, which was 1.43 times more than those placed on the top tier. In the artificial diet group, abalone placed on the bottom tier grew to 21.08 g, which was 1.80 times more than those placed on the top tier (Fig. 4).

In terms of shell length, in the *Gracilaria* group, growth of the abalone on the top tier was significantly less ( $P < 0.05$ ) than those on the middle and bottom tiers after 220 days. There was no significant difference in shell length between the abalone placed on the middle and bottom tiers on every observation time except on the 186th day. In the *Gracilaria* group, the abalone placed on the bottom tier grew to 42.83 mm, which was 1.1 times more than those placed on the top tier. In the artificial diet group, the abalone placed on the bottom tier grew to 51.22 mm, which was 1.2 times more than those placed on the top tier (Fig. 5).

In terms of weight gain per day, in the *Gracilaria* group, the growth rate of abalone varied from 3.0 to 51.8 mg/day, and was highest during the observation period September 1 to October 2, followed by July 12 to August 2, and May 6 to June 9. In the artificial diet group, the growth rate of abalone varied from -8.4 to 71.0 mg/day, and was highest during the first 96 days, followed by the periods of July 12 to August 2 and September 1 to October 2 (Table 1).

In terms of length increase per day, in the *Gracilaria* group, the growth rate of abalone varied from 3.0 to 100.9  $\mu\text{m}/\text{day}$ , and was the highest during the first 33 days, followed by the periods May 6 to June 9 and September 1 to October 2. In the artificial diet group, the growth rate of abalone varied from 12.3 to 162.7  $\mu\text{m}/\text{day}$ , and was highest during the first 96 days, followed by the period February 5 to May 6 (Table 2).

The overall growth rate of abalone fed on artificial diet was 24.41, 47.73, and 47.85 mg/day for the top, middle, and bottom tier, respectively, which was significantly higher ( $P < .05$ ) than those fed on *Gracilaria*. The overall growth rate of abalone fed on artificial diet was 42.05, 62.81, and 61.59  $\mu\text{m}/\text{day}$  for the top, middle, and bottom tier, respectively, which was significantly higher ( $P < .05$ ) than those fed on *Gracilaria* (Table 4). Among the 6 treatments, *H. diversicolor supertexta* placed on the middle and bottom tiers and fed on artificial diet grew the most, whereas the abalone placed on the top tier and fed on *Gracilaria* grew the least (Table 3).

The harvest weight of abalone fed on artificial diet and *Gracilaria* after 395 days of rearing from 2 baskets was 646, 985, 907 g and 357, 532, 484 g for those placed on the top, middle, and bottom tier, respectively. The FCR of abalone placed on the top, middle, and bottom tier and fed on artificial diet was 3.10, 2.68, and 2.76, whereas the FCR of abalone placed on the top, middle, and bottom tier and fed on *Gracilaria* was 4.43, 3.03, and 3.04, respectively (Table 4).

## DISCUSSION

About 13 years ago, Uki et al. (1986a) evaluated the dietary values of 57 species of marine algae (25 Phaeophyta, 25 Rhodophyta, and 7 Chlorophyta) on the growth of *H. discus hannai*. They reported that 12 species of algae were shown to be of superior value with growth rate ranging from 41 to 137  $\mu\text{m}/\text{day}$  (Table 5).



TABLE 1.

Growth rate (in mg/day) for *Haliotis diversicolor supertexta* placed on the top (T), middle (M), and bottom (B) in a 7-tier system, and fed *Gracilaria* and artificial diet during different time periods. Data in the same column having different letters are significantly different ( $P < .05$ ) among 6 treatments.

Diet	Tier	Time Period (Month/Day-Month/Day)												
		1996						1997						
		11/1- 12/4	12/4- 1/6	1/6- 2/5	2/5- 3/7	3/7- 4/6	4/6- 5/6	5/6- 6/9	6/9- 7/12	7/12- 8/2	8/2- 9/1	9/1- 10/2	10/2- 11/1	11/1- 12/1
<i>Gracilaria</i>	T	11.8 <sup>d</sup>	12.0 <sup>c</sup>	8.3 <sup>d</sup>	11.2 <sup>d</sup>	10.4 <sup>c</sup>	3.0 <sup>d</sup>	21.1 <sup>c</sup>	20.2 <sup>bc</sup>	23.0 <sup>c</sup>	4.0 <sup>a</sup>	24.7 <sup>d</sup>	25.8 <sup>d</sup>	6.6 <sup>d</sup>
	M	24.4 <sup>c</sup>	11.3 <sup>c</sup>	16.2 <sup>c</sup>	19.5 <sup>c</sup>	17.1 <sup>d</sup>	13.6 <sup>b</sup>	27.7 <sup>b</sup>	16.4 <sup>cd</sup>	27.4 <sup>d</sup>	6.5 <sup>a</sup>	45.2 <sup>b</sup>	30.4 <sup>cd</sup>	22.7 <sup>bc</sup>
	B	21.9 <sup>c</sup>	10.0 <sup>c</sup>	15.0 <sup>cd</sup>	18.3 <sup>c</sup>	10.8 <sup>c</sup>	8.2 <sup>c</sup>	38.5 <sup>a</sup>	22.7 <sup>b</sup>	34.3 <sup>c</sup>	4.5 <sup>a</sup>	51.8 <sup>b</sup>	33.2 <sup>c</sup>	19.5 <sup>c</sup>
Artificial diet	T	42.1 <sup>b</sup>	45.5 <sup>b</sup>	36.0 <sup>b</sup>	20.6 <sup>c</sup>	36.0 <sup>c</sup>	3.0 <sup>d</sup>	3.7 <sup>d</sup>	14.8 <sup>d</sup>	32.2 <sup>c</sup>	-8.4 <sup>b</sup>	33.8 <sup>c</sup>	33.6 <sup>c</sup>	29.3 <sup>b</sup>
	M	56.5 <sup>a</sup>	65.1 <sup>a</sup>	59.8 <sup>a</sup>	40.8 <sup>a</sup>	55.7 <sup>b</sup>	33.4 <sup>a</sup>	36.4 <sup>a</sup>	21.4 <sup>b</sup>	64.0 <sup>b</sup>	6.9 <sup>a</sup>	74.9 <sup>a</sup>	59.9 <sup>a</sup>	51.1 <sup>a</sup>
	B	57.3 <sup>a</sup>	68.0 <sup>a</sup>	65.7 <sup>a</sup>	35.4 <sup>b</sup>	63.0 <sup>a</sup>	35.8 <sup>a</sup>	29.7 <sup>b</sup>	28.4 <sup>a</sup>	71.0 <sup>a</sup>	-0.8 <sup>ab</sup>	69.4 <sup>a</sup>	50.2 <sup>b</sup>	56.5 <sup>a</sup>

TABLE 2.

Growth rate (in  $\mu\text{m}/\text{day}$ ) for *Haliotis diversicolor supertexta* placed on the top (T), middle (M), and bottom (B) in a 7-tier system, and fed *Gracilaria* and artificial diet during different time periods. Data in the same column having different letters are significantly different ( $P < .05$ ) among 6 treatments.

Diet	Tier	Time Period (Month/Day-Month/Day)												
		1996						1997						
		11/1- 12/4	12/4- 1/6	1/6- 2/5	2/5- 3/7	3/7- 4/6	4/6- 5/6	5/6- 6/9	6/9- 7/12	7/12- 8/2	8/2- 9/1	9/1- 10/2	10/2- 11/1	11/1- 12/1
<i>Gracilaria</i>	T	60.6 <sup>c</sup>	42.1 <sup>d</sup>	30.3 <sup>f</sup>	24.3 <sup>d</sup>	19.7 <sup>d</sup>	3.0 <sup>d</sup>	54.1 <sup>c</sup>	47.3 <sup>a</sup>	29.5 <sup>c</sup>	25.7 <sup>b</sup>	28.4 <sup>c</sup>	35.7 <sup>a</sup>	27.0 <sup>c</sup>
	M	100.9 <sup>b</sup>	40.0 <sup>d</sup>	38.0 <sup>e</sup>	43.0 <sup>b</sup>	38.7 <sup>b</sup>	36.7 <sup>b</sup>	58.5 <sup>b</sup>	34.2 <sup>b</sup>	11.4 <sup>c</sup>	21.3 <sup>b</sup>	44.5 <sup>b</sup>	29.7 <sup>b</sup>	27.3 <sup>c</sup>
	B	95.5 <sup>b</sup>	34.8 <sup>c</sup>	44.7 <sup>d</sup>	29.0 <sup>f</sup>	26.3 <sup>c</sup>	24.0 <sup>c</sup>	88.2 <sup>a</sup>	44.5 <sup>a</sup>	18.6 <sup>d</sup>	22.0 <sup>b</sup>	53.9 <sup>a</sup>	35.3 <sup>a</sup>	21.3 <sup>d</sup>
Artificial diet	T	105.2 <sup>b</sup>	104.2 <sup>c</sup>	52.3 <sup>c</sup>	30.0 <sup>c</sup>	36.3 <sup>b</sup>	37.0 <sup>b</sup>	18.5 <sup>c</sup>	28.8 <sup>c</sup>	26.2 <sup>c</sup>	22.3 <sup>b</sup>	12.3 <sup>e</sup>	24.7 <sup>bc</sup>	37.0 <sup>ab</sup>
	M	157.9 <sup>a</sup>	140.0 <sup>b</sup>	91.3 <sup>a</sup>	50.0 <sup>a</sup>	46.0 <sup>a</sup>	67.0 <sup>a</sup>	50.3 <sup>c</sup>	25.5 <sup>c</sup>	41.4 <sup>b</sup>	32.3 <sup>a</sup>	27.1 <sup>c</sup>	29.3 <sup>bc</sup>	41.3 <sup>a</sup>
	B	162.7 <sup>a</sup>	148.5 <sup>a</sup>	83.0 <sup>b</sup>	43.6 <sup>b</sup>	49.7 <sup>a</sup>	69.3 <sup>a</sup>	43.8 <sup>d</sup>	29.4 <sup>bc</sup>	49.5 <sup>a</sup>	25.7 <sup>b</sup>	21.0 <sup>d</sup>	24.0 <sup>c</sup>	35.0 <sup>b</sup>

The growth rate of *H. discus hannai* fed on *Eisenia bicyclis*, *Ulva pertusa*, and *Gracilaria verrucosa* was 98, 41, and 24  $\mu\text{m}/\text{day}$ , respectively (Uki et al. 1986a). However, the growth rate of Taiwan abalone fed on *Gracilaria* is higher than those fed on *Ulva* (Chen 1984). The growth rate of *H. iris* (15–25 mm) fed on *Gracilaria chilensis* was 45.5  $\mu\text{m}/\text{day}$ , higher than 0.8  $\mu\text{m}/\text{day}$  for the same species fed on *Ulva lactuca* (Stuart and Brown 1994). The growth rate of *H. asinina* (14.5 mm) fed on *Gracilaria heteroclada* was 193  $\mu\text{m}/\text{day}$ , probably the highest record (Capinpin and Corre 1996).

Our research indicates that the growth rate of *H. diversicolor supertexta* (26 mm) fed on *G. tenuistipitata* was the highest (101  $\mu\text{m}/\text{day}$ ) during the first 33 days. The nutritional value of *G. tenuistipitata* used as food for *H. diversicolor supertexta* was superior to 57 species of marine algae except Phaeophyta *Alcaria crassifolia* for *H. discus hannai* (Uki et al. 1986a), and superior to *G. chilensis* for *H. iris* (Stuart and Brown 1994) and *G. confervoides* (Morrison and Whittington 1991), but was inferior to *G. heteroclada* for *H. asinina* (Capinpin and Corre 1996).

TABLE 3.

The overall growth of *Haliotis diversicolor supertexta* placed on the top (T), middle (M), and bottom (B) in a 7-tier system, and fed on *Gracilaria* and artificial diet after 395 days. Data in the same column having different letters are significantly different ( $P < .05$ ) among 6 treatments.

Diet	Tier	Weight Gain (%)	Growth Rate (mg/day)	Length Increase (%)	Growth Rate ( $\mu\text{m}/\text{day}$ )
<i>Gracilaria</i>	T	261.1 <sup>d</sup>	13.91 <sup>c</sup>	50.6 <sup>c</sup>	33.57 <sup>c</sup>
	M	377.8 <sup>c</sup>	21.31 <sup>b</sup>	60.9 <sup>b</sup>	41.59 <sup>b</sup>
	B	427.2 <sup>b</sup>	22.07 <sup>b</sup>	65.2 <sup>b</sup>	42.81 <sup>b</sup>
Artificial diet	T	458.4 <sup>b</sup>	24.41 <sup>b</sup>	61.6 <sup>b</sup>	42.05 <sup>b</sup>
	M	896.9 <sup>a</sup>	47.73 <sup>a</sup>	92.4 <sup>a</sup>	62.81 <sup>a</sup>
	B	865.8 <sup>a</sup>	47.85 <sup>a</sup>	90.5 <sup>a</sup>	61.59 <sup>a</sup>

TABLE 4.

Growth data of *Haliotis diversicolor supertexta* placed on the top (T), middle (M), and bottom (B) tier in a 7-tier system, and fed on *Gracilaria* and artificial diet after 395 days.

Diet	Tier	Initial Number	Initial <sup>a</sup> Total Weight (g)	Final Number	Final <sup>b</sup> Total Weight (g)	Feed <sup>c</sup> Consumed (g)		FCR (Wet)	FCR (Dry)
						Wet	Dry		
<i>Gracilaria</i>	T	70	147.35	46	357.30	9805.4	929.6	46.70	4.43
	M	70	155.95	50	532.32	12022.9	1139.8	31.94	3.03
	B	70	142.86	45	484.19	10927.5	1035.9	32.01	3.04
Artificial feed	T	70	147.23	55	645.94	1775.0	1544.4	3.56	3.10
	M	70	147.15	47	984.84	2582.0	2246.6	3.08	2.68
	B	70	152.80	43	906.59	2392.3	2081.5	3.17	2.76

FCR =  $c/(b-a)$

Concerning the growth rate of abalone feeding on artificial diets, about 4 years ago, Fleming et al. (1996) sent a questionnaire to farmers, researchers, and feed companies. They reported that the growth rate of large juveniles (25–50 mm) fed on artificial diet was 50–138  $\mu\text{m}/\text{day}$  with an average of 85  $\mu\text{m}/\text{day}$ . The growth rate of *H. discus hannai* (7–20 mm) feeding on Nie's diet was 103–211  $\mu\text{m}/\text{day}$ . The growth rate of small juveniles (17–20 mm) feeding on a diet manufactured by Nihon Nosan Kogyo K. K., the largest producer of artificial diet in Japan, was 160  $\mu\text{m}/\text{day}$  (Fleming et al. 1996); but they did not state the duration of the trial or species tested. Capinpin and Corre (1996) reported that the growth of *H.*

*asinina* (14.5 mm) fed on Nosan No. 3 diet (32% protein) (Nihon Nosan Kogyo K. K., Japan) was 192  $\mu\text{m}/\text{day}$  over 90 days. This growth rate was probably the highest record for abalone feeding on artificial diet.

Table 6 summarizes the reported data about the growth rate of abalone fed on artificial diets. About 40 years ago, Ogino and Ohta (1963) reared *H. discus* using white fishmeal as the main protein source, and observed a growth rate of 4.3 mg/day in 78 days. Uki et al. (1985) evaluated the quality of several protein sources in diets for *H. discus hannai* (34 mm). They found that the growth rate of abalone fed on casein-based diet (30% protein), soybean-

TABLE 5.

Growth of different species of *Haliotis* fed on different algae.

Species	Size (mm)	Diet	Duration (day)	Growth Rate ( $\mu\text{m}/\text{day}$ )	Reference
<i>H. discus hannai</i>	24	<i>Eisenia bicyclis</i>	30	98	Uki et al. (1986a)
	27	<i>Laminaria religiosa</i>	30	83	
	27	<i>Costaria costata</i>	30	90	
	27	<i>Undaria pinnatifida</i>	30	90	
	27	<i>Alcaria crassifolia</i>	30	137	
	27	<i>Desmarestia ligulata</i>	30	79	
	24	<i>Porphyra yezoensis</i>	30	91	
	27	<i>Palmaria palmata</i>	30	99	
	30	<i>Chondria crassicaulis</i>	30	90	
	24	<i>Ulva pertusa</i>	30	41	
	30	<i>Enteromorpha</i> sp.	30	62	
	24	<i>Codium divaricatum</i>	30	43	
	30	<i>Gracilaria verrucosa</i>	30	24	
	<i>H. laevigata</i>	15	<i>Gracilaria confervoides</i>	—	
<i>H. iris</i>	15	<i>Ulva lactuca</i>	56	0.8	Stuart and Brown (1994)
	15	<i>Gracilaria chilensis</i>	56	45.5	
	15	<i>Macrocystis pyrifera</i>	56	34.4	
	15	Mixture of three algae	56	57.2	
<i>H. tuberculata</i>	15	<i>Plamaria palmata</i>	226	58	Mgaya and Mercer (1995)

TABLE 6.  
Comparative growth of different species of *Haliotis* fed on algae and artificial diet.

Species	Size (mm)	Diet	Duration Day	Growth Rate ( $\mu\text{m}/\text{day}$ )	Reference	
<i>H. discus hannai</i>	34	Casein-based diet (30% protein)	40	133	Uki et al. (1985)	
	34	Fishmeal-based diet (32% protein)	40	74		
	34	Soybean-based diet (31% protein)	40	106		
	31	<i>Eisenia bicyclis</i>	50	54	Uki et al. (1986b)	
	31	Casein-based diets (4.8–43.1% protein)	50	32–82		
	31	Fishmeal-based diets (5.6–43.1% protein)	50	32–47		
	<i>H. asimia</i>	7	Artificial diet	70	135	Nie et al. (1986)
13		Artificial diet	70	101		
14.5		<i>Gracilaria heteroclada</i>	90	193	Capinpin and Corre (1996)	
<i>H. fulgens</i>	14.5	<i>Kappaphycus alvarezii</i>	90	59	Viana et al. (1993)	
	14.5	Artificial diet (32% protein)	90	192		
	13	<i>Macrocystis pyrifera</i>	90	25		
<i>H. midae</i>	13	Casein-based diet (44% protein)	90	98	Knauer et al. (1996)	
	13	Fishmeal-based diet (35% protein)	90	101		
	7.4	Diatoms	30	50		
<i>H. diversicolor supertexta</i>	7.9	Artificial diet (35.5% protein)	30	59	Britz (1996a)	
	20	Fishmeal-based diet (27–47% protein)	95	83–96		
	21	<i>Plocamium corallorhiza</i>	124	29		
	21	Casein-based diet (31% protein)	124	45	Britz (1996b)	
		Fishmeal-based diet (29% protein)	124	65		
		26	<i>Gracilaria tenuistipitata</i>	33		60–101
26	<i>Gracilaria tenuistipitata</i>	Artificial diet	395	33–42	Present study	
			26	Artificial diet		105–163
			26	Artificial diet		42–68
			26	Artificial diet		42–68

based diet (31% protein), and fishmeal-based diet (32% protein) was 133, 106, and 74  $\mu\text{m}/\text{day}$ , respectively, in 40 days. Uki et al. (1986b) reported that the growth rate of *H. discus hannai* (31 mm) fed on casein-based diets (4.8–55.4% protein), fishmeal-based diets (5.6–43.1% protein), and *Eisenia bicyclis* was 32–82, 32–47, and 54  $\mu\text{m}/\text{day}$ , respectively, in 50 days. Casein was considered the most suitable protein for inclusion in artificial diets.

On the contrary, Bretz (1996b) reported that the growth rate of *H. midae* fed on casein-based diet (31% protein) and fishmeal-based diet (29% protein) was 45 and 65  $\mu\text{m}/\text{day}$ , respectively, in 124 days. Viana et al. (1993) reported that the casein-based diet (44% protein) and the fishmeal-based diet (35% protein) produced similar growth rate in *H. fulgens* (13 mm): 98 and 101  $\mu\text{m}/\text{day}$ , respectively, which was about 4 times higher than those feeding on *Macrocystis pyrifera* in 90 days. The source and the processing of

the fishmeal used in the studies may account for these differences. Casein is unlikely to be widely used as a primary protein source in practical diets due to its high cost. Nie et al. (1986) reported that the growth rate of *H. discus hannai* (13 mm) fed on a diet prepared from equal amounts of soybean meal and fishmeal as protein source was 101  $\mu\text{m}/\text{day}$ . Our research indicates that the growth rate of *H. diversicolor supertexta* fed on an artificial diet (30% protein) that used soybean as the main protein source was 163 and 149  $\mu\text{m}/\text{day}$  during the first 33 days and 66 days, respectively. Our research also indicates that the growth of Taiwan abalone juveniles fed on artificial diet was 3.3 times higher than those fed on *Gracilaria* during the first 66 days.

*H. diversicolor supertexta* fed on *Gracilaria* increased its body weight during September 1 to October 2, July 12 to August 2, and May 6 to June 9, indicating that they prefer a temperature of 24–30

°C. This observation was identical to that of Chen (1984) who suggested that suitable temperature for the growth of *H. diversicolor supertexta* was 24–30 °C. During August when consistently high temperatures (> 28 °C) were recorded, abalone growth was low compared to the other periods. The reduction in growth rate was ascribed to reduction in the feeding rate of the animals due to typhoon strikes. Low growth rate may also relate to sexual maturation (Ohba 1964). However, we did not observe the maturation status of abalone in this study.

In the present study, despite the low temperatures (16–24 °C) recorded during November to February, there was consistently higher growth in abalone fed on artificial diet. This indicates that *H. diversicolor supertexta* fed on artificial diet grew significantly faster during in the first 96 days. The larvae of Taiwan abalone are generally available during October to March. We suggest that the larvae can be fed on artificial diets when they are just removed from the plastic plates. In fact, it has become a common practice to raise larvae to 20–30 mm in nursery ponds using artificial diets for the juvenile growers, when larvae are placed inside cement plates as shelters in concrete ponds. Our research indicated that *H. diversicolor supertexta* fed on artificial diet still grew well when the temperature dropped to below 20 °C.

Uki and Watanabe (1992) reported that the growth rate of abalone increased with increasing protein level in diets. They also reported that the optimal level of protein for *H. discus hannai* was 28%. Mai et al. (1995) reported that the optimum protein level was 22.3–32.3% and 23.3–35.6% for *H. tuberculata* and *H. discus hannai*, respectively. The protein content in dry weight used in the present study was 26% in *Gracilaria tenuistipitata* and 30% in the artificial diet. The fact that growth of the *H. diversicolor* fed on

*Gracilaria* was inferior to those fed on artificial diet is considered to be due to a deficiency of essential nutrients, lower content of protein, lipid and nitrogen-free extract, and higher content of crude fiber and ash in *Gracilaria* as compared to artificial diet.

In the present study, the abalone placed in the multiple-tier basket system were reared in the same tank and were exposed to the same environmental conditions, with the exception of light intensity. The abalone placed on the top tier that received more light grew significantly less than those placed on the middle and the bottom tiers. Hahn (1989) reported that abalone are easily stressed by light. Low growth rate of abalone in the top tier may also be attributed to the water flow passed from the bottom through airlift.

In conclusion, Taiwan abalone fed on artificial diet performed better than those fed on *Gracilaria tenuistipitata*, displaying higher rates of weight gain and length increase, and lower FCR. The present study indicated that growth of the abalone placed on the top tier in a multiple-tier basket system was inferior to those placed on the other tiers. The present study demonstrated that Taiwan abalone *H. diversicolor supertexta* fed on artificial diet in a multiple-tier basket system could grow to market size in less than half the time in comparison to those fed on *Gracilaria*.

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## MORPHOLOGICAL CHANGES IN THE RADULA OF ABALONE (*HALIOTIS IRIS*) DURING POST-LARVAL DEVELOPMENT

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**ABSTRACT** *H. iris* Gmelin post-larvae fed with diatoms (*Cylindrotheca closterium*) were sampled weekly from 10–60 days post-settlement. Radula morphology was examined by SEM, and compared with that of competent larvae (260–280  $\mu\text{m}$  shell length (SL)) and adults (125–130 mm SL). Larvae (130 °C.days old) had a well-formed radula with ~10 rows of teeth. Each row comprised 1 rachidian tooth, 2 pairs of lateral teeth, and 1 pair of marginal teeth. The number of rows of teeth increased to 26–29 by ~500  $\mu\text{m}$  SL (Day 10 post-settlement) and stayed at that level throughout the post-larval period (maximum size/age observed was 2.7 mm SL at Day 60). Marginal teeth were added steadily to give ~30 pairs per row at ~2.5 mm SL (compared to 60–80 in adults). Lateral teeth (L3–L5) were added between 1.0 and 1.7 mm SL to complete the adult complement. Progressive changes in the post-larval radula above 1–1.5 mm SL included: (1) reduction of serrations on the margins of rachidian and lateral teeth, particularly the more central (R, L1, L2) teeth; (2) increased spacing between adjacent rows of teeth; (3) lengthening of the outer lateral teeth (L3–L5) making them larger and taller than the central (R, L1, L2) teeth. The clearance angle of rachidian and lateral teeth was variable, but generally increased as post-larvae grew. Post-larvae <1 mm SL had highly curved teeth and apparent clearance angles around or below 0°. Larger post-larvae had positive clearance angles. These developments suggest that the teeth of post-larvae <1 mm SL probably function as “scoops” which slide across the surface collecting small diatoms and other fine, loose particles. Radulae of post-larvae > 1mm SL become more suitable for collecting larger particles, and gouging feeding substrata.

**KEY WORDS:** benthic diatom; radula; development; feeding; growth; post-larval abalone

### INTRODUCTION

Post-larval abalone have low survival in both hatcheries (e.g., Searcey-Bernal et al. 1992, Roberts et al. 1998) and natural habitats (eg. Sasaki and Shepherd 1995, McShane and Naylor 1995, Preece et al. 1997). The nature and quantity of their food is likely to be a critical factor controlling survival. As post-larvae grow, their food consumption increases rapidly (Roberts et al. 1999) and there is evidence that post-larval survival rates can be density-dependent (McShane 1992, Preece et al. 1997). As post-larval abalone grow, their sources of nutrition change, both on artificial surfaces and on coralline algae (Kawamura et al. 1998a).

Benthic diatoms are a principal food of post-larval abalone (Kawamura et al. 1998a). Post-larval growth and survival differ among diatom diets, due largely to differences in the post-larva's ability to ingest and digest the diatoms (Kawamura et al. 1998a,b, Roberts et al. 1999). Both ingestion and digestion appear to be affected by the radula. Physical rupturing of diatom cells probably relies solely on the radula (Kawamura et al. 1995) or other buccal apparatus, as the abalone gut lacks any grinding mechanism (Crofts 1929). The ability of post-larvae to ingest large (or stalked) diatoms increases as post larvae grow (Kawamura et al. 1998b, Roberts et al. 1999). This appears to be a function of food handling by the radula rather than mouth size (Roberts et al. 1999).

Diatoms in the genus *Cocconeis* are particularly interesting in the context of abalone development. *Cocconeis* is often the dominant diatom on coralline algae (authors' unpubl. data) and on pre-grazed abalone settlement plates. However, the food value of *Cocconeis* spp. for abalone post-larvae is dependent on the stage of post-larval development (Kawamura et al. 1995, Matthews and Cook 1995, Daume et al. 1997, Roberts et al. 1999). Newly settled post-larvae are unable to effectively ingest *Cocconeis* cells, and growth curves may flatten out at 500–700  $\mu\text{m}$  shell length (SL) unless other sources of food are available (Kawamura and Takami 1995, Takami et al. 1997). However, once post-larvae reach ~800  $\mu\text{m}$  SL they can efficiently ingest *Cocconeis* cells, and grow rapidly. This transition in feeding efficiency on *Cocconeis* may be gradual (Roberts et al. 1999) or more sudden (Takami et al. 1997, Daume et al. 1997), perhaps depending on the specific characteristics of the culture.

The characteristics of *Cocconeis* species that make them resistant to grazing are their very high attachment strength and their low profile. Grazing efficiency could be affected by either the detailed morphology of the feeding apparatus, or the force with which it is used. In post-larval abalone, the radula is the organ of key interest, and changes in feeding efficiency are likely to relate to radula morphology, rather than the strength of the radula action. If post-larval strength was inadequate, then radula strokes would falter. This was not seen in small post-larval *Haliotis iris* Gmelin, which grazed smoothly over *Cocconeis scutellum* Cleve (Roberts et al. 1999).

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The radula begins to develop during the larval phase (Tong and Moss 1992). It is used for feeding within a day or two of settlement, and is capable of collecting small diatoms and other particles at this time (Hahn 1989, Roberts et al. 1999). However, Kitting and Morse (1997) found that post-larval *Haliotis rufescens* Swainson grazing on coralline algae did not ingest particles until 10 days after metamorphosis. This contrast suggests that feeding is strongly influenced by the interaction between available food, and the developing radula.

There have been few observations of radulae in post-larvae of abalone or other molluscs. Several reports present a photograph and limited information on the abalone radula at a single point of larval/post-larval development (Tong 1984 - *H. iris*, Garland et al. 1985 - *Haliotis ruber* Leach, Dinamani and McRae 1986 - *H. iris*, Daume et al. 1997 - *Haliotis rubra* Leach, Kitting and Morse 1997 - *H. rufescens*). None of these studies describes the progressive development of the radula throughout the post-larval period. Studies of radula development in other larval/post-larval molluscs are limited to chitons, aplacophorans, opisthobranchs and pulmonates (Eernisse and Kerth 1988).

In this paper we describe changes in radula morphology of *H. iris* throughout the post-larval stage, and compare the post-larval radulae with those of larvae and adults. Radula morphology is discussed in relation to changes in post-larval feeding.

## MATERIALS AND METHODS

### Abalone Rearing

*H. iris* larvae were obtained as described previously (Roberts et al. 1999). Competent larvae were transferred to tissue culture dishes (Falcon 3046) with 10 ml of 0.2  $\mu\text{m}$  filtered natural seawater (FSW) containing 150  $\mu\text{g/ml}$  each of Penicillin G sodium (Biochemie) and Streptomycin sulphate BP (Sigma). These larvae were induced to attach and metamorphose by the addition of 1  $\mu\text{M}$  GABA (Roberts and Nicholson 1997). Two days after settlement induction, a diatom culture (*Cylindrotheca closterium* (Ehrenberg) Reimann and Lewin 1964) was added to the wells as a food source. These post-larvae were held at  $17.5 \pm 1.0$  °C with supplementary diatom (*C. closterium*) added as necessary, and water replaced each 3–4 days with new FSW without antibiotics. We measured post-larval size as the longest shell dimension (shell length = SL) and post-larval age as days since settlement (settlement induction = Day 0).

### Observations of Radulae

Samples of post-larvae were preserved in 4% formalin in seawater approximately weekly from 10–60 days post-settlement. Shell length of each individual was recorded. A sample of larvae was taken from the rearing vessel after 8 days of development at 16 °C, and preserved as above. To obtain clean radulae, we dissolved larval/post-larval tissues by bathing the abalone in sodium hypochlorite (0.6% final Cl concentration, Wako Pure Chemical Industries Ltd., Osaka) for several minutes. When free of surrounding tissues, the radula was removed with a pipette and transferred through several distilled water baths before the radula length, width and number of rows of teeth were determined using a light microscope and video micrometer. Adult radulae were dissected from fresh animals and rinsed with distilled water. Radulae were then transferred to SEM stubs, laid flat with teeth upwards,

and allowed to air dry before sputter coating with gold for SEM observation.

The radula formulae, terminology and measurements used are illustrated in Figure 1. The length of the rachidian and L3 teeth was measured along the centre line of the cusp in plan view (Fig 1A). Whole radula width was measured with marginal teeth in resting configuration (not splayed) and whole radula length was measured along the centre line of the radula (Fig. 1D). Clearance angle of rachidian and lateral teeth, as defined by Padilla (1985), was measured by viewing flat-lying radulae from the side and averaging the angle of 10–14 teeth. On printed photos, a line was drawn representing the approximate slope along the midline of the back surface of the tooth. The angle between this line and the long axis of the radula was the clearance angle (Fig. 1B, 1C). Imperfect view angles and highly curved teeth made precise measurements difficult, so data should be treated as indicative rather than absolute. Rake angles could not be measured directly on most teeth due to their shape and orientation. However, rake angle can be calculated from clearance angle if the angle across the tooth tip is known ( $\text{Rake angle} = 90 - \text{Tooth tip angle} - \text{Clearance angle}$ ) (Fig. 1B). Accurate counts of marginal teeth were only possible in small

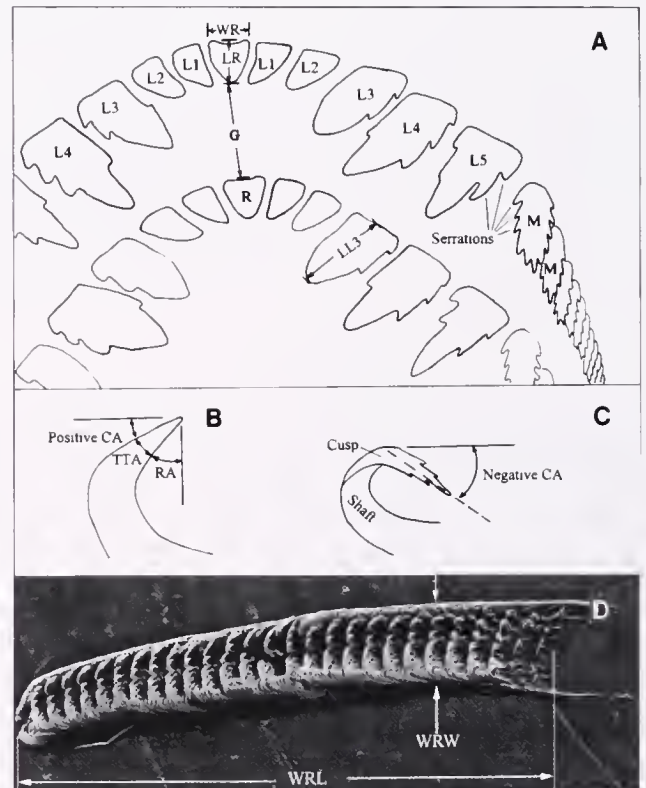


Figure 1. Illustration of terms used in the text to describe radula morphology. (A) Two transverse rows of radula teeth in plan view. Tooth types: R = rachidian, L1–L5 = lateral teeth 1–5. M = marginals (not all shown). Measurements: WR = width of rachidian tooth, LR = length of rachidian tooth, LL3 = length of L3 tooth, G = gap between rachidian teeth of adjacent transverse rows. (B) A single radula tooth in profile illustrating: CA = clearance angle (positive), RA = rake angle (both as defined by Padilla 1985), TTA = tooth tip angle. (C) A single tooth in slightly oblique profile showing tooth components (after Hickman 1980) and the negative clearance angle of a strongly curved tooth. (D) Whole radula laid flat showing 27 transverse rows of teeth projecting upwards, WRW = whole radula width (marginals are in resting position), WRL = whole radula length.



post-larvae with few marginals, and in specimens with the marginal teeth fortuitously splayed.

## RESULTS

During the post-larval period the radula underwent various morphological developments toward the adult form. Most developments correlated slightly more strongly with post-larval shell length than post-larval age (Table 1), so the following description relates primarily to shell length.

The overall length of the radula increased linearly with post-larval shell length (Fig. 2A). This length increase was caused by different factors at different stages of post-larval development. The number of rows of radula teeth increased only during the first 10 days after settlement. The larval radula contained 9–10 rows of fully formed teeth, and an additional 2–3 rows of developing teeth. By Day 10 post-settlement, the number of rows of teeth had increased to 26–28, and remained between 26 and 29 throughout the post-larval period correlating very weakly with shell length (Fig. 2A). From 500–1700  $\mu\text{m}$  SL the increase in radula length reflected both an increased gap between rows of teeth, and increased length of the rachidian and L1–L2 teeth (Fig. 2B). Above 1700  $\mu\text{m}$  SL the gap between adjacent rows of teeth increased rapidly (Fig. 2B) as differentiation of the L3–L5 teeth developed (see below).

Radula width also increased linearly with post-larval shell length (Fig. 3A) due to an increase in the number of teeth per row (Fig. 3B), and to a lesser extent, the width of individual teeth (Fig. 3A). The number of marginal teeth per row increased from just one pair in competent larvae to ~30 pairs in post-larvae of 2000–2700  $\mu\text{m}$  SL (Fig. 3B). The adult radula contained ~60–80 pairs of marginal teeth per row (data not presented).

Lateral teeth were added relatively late in post-larval development. In the larval radula, lateral and marginal teeth were not clearly differentiated, but the radulae appeared to contain 2 pairs of lateral teeth and 1 pair of marginal teeth per row. All post-larvae less than 1 mm SL contained only the two pairs of lateral teeth (L1 and L2) present in the larval radula. An additional 3 pairs of lateral teeth (L3–L5) were added progressively as post-larvae grew from ~1.0 to ~1.7 mm SL (Fig. 3B) completing the adult complement of laterals.

TABLE 1.

Correlation of radula variables with post-larval age and post-larval shell length. Post-larvae were chosen to include a range of sizes for each age. Data are Pearson's correlation coefficients. All correlations were significant ( $P < 0.01$ ) except where marked (NS).

Variable	Post-larval		n
	age	shell length	
Overall length of radula	0.878	0.967	55
Overall width of radula at rest	0.866	0.942	48
No. of rows of teeth in radula	0.260 (NS)	0.363	51
Width of rachidian tooth	0.706	0.847	32
Length of rachidian tooth	0.855	0.891	28
Length of third lateral (L3) tooth	0.776	0.875	18
Gap between adjacent rachidian teeth	0.820	0.954	28
Clearance angle of rachidian/lateral teeth	0.880	0.810	21
Number of lateral teeth per row	0.907	0.891	29
Number of marginal teeth per row	0.981	0.976	8

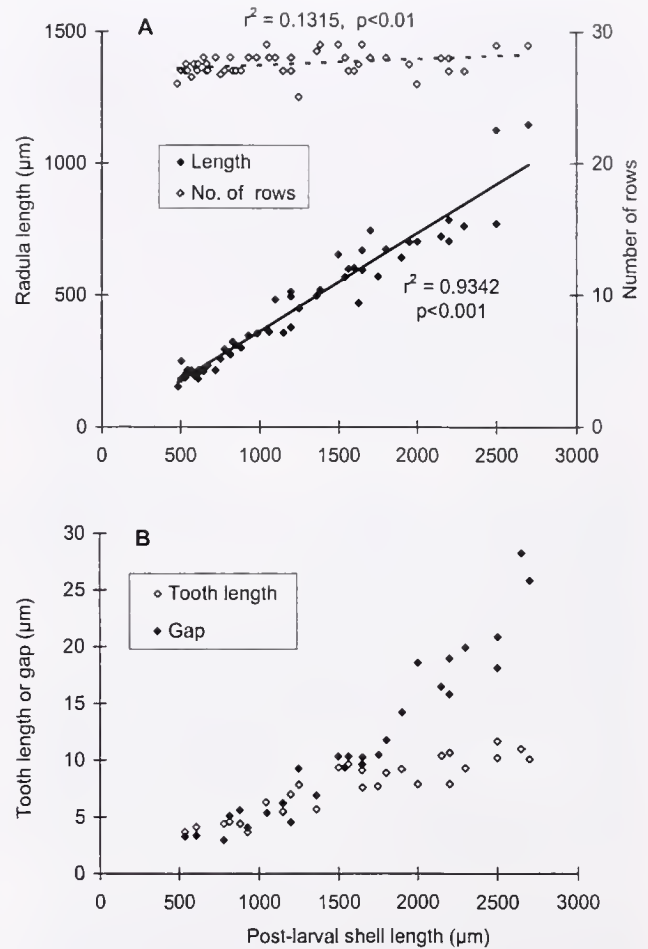


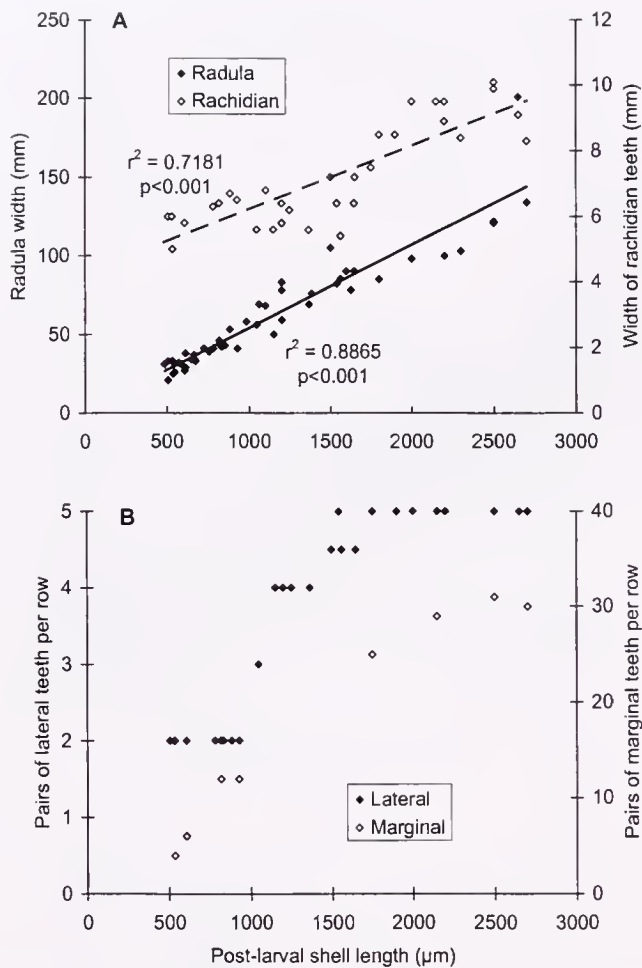
Figure 2. Relationship between post-larval size and factors relating to radula length: (A) whole radula length and number of rows of transverse teeth in radula; (B) length of the rachidian tooth and gap between rachidian teeth in adjacent transverse rows.

### Morphology of Individual Radula Teeth

The serrations on the working edges of the rachidian and lateral teeth became less pronounced as post-larvae grew. The rachidian and lateral teeth initially had long, pointed serrations along both lateral margins (Fig. 4A, B). Above 1 mm SL, these serrations became progressively shallower, first on the rachidian and L1 teeth (Fig. 4C, D), and later on L2, and to some extent, L3 and L4 (Fig. 4E). By about 2 mm SL, post-larvae had lost nearly all serrations from R–L2 teeth, but retained them on L3, and particularly L4–L5. Marginal teeth remained finely serrated throughout the post-larval period. The adult radula lacked serrations on the rachidian and lateral teeth (Fig. 4F), but retained them on marginal teeth.

Differentiation of the lateral teeth was evident in larger post-larvae. The rachidian and L1–L2 teeth were similar to one another throughout the post-larval period (but some differentiation was evident in the adult radula). The L3–L5 teeth were longer than R, L1, and L2 from the time they first appeared, but the size difference became more marked in larger post-larvae, with rapid lengthening of the L3–L5 teeth (Fig. 5 for L3, data for L4 and L5 similar but not shown).

The morphology of marginal teeth did not change markedly over the post-larval period. Marginal teeth were long, narrow and



**Figure 3.** Relationship between post-larval size and factors relating to radula width: (A) whole radula width and width of the rachidian teeth; (B) number of lateral and marginal teeth in each transverse row.

comb-like, with many long, fine cusps (Fig. 4). The size of marginal teeth within a row decreased away from the lateral teeth in larger post-larvae (Fig. 4E).

There was large variation in the clearance angle of the rachidian and lateral teeth within and between radulae. The curvature of post-larval radula teeth, and difficulty in obtaining a perfect profile of teeth made precise measurements impossible. Despite these difficulties it was apparent that the clearance angle of radula teeth in our SEM preparations increased with post-larval shell length (Fig. 6). Teeth of post-larvae less than 1 mm SL were generally strongly curved (Fig. 7A) and had clearance angles of around  $0^\circ$  or less (Fig. 6). Larger post-larvae had a flatter working surface on the tooth and much higher clearance angles (Fig. 6, Fig. 7B). The angle across the tip of the radula teeth was about  $10\text{--}15^\circ$ , thus rake angles in post-larvae over 1 mm averaged about  $40\text{--}70^\circ$ .

## DISCUSSION

### Changes In Radula Formula and Morphometry

The number of rows of radula teeth increased rapidly between the larval stage and 500  $\mu\text{m}$  SL (Day 10 post-settlement), but then remained relatively constant for the remainder of the post-larval

period. However, radula length and width continued to increase throughout the larval period due to addition and enlargement of teeth (Figs. 2–4). Marginal teeth were added steadily throughout the post-larval period, but lateral teeth were added only above 1 mm SL. Thus, radula development initially increased the length of the collecting organ and the sweeping action provided by the marginal teeth. Later addition of lateral teeth was followed by progressive changes in various aspects of radula morphology, that appeared to create specialisation of the teeth, and alter feeding capability.

We observed progressive differentiation of the lateral and rachidian teeth above 1.5 mm SL. There was a rapid increase in the gap between adjacent rows of teeth (Figs 2B, 4) and in the length of the L3–L5 teeth (Fig. 5). With this change, L3–L5 stood much higher above the radula membrane than R-L2 suggesting that they would be the main excavating teeth. The increased space between adjacent rows of teeth may allow the radula to handle larger particles efficiently. In post-larvae of less than 1 mm SL the gap between rows of teeth was  $\sim 3\text{--}5\ \mu\text{m}$  which is less than the cell length of most diatom strains, and less than the width of many. By 2.5–2.7 mm SL the gap between adjacent rows of teeth was about  $20\text{--}30\ \mu\text{m}$ , which is greater than the cell width of most diatoms.

### Reduction of Serrations on Teeth

Hickman (1980) suggested that the detailed morphology of the cusp is most likely to reflect the precise nature of the feeding substratum (but also warned that examples of this are not common). The radula teeth of *H. iris* post-larvae contained deep serrations initially, but these were progressively lost as the L3–L5 teeth differentiated from R-L2. This may suggest that the radula is developing away from handling very small food particles such as bacteria and small diatoms.

### Clearance and Rake Angle of Teeth

By drawing parallels between radula teeth and engineering cutting tools, Padilla (1985) suggested that the rake angle and clearance angle provided information on the function of radula teeth, even in radulae dissected out of gastropods. Low rake angles can result in ploughing rather than cutting teeth, and clearance angles of zero or less can prevent the leading tip of the tooth from contacting the substratum, so the tooth will slide on the back surface of its cusp (Padilla 1985). The orientation of post-larval radula teeth prevented us from measuring rake angle on most teeth, so only clearance angle was measured. It was not possible to observe the clearance angles in live animals so our measurements were limited to radulae extracted from post-larvae with sodium hypochlorite, air dried and laid flat for SEM. We do not know how relevant these measurements are to the feeding action of the radula, so quantitative interpretation of the data can only be conjectural. The curved nature of cusp adds a further complication to the measurement and interpretation of clearance angles. Whether clearance angles should be measured across the back face of the cusp (as we did), or right at the tip of the tooth, probably depends on the precise nature of the feeding surface, and the likely wear pattern of teeth.

In our data, clearance angle was variable within and between radulae, but generally increased as post-larvae grew. Post-larvae  $< 1\ \text{mm}$  SL had clearance angles around  $0^\circ$  or less. If these data are representative of the tooth position during feeding, then these teeth would tend to slide across the surface rather than dig in. Their

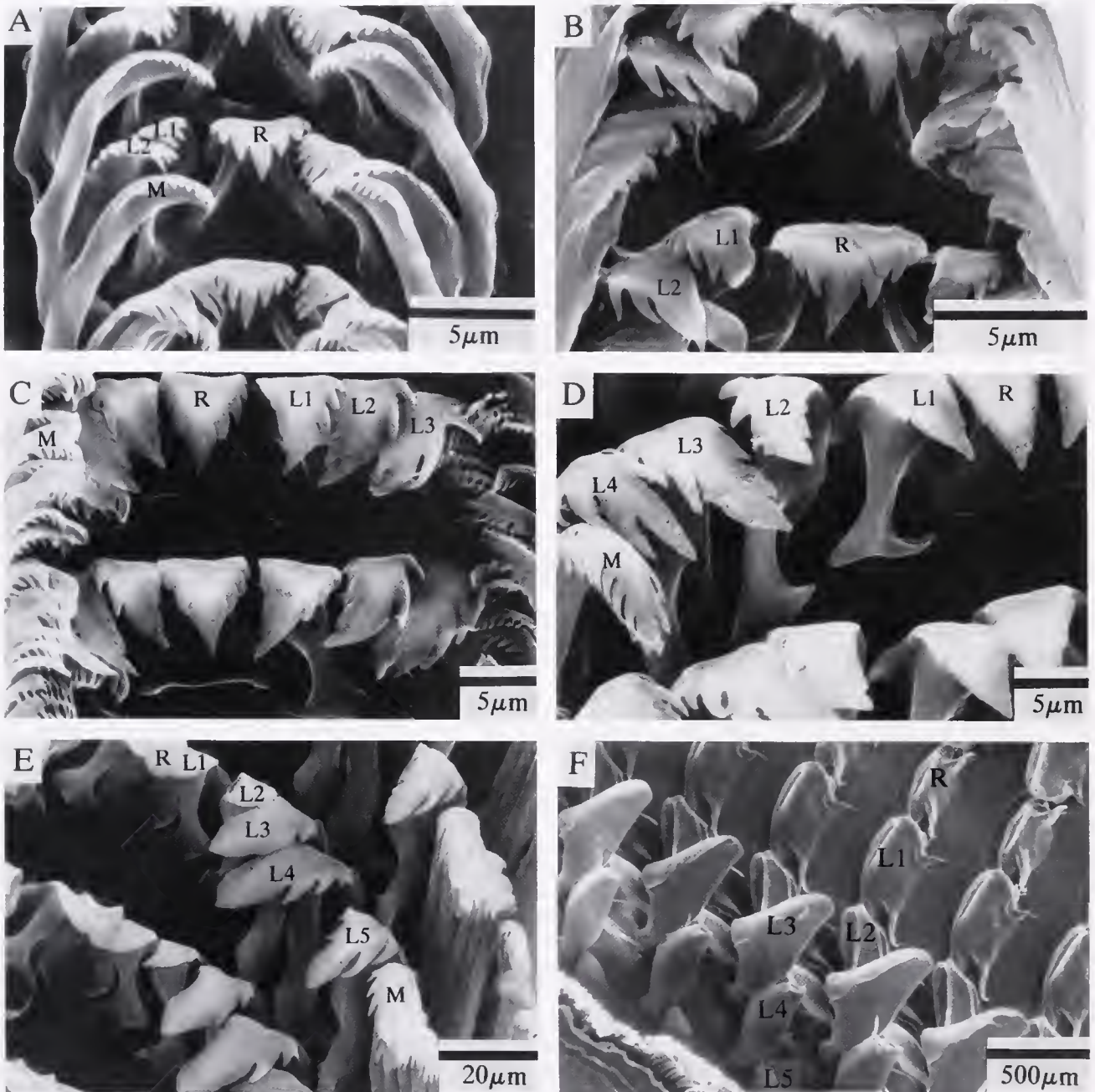


Figure 4. Developmental stages of the post-larval radula, as seen by SEM. R = rachidian tooth, L1-L5 = lateral teeth, M = marginal teeth. Radula formulae represent numbers of M+L+R+L+M (Voltzow 1994). (A) Competent larva 270  $\mu$ m SL after developing for 8 days at 16  $^{\circ}$ C, radula formula = 1+2+R+2+1. (B) Post-larva of 530  $\mu$ m SL at Day 10 post-settlement, -4+2+R+2+-4. (C) Post-larva of 1050  $\mu$ m SL at Day 31, -12+3+R+3+-12. (D) Post-larva of 1.2 mm SL at Day 53. Note that the laterals have started to differentiate, -14+(2+2)+R+(2+2)+-14. (E) Post-larva of 2.7 mm SL at Day 60, with L3-L5 now much larger than R-L2, and the latter having lost their coarse serrations. (F) Adult of 130 mm shell length. Marginals are numerous and small (not all visible) and L3-L5 are specialised gonging teeth without serrations; -80+(3+2)+R+(2+3)+-80.

strongly curved shape may make them effective "scoops" for small particles. Larger post-larvae had positive clearance angles (averages of 15-35 $^{\circ}$ ) which would allow the tip of the tooth to dig in, meaning that rake angle becomes important. Tooth rake angles in post-larvae > 1 mm SL were around 40-70 $^{\circ}$ , which is probably adequate to make them "cut" rather than "plough" (*sensu* Padilla 1985). Daume et al. (1997) reported a low clearance angle of -8 $^{\circ}$

on the radula teeth of 53-day-old post-larval *H. rubra*. They made no mention of variation within/between radulae.

#### Hardness and Flexibility of Radula Teeth

In adult gastropods, newly formed radula teeth are initially soft, consisting of chitin and proteins (Runham 1961). The hardness of

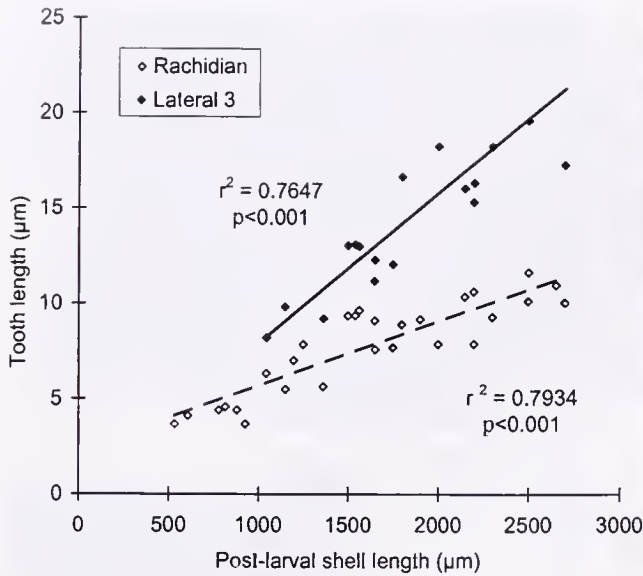


Figure 5. Relationship between post-larval size and the length of the rachidian and L3 teeth. Each data point represents the mean of 3–6 teeth on one radula.

the teeth develops through precipitation of inorganic salts (Ca, Fe, P in the rhipidoglossa) via a complex biomineralisation process (Sollas 1907, Rinkevich 1993). There have been few observations of biomineralisation of post-larval radulae. Eernisse and Kerth (1988) observed dark-capped lateral teeth in even the most recently formed radulae of chitons. They analytically confirmed mineralisation of the teeth in juvenile chitons 16 days post-metamorphosis. No such dark caps are present in abalone, and there are no data on the mineralisation of post-larval abalone teeth. We guess that the teeth of young post-larval abalone would be relatively soft and flexible (especially those most recently formed) and that this would affect diatom ingestion, especially with respect to tightly attached and/or low-profile diatoms, such as *Cocconeis* spp. (see below). It would also limit the ability of the radula to penetrate firm substrata such as foliose macroalgae or crustose

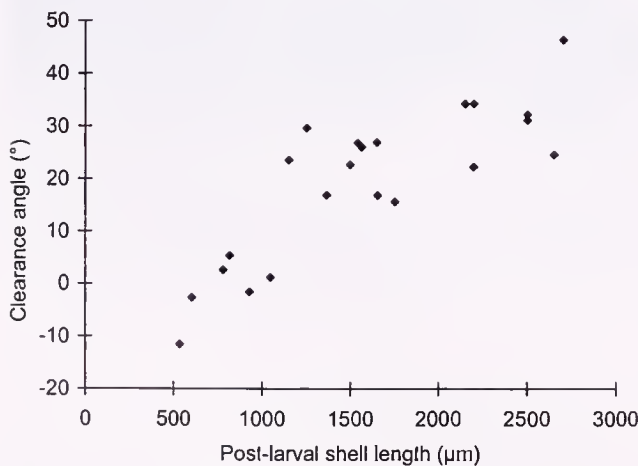


Figure 6. Relationship between clearance angle of teeth (see Fig. 1) and post-larval shell length. Each point represents the mean of 10–14 teeth on one radula.

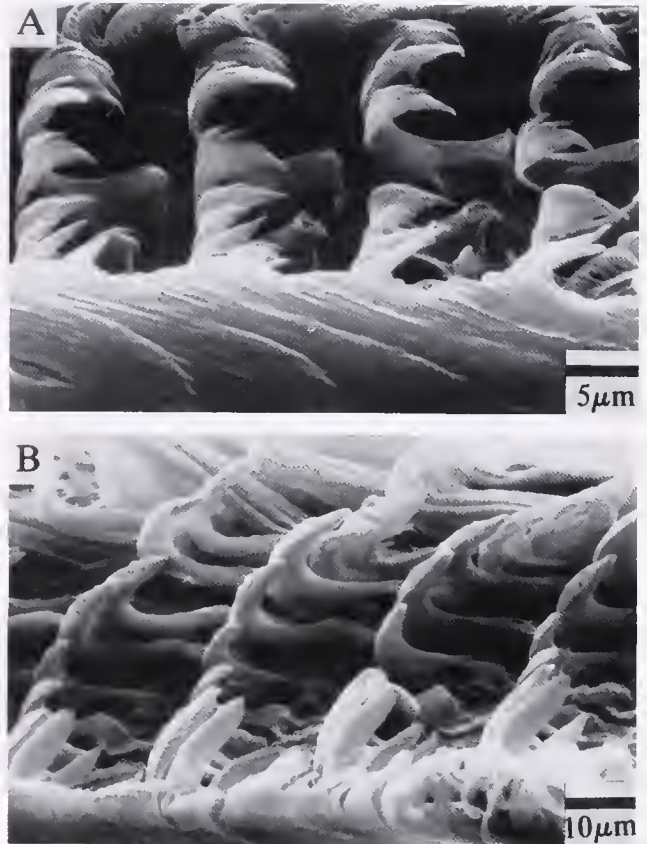


Figure 7. SEM photographs of the post-larval radula showing: (A) Strongly curved cusps on radula teeth, producing clearance angles around 0° or less. These were typical in small post-larvae (<1 mm SL). (B) Straight-cusped radula teeth with positive clearance angles - typical of larger post-larvae (>1.5 mm SL).

corallines. While the radula teeth are still soft and flexible, it would make sense for them to act as scoops rather than excavators.

*Grazing on Cocconeis spp.*

Ingestion of *Cocconeis* spp. by post-larval abalone is of particular interest in the context of hatcheries and natural recruitment (see Introduction). In feeding and growth experiments conducted in parallel with this study, the post-larvae became progressively more efficient at ingesting *Cocconeis scutellum* between shell lengths of 0.5 and 1.2 mm (Roberts et al. 1999). Faeces of 0.5 mm post-larvae contained few *C. scutellum* cells, and post-larvae were observed grazing rapidly and smoothly over *C. scutellum* cells without removing or rupturing them. By 0.8 mm SL, post-larval grazing was efficiently removing *C. scutellum*. An increase in clearance angle would result in a gouging, rather than sliding, tooth (Padilla 1985) and could explain the ingestion pattern seen on *C. scutellum*. Our data suggested that clearance angle does increase (and become positive) during early post-larval development, but the change was relatively small in post-larvae less than 1 mm SL, and variability was high. Furthermore, the data are based on radulae removed from post-larvae and prepared for SEM, so any discussion of the feeding implications of increasing clearance angle is conjectural. The hardness and flexibility of the radula teeth (see above) may also explain the change in ingestion of *Cocconeis* cells.

### Changing Radula Function and Diet

Based on the observed changes in radula morphology, we can speculate about changes in food sources for post-larval *H. iris*. The radula teeth of post-larvae <1 mm SL probably function as scoops, which slide across the feeding substratum and collect small and loosely attached particles. On hatchery surfaces these will be predominantly small to medium sized naviculoid diatoms and other biofilm components such as bacteria and extracellular secretions. On coralline algae in natural habitats the food particles may include biofilm components (e.g., bacteria, loosely attached diatoms and extracellular secretions) or loose epithelial cells.

Above 1 mm SL the apparent clearance and rake angles mean that the radula teeth may begin to penetrate at least soft foods, rather than just sliding across the substratum. The reduction of tooth serrations may indicate less emphasis on very small food items, and the increasing spacing of tooth rows may render the radula more capable of handling larger food particles. The L3-L5 teeth appear to become specialised "gouging" teeth, and the increased number of marginals improves the particle collecting action of the radula. These changes are consistent with observations that larger abalone post-larvae consume even large or tightly attached diatoms (e.g., Roberts et al. 1999), and can ingest delicate red seaweeds (Crofts 1937) and the surface cuticle/cells of coralline algae (Garland et al. 1985, Kitting and Morse 1997).

Steneck and Watling (1982) regarded rhipidoglossan radulae as "brooms" with very limited excavating force. They stated that no rhipidoglossans feed on calcareous algae (which includes the corallines). However, Shepherd and Cannon (1988) found a predominance of coralline algal fragments in the guts of 5–10 mm *Haliotis laevigata* Donovan and *H. scalaris* Leach. Garland et al. (1985) showed that even 6–13 week old *H. rubra* (average of 700–2400 µm SL respectively) can ingest the cuticle and some cytoplasm from coralline algae. Thus, abalone are capable of ingesting at least the outer layers of coralline algae from an early age, but determination of the relative importance and food value of coralline algae requires quantitative studies of feeding and food utilisation.

Many of the structural developments of the abalone radula occurred within 60 days post-settlement but the radula of 2.5 mm juveniles was still quite different from that of the adult. The adult *H. iris* radula has the same number of rachidian and lateral teeth, but at least twice as many marginal teeth per row. The adult's L3-L5 teeth lack serrations and are pointed blocks (apparently specialised for gouging macroalgae) (Fig. 4F) and the L2 teeth were differentiated from the R and L1 teeth.

The sequence of radula development we observed was for post-larvae feeding on a film of loosely attached diatoms at 17 °C. Development correlated more strongly with post-larval size than

post-larval age (Table 1), so we would expect slower radula development with lower temperatures and poor food sources. Grazing resistant food sources (such as coralline algae) may accelerate tooth wear and tooth loss, resulting in differences in radula length, number of rows of teeth, and tooth serrations.

### Ontogeny of Radula Development

Previous work on radula development in larval and post-larval molluscs was reviewed by Eernisse and Kerth (1988). In all gastropods studied (two opisthobranch species and seven Pulmonate families) lateral teeth developed before a median (=rachidian) tooth was added. The same pattern was observed for four species of chiton (Eernisse and Kerth 1988). In *H. iris*, by contrast, the rachidian tooth was present from the time that the radula first became visible in the larva.

Eernisse and Kerth (1988) found no evidence of radula formation in the larvae of four chiton species, but they state that Minichev and Sirenko (1974) reported primordial radulae in unidentified chiton trochophores. In contrast, the radula of abalone is well developed by the time larvae are competent to settle (Fig. 4A of this paper, Moss and Tong 1992, Roberts and Nicholson 1997, Salas-Garza et al. 1994). If metamorphosis is delayed, *H. iris* larvae continue to rapidly add rows of teeth to the radula reaching ~15 rows after 15 days at 16 °C, and up to 30 or more rows after ~30 days (R. Roberts, unpublished). The development of the radula in abalone larvae provides a functional food-collecting organ within a day or two of settlement (Hahn 1989, Kitting and Morse 1997, Roberts et al. 1999).

In *H. iris*, additional lateral teeth were added "outside" (=lateral to) the existing lateral teeth, but "inside" (=central to) the marginal teeth. This pattern is consistent with other gastropods, but differs from chitons, which add new lateral teeth between existing laterals during post-larval development (Eernisse and Kerth 1988). When additional pairs of lateral teeth are forming in *H. iris*, they are similar to marginal teeth in their drooping, comb-like form (Fig. 2D). This, and their place of formation, suggest that new lateral teeth may be added by progressive differentiation of the inner-most marginal teeth.

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## THE EXTRACELLULAR MINERAL CONCRETIONS IN *ANODONTA CYGNEA* (L.): DIFFERENT TYPES AND MANGANESE EXPOSURE-CAUSED CHANGES

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**ABSTRACT** The mantle and gill extracellular mineral concretions of normal unpolluted and manganese-incubated specimens of the freshwater mussel, *Anodonta cygnea* (L.), were studied for their elementary composition and morphology. The mineral concretions of the gills, as well as those analyzed on the haemolymph side of the intrapallial epithelium, revealed a phosphate nature, being related to manganese accumulation. On the contrary, mineral formations located on the haemolymph side of the extrapallial epithelium are mainly built of carbonate salts, probably being more related to the shell biomineralization process than to the toxic metal detoxification. The concretions also differ markedly in their morphological aspects, with the phosphate formations being spherical, and the carbonate formations quite irregularly shaped.

**KEY WORDS:** biomineralization, detoxification, mussel, gill, mantle, concretion, manganese

### INTRODUCTION

Many invertebrate phyla are known to produce calcium concretions in one or more of their tissues (George 1982). The freshwater bivalve, *Anodonta cygnea*, has been reported to use this insoluble store of calcium for shell deposition (Istin and Girard 1970a).

The extracellular concretions of *Anodonta* and other related species have been described in the mantle (Pekkarinen and Valovirta 1997) and gill (Silverman et al. 1983, Silverman et al. 1985, Silverman et al. 1987a). The composition and structure of these concretions has been studied in *Ligumia subrostrata* (Silverman et al. 1983), *Margaritifera margaritifera* (Pekkarinen and Valovirta 1997), and *Anodonta* sp. (Pynnönen et al. 1987), through histochemistry, electron microscopy, and chemical analysis.

We now report the results of an annual monitoring of these extracellular concretions in normal specimens of the freshwater mussel, *Anodonta cygnea*, with respect to their morphology and mineral composition. Both concretions from the mantle and gill epithelia were examined, as well as the alteration of their normal characteristics after manganese exposure, to establish a connection between these microstructures and their possible physiological roles, namely those related to shell production and heavy metal detoxification.

### MATERIALS AND METHODS

Lake mussels, belonging to the species *Anodonta cygnea* (L.), were collected through dragging from the clay bottom of the Mira lagoon (Aveiro, Portugal). The length of the animals treated varied from 12.0 to 18.0 cm, with a mean of 15.0 cm.

Six specimens were collected each month, were transported to the Institute laboratory facilities in iceboxes containing natural pond water; kept in the laboratory in aerated pond water without feeding; and processed within 24 h of collection. The animals were

considered healthy if they showed active ventilation, powerful valve closing or water ejection upon disturbance, and if their nacre presented a smooth and shiny look.

For the manganese-exposure assay, 12 specimens of about 200-g body weight, collected in the same way during March, were randomly assigned to two treatment categories, control and heavy metal exposure, and kept in plastic tanks with naturally collected water at a temperature similar to natural conditions. The tanks were placed in relative darkness and the water was aerated and used without any pretreatment.

For the heavy metal treatment, manganese, as  $MnCl_2$ , was added to the tank water to reach a final concentration of around  $10^{-6}$  M (between 1 and 2 ppm) from a stock solution previously prepared with distilled water. Manganese was the heavy metal chosen because of the well-known relationship with the mineral structures (Silverman 1989). The exposure period lasted for 16 h, during which the mussels showed normal ventilation. After exposure, both manganese-exposed and control animals were subjected to the same treatment as the specimens collected each month.

Gill and mantle fragments were extracted by cutting the mussel adductor muscles, opening the shell, and cutting the middle region of the tissue free from the body. After dissection, small (3-4 mm) mantle pieces were separated into their two composing epithelia, i.e., the one facing the pallial cavity (intrapallial epithelium) and the other facing the shell side (extrapallial epithelium). These fragments were then placed in aluminum cylinders with their haemolymph-bathed side up (showing the basal face of both epithelia), and left to dry at room temperature.

The composition of concretions from both mantle epithelia, on the haemolymph side, was analyzed on the assembled material by energy dispersive X-ray spectroscopy (EDS), with a spectrum collection time of 60 seconds. The elements chosen for analysis were Ca, P, C, Cl, Na, Mg, and Mn, from pilot determinations. Gill tissue fragments, on their haemolymph-bathed side and equally assembled, were also analyzed with this technique, to establish

eventual differences between the concretion populations from both organs. The morphology of the different mineral concretions, determined by their elemental composition, was then studied by scanning electron microscopy (SEM), also using the dried tissue samples without any other treatment.

## RESULTS

Results of the mineral concretion composition reveal no major variations, either between individuals or throughout the year, the only significant differences being those between tissue-type analyzed, and those exposed to manganese. Therefore, comments will focus on these elements, using the EDS records of each group of animals (i.e., normal and metal-exposed) and tissues (i.e., gill, extrapallial and intrapallial epithelia).

Using the March records, which also correspond to the control group for the contamination study, we find high amounts of phosphate-like concretions on both the gill (see Fig. 1) and the intrapallial epithelium, on its haemolymph side (Fig. 2). The major phosphate composition of these mineral formations is demonstrated by their higher content of phosphorus, compared to carbon—which is probably more related to the surrounding tissue present—as shown by the record of the intrapallial epithelium itself (Fig. 4). In these phosphate concretions, and throughout the whole year, there seems to be a good correlation with the manganese level, suggesting a constant relationship between them.

On the other hand, at the haemolymph side of the shell-facing extrapallial epithelium, the mineral concretions reveal a carbonate nature (Fig. 3), although with some phosphate, which is usually rather low (residual). This second group of concretions does not show the same tight relationship with the manganese composition, with a lower level being the general rule.

The two kinds of mineral concretions, now detected also show morphological differences (Fig. 5), with the carbonate concretions (3) being usually larger and more irregular. The phosphate formations (1,2) are present in higher amounts and show a regular spherulitic morphology, although with important size variation. They are also tightly covered by organic material presenting a net-like structure, in which they seem to be formed. Both concretion types are well attached to the respective epithelium, always giving the same distribution results when both tissues are separated from each other (see Fig. 6 for better a understanding of the overall arrangement).

These general features were not significantly changed in the manganese-incubated specimens. These results show that the manganese is preferentially bound by the phosphate-bearing granules, being increased only in those formations in response to the contamination situation, compared to the normal control animals. The association between manganese and phosphate minerals is also supported by our observations on the carbonate-composed concretions, where higher amounts of this metal were associated with the samples with the highest level of residual phosphate.

## DISCUSSION

*Anodonta cygnea* uses calcium concretions as calcium stores for shell growth (Istin and Girard 1970b), associated with anhydase activity as a regulatory feature (Istin and Girard 1970a). Concretions are reported both in the gills (Silverman et al. 1983, Silverman et al. 1985, Silverman et al., 1987a) and in the mantle (Pekkarinen and Valovirta 1997) connective tissues of this and other related (i.e., freshwater) species.

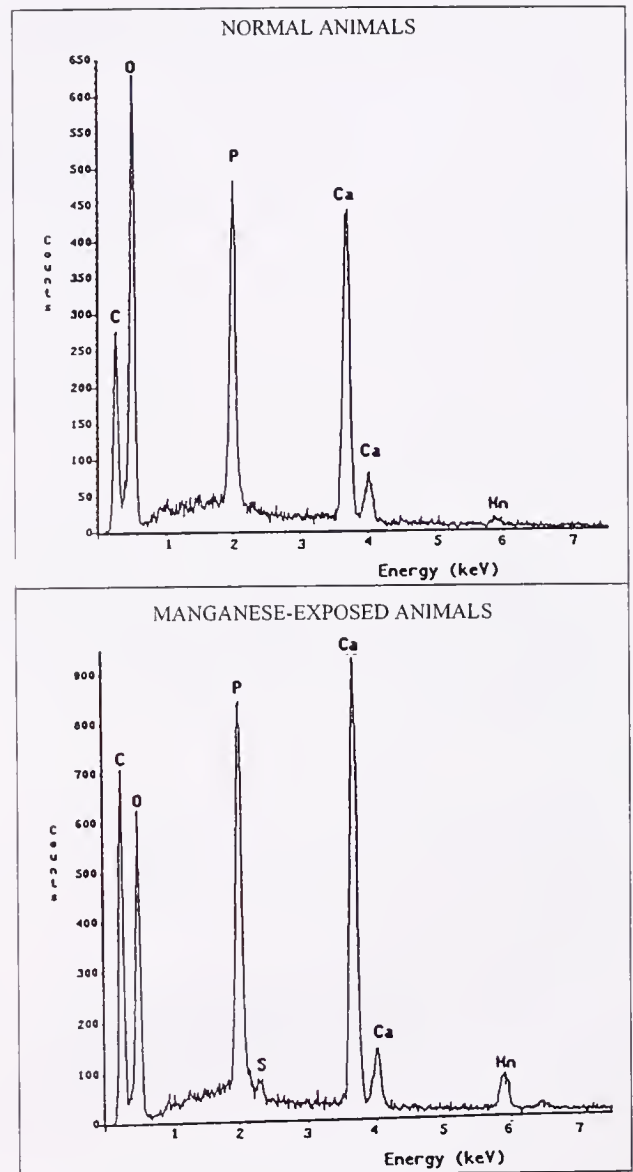


Figure 1. EDS records from analyses of mineral concretions in the gills of *Anodonta cygnea* specimens collected in March, from the natural environment (above) or after manganese-contamination (below).

In the unionid, the calcium of gill concretions is bound to inorganic or organic phosphate (Silverman et al. 1983, Silverman et al. 1987b; Pynnönen et al. 1987, Lautié et al. 1988) and is associated with an organic matrix (Silverman et al. 1983; Silverman et al. 1987b). According to Silverman et al. (1983), 25% of the concretion weight is organic, calcium makes up 25%, and phosphate represents 36%–39%. These extracellular concretions are composed of phosphorus, calcium, manganese, and iron, with smaller amounts of Mg, Al, S, Cl, Zn, and Ba (Lautié et al. 1988). No carbonate can be detected in these concretions by Raman analysis (Lautié et al. 1988).

These features resemble those reported here for the gill and intrapallial epithelium, except for the trace element composition, which were not studied in the present work. The phosphate concretions we studied are probably identical to the ones reported by Lautié et al. (1988) and, therefore, the carbon amount de-



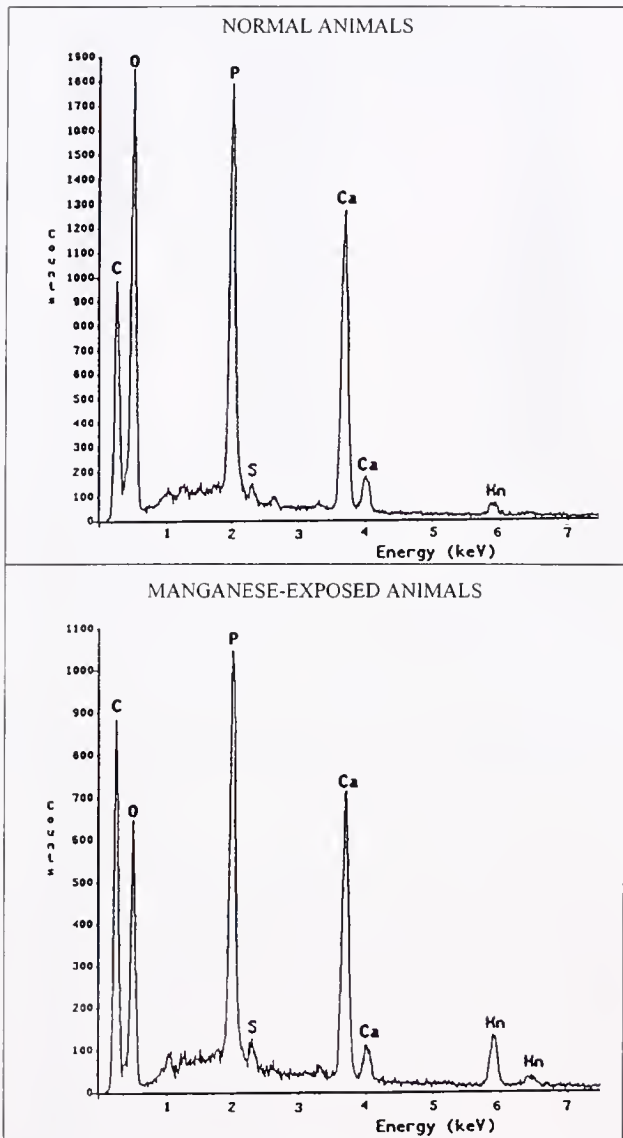


Figure 2. EDS records from analyses of mineral concretions on the intrapallial epithelium of *Anodonta cygnea* specimens collected in March, from the natural environment (above) or after manganese-contamination (below).

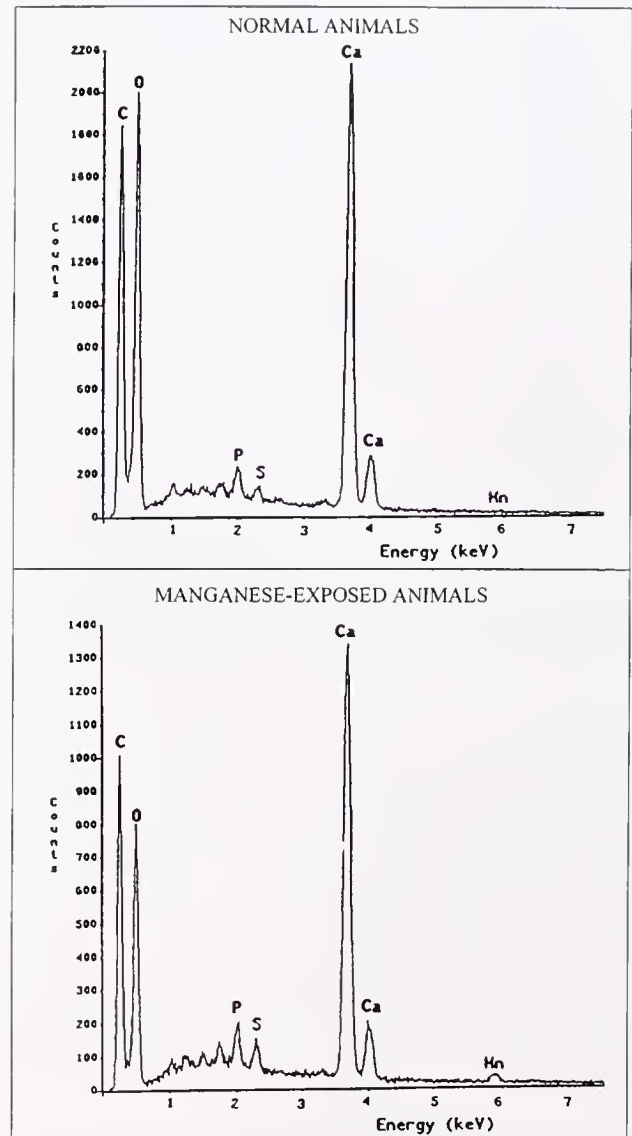


Figure 3. EDS records from the analyses of mineral concretions on the extrapallial epithelium of *Anodonta cygnea* specimens collected in March, from the natural environment (above) or after manganese-contamination (below).

tected could be related to the organic material present. The calcium/phosphate proportions are, in fact, parallel to the data now presented.

The phosphate spherules react positively to PAS following amylase reaction, suggesting the presence of polysaccharides (Silverman et al. 1983). However, although being partially composed of polysaccharides, the organic core of the concretions described by these authors does not appear to have sulfate groups, in contrast to the results of Davis et al. (1982), who found sulfur with X-ray microanalysis.

The negative reaction for the carboxyl and sulfhydryl radicals of the organic matter from concretions has been explained by the tightly bound metal amounts, which can keep the active groups from reacting (Lautié et al. 1988). Alternatively, these contradictory results can be explained by the presence of two kinds of

mineral concretion differently located, and separated from each other, one having sulfated organic material and the other being poorer in that respect. This is, in fact, what we found in both epithelia of *Anodonta cygnea* mantle.

The extracellular spherocrystals of the mantle are usually shown to be composed of calcium carbonate (Istin and Girard 1970b), a conclusion taken after studying the action of the pH, CO<sub>2</sub> partial pressure, and carbonic anhydrase activity in the calcium movements, and the location of Ca and this enzyme in the concretions. Alternatively, Pekkarinen and Valovirta (1997) and others reported the presence, in this same tissue, of calcium phosphate concretions in both *Anodonta* and *Margaritifera*, similar to those found in the gills of the same animals. The present work demonstrates, for the first time to our knowledge, that both types of concretion composition are present simultaneously in the mantle

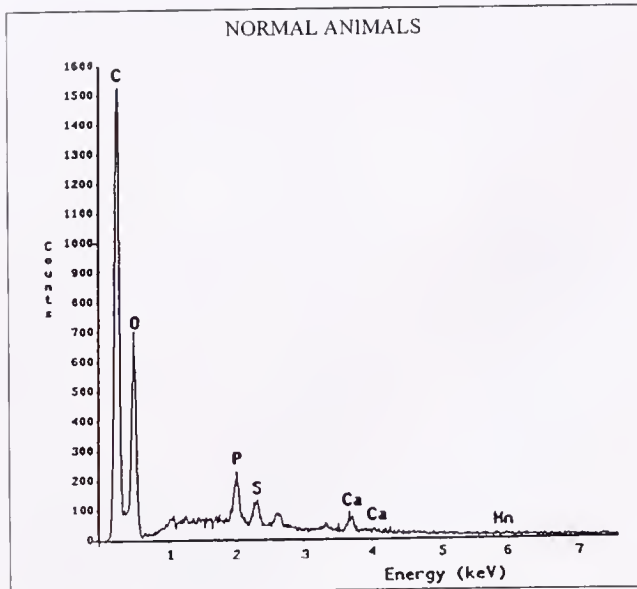


Figure 4. EDS records from the analyses of the intrapallial epithelium from normal *Anodonta cygnea* specimens collected in March.

of *Anodonta cygnea*, which explains these contradicting results, and confirms the hypothesis proposed by Pekkarinen and Valovirta (1997).

On the other hand, lamellar concretions have been described singly or clustered (Silverman et al. 1983), both in muscle (Kapur and Gibson, 1968) and mantle (Davis et al. 1982) tissues; they are related to the biomineralization process of the shell, because they disappear during shell regeneration (Watabe et al. 1976).

The morphological characteristics reported for mantle concretions can also be explained by the presence of both kinds of mineral formations. We found spherulitic concretions together with more lamellar-shaped formations, both described in an isolated fashion by these authors. Perhaps each different kind show, in fact, some variation in their amounts inside the mantle (as those re-

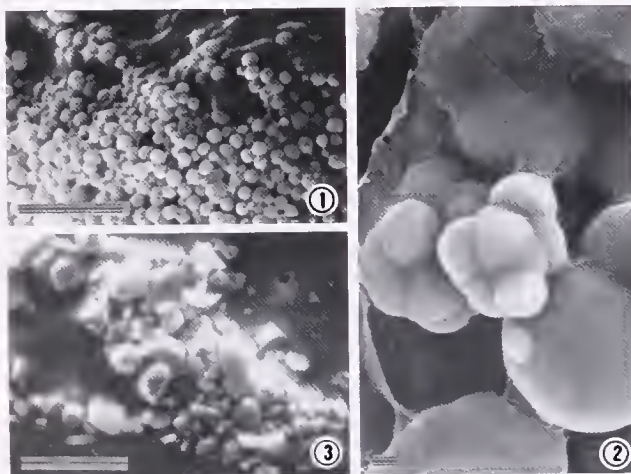


Figure 5. SEM photographs showing the morphology of *Anodonta cygnea* mineral concretions. (1) Spherical concretions, located on the surface of the intrapallial epithelium, on its haemolymph side. Bar 10  $\mu\text{m}$ . (2) Higher magnification of the structures seen in (1). Bar 1  $\mu\text{m}$ . (3) Irregular-shaped concretions, located on the surface of the extrapallial epithelium, on its haemolymph side. Bar 10  $\mu\text{m}$ .

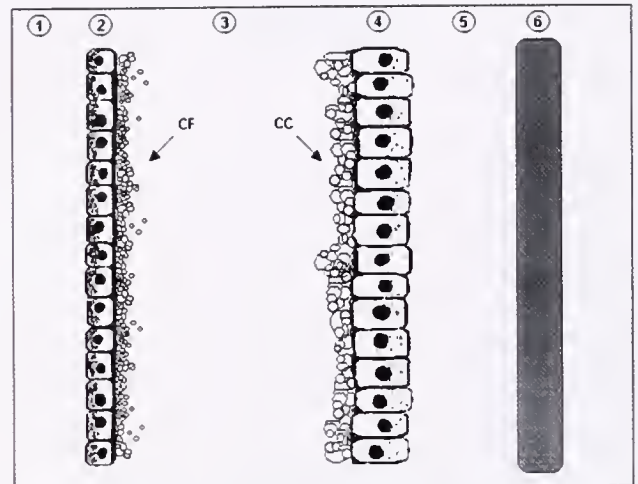


Figure 6. Schematic representation showing the localization of the different pallial concretion types of *Anodonta cygnea*. (1) external medium; (2) intrapallial epithelium; (3) haemolymph compartment; (4) extrapallial epithelium; (5) extrapallial compartment; (6) shell; CF = spherical calcium phosphate concretions; CC = irregularly shaped calcium carbonate concretions.

ported for the shell regenerating specimens, with respect to the lamellar formations), which can explain why both types were not found by each worker mentioned. Such a fluctuation is not detected in our results, where, although with some variations, both types coexist throughout the year.

The hypothetical functions of these mineral concretions have been studied by several authors (e.g., Istin and Girard 1970a). Concretions may be involved with calcium storage for shell regeneration or normal growth (Vaidya and Nagabhusanam 1980), pH buffering during metabolic and/or respiratory acidosis (Istin and Girard 1970b), and detoxification of heavy metals (Mason and Simkiss 1982).

During acidosis, bivalves dissolve their calcium reserves to buffer the pH (as reviewed by Burton 1983), causing a significant increase of the circulating levels of calcium (e.g., Pynnönen, 1990a, Byrne and McMahon 1991, Pekkarinen and Suoranta, 1995), carbonates and  $\text{P}_{\text{CO}_2}$  (Burton 1983, Pynnönen 1994), while the internal pH stays only slightly decreased (Collip 1921).

The excessive calcium arising from this situation, on the other hand, can partially leak to the external mantle cavity (Pekkarinen and Suoranta 1995) and be lost to the environment, or be again absorbed after the acidic stress period. The calcium remaining in the biological fluids can be incorporated into the shell (Pekkarinen and Suoranta 1995), in the same way as the one seasonally liberated by the succinic acid formed in the summer (Machado et al. 1990).

The reported high permeability of the mantle to calcium ions (Coimbra et al. 1988) will allow this excess calcium to be rapidly transported to the mineralizing front, causing the fast formation of a calcified pellicle on the inner side of the shell (Machado et al. 1988).

A different model is proposed by other authors, in which the pH is buffered with the dissolution of calcium carbonate from the shell itself (Sorokina and Zelenskaya 1967). However, their studies dealt mainly with marine species that present much smaller amounts of calcium microspherules (Machado et al. 1988). In addition, freshwater organisms do not have an abundant supply of

calcium and, therefore, try to avoid any loss. During a metabolic hypoxia, for example, the animal needs a mechanism to retain the calcium liberated from dissolved minerals. Accordingly, Machado et al. (1988) propose significant differences between marine and freshwater mollusks with respect to the origin of calcium to be dissolved for pH buffering purposes.

The calcium carbonate of the shell would be more susceptible to leaching by acidic metabolites than the calcium phosphate of the concretions (Howard et al. 1981), because it is more soluble. This agrees with the fact that these concretions do not seem to be implicated with pH buffering during anoxia in these animals (Silverman et al. 1983), thus different bivalves may have different calcium reserves to be used in this mechanism (Pynnönen et al. 1987).

Keeping *Anodonta cygnea* in acidic water leads to further shell growth, demonstrated by the formation of a calcified new pellicle on the inner surface of the shell (Machado et al. 1988). Therefore, it was suggested that calcium may come from the phosphate concretions (Pekkarinen and Suoranta, 1995), at least in this species.

In other works, however, calcium concretions were not mobilized under the most severe hypoxia or acidic conditions (Silverman et al. 1983, Pynnönen, 1990a, 1990b). On the contrary, the increase of calcium in the blood that follows hypoxia was accompanied by an increase of the concretions level, showing that the concretions in the gill of these mussels were gaining calcium from the circulating fluid during a time of increased blood calcium, which would serve to avoid calcium loss to the environment following its liberation from the shell, as a result of hypoxia (Silverman et al. 1983).

Radiolabeled studies also showed that the source of calcium for the elevated haemolymph levels seems to be the shell (Crenshaw and Neff, 1969). In the freshwater bivalve, *Ligumia subrostrata*, the calcium released from the shell during hypoxia (McMahon, 1979; Silverman et al. 1983) is deposited into calcium phosphate concretions in the gill tissue (Silverman et al. 1983).

Finally, these contradicting results lead to the hypothesis that a fraction of the calcium stored in the concretions is associated with carbonate, being more easily solubilized than calcium phosphate (Pekkarinen and Valovirta, 1997).

All previous results, together with the present observations of gill and mantle mineral concretions in *Anodonta cygnea*, can be explained if we consider this dual nature of the mantle mineral formations. In fact, the phosphate concretions are most probably related to the other possible functions usually assigned to these

structures, namely the metal sequestering and detoxification processes, as suggested by the higher incorporation of manganese reported here and in other works (Silverman et al. 1987b). This detoxification mechanism seems to be more important in freshwater species, because this environment is subjected to higher heavy metal fluctuations, according to Mersch et al. (1996). As explained by this author, this type of concretion probably evolved as a form of retaining the calcium that would otherwise be lost during acidosis. Its lower solubility and higher calcium binding ability (Silverman et al. 1987b) allowed for its later use as a detoxifying agent, in view of the higher binding of metal, as calcium analogues (Silverman et al. 1987b). The phosphate granules are also closely related to the reproduction cycle, being rapidly mobilized for the glochidia shell formation (Silverman et al. 1985), in what seems to constitute its primer function (Silverman et al. 1985; Silverman et al. 1987b), an idea also confirmed by the fact that these are the only concretions found in the gills of unionid bivalves, their marsupial organs.

On the other hand, there are the carbonate lamellar granules, formed only by the extrapallial mantle epithelium, directly related to the inner shell growth and bearing some resemblance in structure and composition. These formations can, in turn, be related to the deposition cycling of the nacre formations, a situation suggested by Mason and Simkiss (1982). As calcium carbonate entities, these formations are more readily dissolved in response to metabolic or respiratory acidosis than the phosphate ones. Under the normal seasonal internal pH decrease postulated by Machado et al. (1988), these concretions would dissolve before the shell itself, in view of their particular spatial localization, partially sparing the nacre from this dissolution event. The increased calcium, following this dissolution, would then cross the extrapallial fluid, fulfilling the mechanism proposed by Machado et al. (1988).

This, however, in view of the small amounts of carbonate concretions, would be a limited event, particularly if the acidosis situations, like those experimentally caused, were strong and prolonged. Under these conditions, the shell would be the next candidate for the acid action, being dissolved as commonly reported.

#### ACKNOWLEDGMENTS

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## FEEDING ENRICHED ARTEMIA BIOMASS TO *PENAEUS VANNAMEI* BROODSTOCK: ITS EFFECT ON REPRODUCTIVE PERFORMANCE AND LARVAL QUALITY

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**ABSTRACT** Two experiments were conducted co-feeding *Penaeus vannamei* broodstock with frozen *Artemia* biomass. In the first experiment, animals were fed natural diets supplemented with squid (treatment SQ), *Artemia* (A), or enriched *Artemia* (EA). In the second experiment, animals received a supplement of *Artemia* enriched with different products: rich in polyunsaturated fatty acids (PUFA) and cholesterol (treatment L), rich in vitamin c, vitamin e, and astaxanthin (treatment V), or a complete enrichment (treatment LV). In experiment 1, treatment SQ gave poor results for most parameters. Supplementation with *Artemia* resulted in higher survival, higher maturation frequency, a higher incidence of repeated spawns, and an improved larval quality. The best results were obtained in the treatment that received enriched *Artemia*. In experiment 2, the highest reproductive performance was obtained through enrichment of *Artemia* with both lipids and vitamins (LV). By reducing the concentration of PUFA and cholesterol in the enrichment product, a decline in egg fertilization, a lower incidence of repeated spawns, and a lower egg production per female was observed. High vitamin levels played a positive role only when provided in combination with high levels of PUFA and cholesterol. If not, symptoms of oversaturation occurred.

**KEY WORDS:** *Artemia*, reproduction, *Penaeus*, shrimp broodstock, nutrition

### INTRODUCTION

In Ecuador—the world's second largest shrimp producer in 1997—stocking of growout ponds depends largely on wild *Penaeus vannamei* postlarvae (PL) and to a lesser extent on PL grown in hatcheries. Until recently, wild PL were preferred over hatchery PL by all the farm managers. Today, thanks to the progress made in reproduction and larviculture techniques, many managers consider both PL types of equal quality, and have begun to focus on closing the shrimp life cycle. In June 1998, 30 hatcheries had their proper maturation facilities and four more were under construction in Ecuador. This evolution spawned an urgent and increased need for applied research and technical assistance. Cost and availability of maturation diets are among the major problems in shrimp maturation. All maturation units base the nutrition of their reproducers on a mixture of fresh frozen natural diets (squid, mussel, oyster) locally available, with supplements of bloodworm imported from Maine, USA or Panama and relatively small portions of commercial dry diets. Traditionally, bloodworm has been the key to success in *P. vannamei* maturation; however, it is the most expensive component, and quality product is not available yearlong. A search for alternatives pointed toward *Artemia* biomass. Naessens et al. (1997) demonstrated that the replacement of bloodworm with adult *Artemia* does not negatively affect the reproductive performance of *P. vannamei*. In 1997, fresh-frozen enriched biomass of *Artemia* from the USA and occasionally from Peru was available on the Ecuadorian market and substituted completely or partially the bloodworm supplement in many of the hatcheries.

The effect of bloodworm on shrimp maturation has been attributed to its polyunsaturated fatty acid (PUFA) profile (Middleditch et al. 1980, Lytle et al. 1990). Although Browdy et al. (1989) and Naessens et al. (1997) demonstrated the importance of co-feeding *Artemia* to *P. semisulcatus* and *P. vannamei* broodstock, respectively, it remains unclear what constituents are re-

sponsible for triggering maturation. Therefore, more detailed research on *Artemia* enrichment components is needed. The present study consisted of two experiments. The first experiment was run from July until September 1996 and sought to identify which reproductive parameters are affected by *Artemia* biomass and enriched *Artemia* biomass, respectively. The second experiment was run from March until May 1997 and sought to identify the relative importance of certain enrichment nutrients.

### MATERIALS AND METHODS

#### Experiment 1

Wild *P. vannamei* reproducers, captured at night at Jama (Manabi, Ecuador), were transported to the CENAİM research center and kept in maturation tanks for 2 to 3 weeks to acclimate to experimental conditions. After acclimation, female shrimp were unilaterally eyestalk-ablated by cutting and pinching and marking with eye-tags. A unisex system was used as described in Browdy et al. (1996): three tanks were stocked with 40 females each and three tanks with 45 males each. At the time of stocking, the average weights of the male and female shrimps were 47.5 and 63.3 g, respectively. The postablation phase of the experiment lasted 77 days, during which females with fully developed ovaries were transferred to one of the three male tanks. If females mated, they were placed in spawning tanks. If not, they were returned to their maturation tanks.

Animals were fed a base diet that consisted of fresh frozen squid, mussel, oyster, and clam at a ratio of 2.5:1.3:1:1 and at a rate of  $12\% \times d^{-1}$  of the tank live biomass wet weight basis (WWB). Administering two different fresh-frozen diet supplements at a rate of  $6\% \times d^{-1}$  WWB resulted in the following treatments: A and EA received a supplement of adult *Artemia* and enriched *Artemia*, respectively. Treatment SQ did not receive a separate supplement; therefore,  $6\% \times d^{-1}$  WWB more frozen squid was added to the base diet. The *Artemia* were harvested from San Francisco Bay

ponds by San Francisco Bay Brand Co. (CA, USA) and were enriched after harvesting according to their standard procedure with an experimental emulsion provided by the Artemia Reference Center (Gent, Belgium). The booster consisted of an ICES 30/4/E reference emulsion containing 30% PUFA to which 2% cholesterol, 3,000 ppm ascorbic acid (AA) equivalent (ascorbyl palmitate; Roche, Belgium), 1,000 ppm  $\alpha$ -tocopherol ( $\alpha$ -TOH) equivalent (DL- $\alpha$ -tocopherol acetate, ATA, Roche), and 1,000 ppm astaxanthin (AX) equivalent (Carophyl Pink, Roche) were added. Feeds were administered in five daily rations, two of which consisted of the dietary supplement only. The three treatments were applied in the same way for female as for male broodstock.

The maturation tanks were oval-shaped (5 m  $\times$  3 m; 19.6 m<sup>2</sup>) black Fibreglas tanks in which sand-filtered and UV-treated seawater (salinity 33 g  $\times$  L<sup>-1</sup>, pH 7.8–8.2) was exchanged at a rate of 250% daily. Water temperature was 24.0 °C during acclimation and was heated to 28.5–29.0 °C from ablation onward. A timer-controlled, inverted photoperiod of 14 h light:10 h dark was adopted, with gradual transition between light and dark hours. Mated females were transferred to individual 300-L black spawning tanks, and from each spawn, the eggs were hatched out in 20-L buckets. Nauplii were collected after phototaxis selection and stocked in 1-L bottles at a density of 100.L<sup>-1</sup> and a temperature of 29 °C until metamorphosis to zoea 1 (Z1). Eight-day larviculture trials were run in 3-L glass bottles. Late nauplii 5 (N5) were stocked at a density of 100 ind  $\times$  L<sup>-1</sup> in seawater of 33 g  $\times$  L<sup>-1</sup> and 28.5 °C. Water was exchanged 90% daily, and an algae concentration of 100,000 cells  $\times$  mL<sup>-1</sup> *Chaetoceros* sp. was maintained.

The hatching percentage was estimated by concentrating the viable nauplii in a 10-L bucket and counting five subsamples. The percentage of egg fertilization was determined by the presence of a double membrane and/or embryonic development. Zoea 1 length was measured with a profile projector on samples of 30 zoea each. Spermatophore quality was based on sperm count and spermatophore weight (Alfaro and Lozano 1993).

#### Experiment 2

Wild reproducers were captured at San Pablo (Guayas, Ecuador) and transported to CENAIM. Acclimation and ablation techniques were similar to those used in experiment 1. Three tanks with mixed sex were monitored during 55 days postablation. Each tank was stocked with 45 males and 40 females with average weights of 55.6 and 64.5 g, respectively. A similar feeding strategy as in experiment 1 was adopted, but diet supplements were adult frozen *Artemia* enriched with three different boosters. For treatments L and LV, the oil component of the enrichment product consisted of the ICES 30/4/E reference emulsion with inclusion of 2% cholesterol. In treatment V, this oil component was replaced by the ICES 0/0/E reference emulsion based on a PUFA-free coconut oil. Furthermore, boosters V and LV contained high vitamin levels: 3,000 ppm AA equivalent, 1,000 ppm  $\alpha$ -TOH, and 1,000 ppm AX equivalent. All treatments were isocaloric.

The same infrastructure and conditions as in experiment 1 were used, but this time water temperature ranged from 27.0 to 29.5 °C. Larval monitoring was only continued up to stage Z1.

#### Data Processing

Female reproducers were considered as experimental units. For statistical analysis, either animals or spawns were considered as treatment replicates. Infertile spawns were not considered. In the

case of ovarian maturation frequency, daily observations were used as replicates, as in Nascimento et al. (1991). Pearson moment-product correlation was used to determine correlations between the independent variables, female weight and spawn order, and the dependent variables related to spawn size and spawn quality. Data were analyzed with analysis of variance: a two-way ANOVA for experiment 1 with male tank as second variable and a one-way ANOVA for experiment 2. Female weight and spawn order were included as covariates in an analysis of covariance (ANCOVA) for the number of eggs per spawn and Z1 length, respectively, as correlations were found between them. When necessary, data expressed in percentages or fractions were  $\text{asin}^{\sqrt{}}$  transformed to obtain normal distribution, although unadjusted means are presented. Duncan's new multiple range test was used to identify differences among treatments. All references to statistical significance were at the 5% level or lower.

## RESULTS

### Experiment 1

Mean survival rates of 35.0, 62.5, and 82.5% were registered for male reproducers and 35, 37.5, and 47.5% for female reproducers of treatments SQ, A, and EA, respectively. On average, female reproducers survived 41 days out of the total 77 days of the experiment, for which no differences were detected among treatments. In total 9, 25, and 55 fertile spawns were obtained in treatments SQ, A, and EA, respectively.

Spawn size (eggs per spawn) was not affected by dietary treatment (Table 1). On the other hand, ovarian maturation and rematuration as well as repeat spawning differed among treatments. The maturation frequency was higher when *Artemia* (A and EA) was supplemented to the diet. The number of females with repeated spawns increased significantly in the order SQ–A–EA, spawning frequency and total egg production per female followed the same trend. Also, all parameters related to egg or larval quality were significantly better in A and EA as compared to SQ (Table 2). The total nauplii production per tank after 77 days, expressed as percentages of the production in treatment SQ, were 451% and 875% for the *Artemia* and enriched *Artemia* treatments, respectively. No significant effect of the diet on spermatophore quality could be detected (sperm count, spermatophore weight presented in Table 3; egg fertilization presented in Table 1), neither was there a male tank effect in the analysis.

### Experiment 2

During the 55 days postablation mean survival rates of 77% for male and 58% for female reproducers were registered. On average, female reproducers survived 45 days out of the total 55 days of the experiment, for which no differences were detected among treatments. Over the whole period, 91, 61, and 24 fertile spawns were recorded for treatments LV, L, and V, respectively.

Table 4 illustrates how different treatments affected the reproductive performance. The maturation frequency was significantly higher for spawners of treatment L than for spawners of treatment LV. No statistical testing was possible on spawn frequencies (no normal distribution), but a decreasing trend is observed in the order LV–L–V. Spawn size did not differ between treatments. Spawners that received a supplement of *Artemia* enriched with high vitamin levels only (V) exhibited the lowest number of eggs produced per female, the lowest egg fertilization, and the lowest incidence of

TABLE 1.

Effect of different dietary supplements on *P. vannamei* spawners: maturation frequency, spawn frequency, number of females that spawned more than once, fecundity, and fertilization (experiment 1).

	Dietary Supplement		
	SQ	A	EA
Maturation/female/day*	0.023 ± 0.037 <sup>a</sup>	0.067 ± 0.065 <sup>b</sup>	0.082 ± 0.068 <sup>b</sup>
Spawns/female/day	0.003 ± 0.009	0.011 ± 0.024	0.023 ± 0.032
# Females that spawned more than once†	7 <sup>a</sup>	16 <sup>b</sup>	36 <sup>c</sup>
Eggs/spawn (× 10 <sup>3</sup> )	213.0 ± 67.9 <sup>a</sup>	214.4 ± 60.8 <sup>a</sup>	210.1 ± 69.1 <sup>a</sup>
Eggs/female (× 10 <sup>3</sup> )	58.6 ± 142.9 <sup>a</sup>	188.9 ± 388.7 <sup>ab</sup>	337.3 ± 507.327 <sup>b</sup>
Egg fertilization (%)	47.0 ± 26.9 <sup>a</sup>	65.9 ± 21.6 <sup>a</sup>	65.9 ± 23.8 <sup>a</sup>

\* Observation of ovarian maturation stage 3 or 4 according to King (1948).

† Statistical differences detected with  $\chi^2$  test.

repeated spawns. In Table 6, the mean sperm count in both spermatophores of male reproducers is given, a recording that was significantly higher in treatment L as compared with treatment V.

No significant effect of the dietary treatments on egg quality or larval quality was observed (Table 5). However, the number of zoea I produced per spawn (a combination of spawn size and larval survival) was lower in treatment V as compared with treatment PV. The decrease of larval survival with successive spawns (spawner exhaustion) seemed to be more critical in treatment V as in the remaining treatments (Fig. 1).

DISCUSSION

A dietary regime consisting of squid, oyster, clam, and mussel (treatment SQ) gave poor results for most reproductive parameters. This is most probably attributed to the noninclusion of bloodworm (Middleditch et al. 1980, Lytle et al. 1990). Supplementation with *Artemia* biomass resulted in higher survival, improved maturation and reproduction, and better offspring quality. Lavens and Sorgeloos (1991), Cahu et al. (1991), and Palacios et al. (1998) demonstrated with their work on *Macrobrachium rosenbergii*, *P. indicus*, and *P. vannamei*, respectively, that offspring quality is associated with the level of metabolic fuel (mainly lipids) in eggs and nauplii. Because fuel levels in eggs or nauplii depend on the nutritional status of the female broodstock, which is affected by

their dietary regime, we can attribute the positive effect of *Artemia* biomass on egg and larval quality to its good nutritional value. The nutritional composition of adult *Artemia* is well documented in reviews by Léger et al. (1986) and by Lavens and Sorgeloos (1996). It seems to be quite similar to the body composition of penaeid shrimp, and therefore, it is likely to contain appropriate protein and lipid levels. Considering that biomass of adult *Artemia* was used in this study, its effect on ovarian maturation and reproductive activity might also be attributed to hormones or sexual steroids in addition to the nutritional input. Indeed, it is likely that the reproductive hormones within crustaceans are of the same nature and, therefore, could be effective in other species. The findings of Mendoza et al. (1997) on the effect of squid extracts on vitellogenesis in *P. vannamei*, and of Alava and Kanazawa (1991) on the effect of clam on ovarian maturation in *P. japonicus*, suggest a role of methanol-water-soluble extracts (hormones, steroids) on shrimp maturation. Further research on this topic could help with the identification of the *Artemia* component responsible for triggering maturation.

Supplementing the feeding regime with enriched *Artemia* as compared to regular *Artemia* promoted mating and spawning. Also, a positive effect on maturation seems to exist, but because of high within-treatment variation, no significant differences were detected. The booster emulsion was particularly rich in lipids, which provide essential nutrients as well as energy. Our studies on the biochemical composition of wild *P. vannamei* reproducers (Wouters et al. in preparation) as well as studies on other shrimp species (Middleditch et al. 1979; Read and Caulton 1980; Jeckel et al. 1989, Castille and Lawrence 1989, Mourente and Rodriguez 1991) demonstrated a remarkable increase of lipids in the ovaries

TABLE 2.

Effect of different dietary supplements on mean percentage hatch, percent larval survival from nauplii 2 to zoea 1, number of zoea 1 per spawn, zoea 1 length and percentage larval survival from zoea 1 to mysis 2 (experiment 1).

	Dietary Supplements		
	SQ	A	EA
Hatch (%)	31.6 ± 36.4 <sup>a</sup>	60.8 ± 27.5 <sup>b</sup>	61.6 ± 30.2 <sup>b</sup>
Larval survival N2-Z1 (%)	41.1 ± 32.0 <sup>a</sup>	71.8 ± 22.2 <sup>b</sup>	69.4 ± 21.1 <sup>b</sup>
Zoea 1/spawn (× 10 <sup>3</sup> )*	42.8 ± 56.5 <sup>a</sup>	93.4 ± 60.7 <sup>b</sup>	94.0 ± 60.8 <sup>ab</sup>
Zoea 1 length (µm)	804.7 ± 116.3 <sup>a</sup>	900.5 ± 36.5 <sup>b</sup>	884.4 ± 36.4 <sup>b</sup>
Larval survival Z1-M2 (%)	8.3 ± 4.7 <sup>a</sup>	43.9 ± 23.7 <sup>b</sup>	48.4 ± 19.5 <sup>b</sup>

\* Calculated data (number of nauplii per spawn × larval survival/100).

TABLE 3.

Spermatophore quality of *P. vannamei* male reproducers at the end of experiment 1 estimated by mean sperm count (number of sperm cells in both spermatophores) and mean spermatophore weight.

	Dietary Supplements		
	SQ	A	EA
Sperm count (× 10 <sup>6</sup> )	12.97 ± 12.89 <sup>a</sup>	13.44 ± 11.59 <sup>a</sup>	13.56 ± 6.75 <sup>a</sup>
Spermatophore weight (g)	0.0397 ± 0.0161 <sup>a</sup>	0.0827 ± 0.0468 <sup>a</sup>	0.0682 ± 0.0208 <sup>a</sup>

TABLE 4.

Effect of different enriched *Artemia* supplements on maturation frequency, spawn frequency, number of females that spawned more than once, fecundity, and fertilization of *P. vannamei* spawners (experiment 2).

	<i>Artemia</i> Supplements		
	LV	L	V
Maturation/ female/day*	0.141 ± 0.066 <sup>a</sup>	0.173 ± 0.079 <sup>b</sup>	0.140 ± 0.080 <sup>ab</sup>
Spawns/female/day	0.064 ± 0.052	0.048 ± 0.052	0.025 ± 0.035
# Females that spawned more than once†	28 <sup>a</sup>	19 <sup>a</sup>	11 <sup>b</sup>
Eggs/spawn (× 10 <sup>3</sup> )	288.1 ± 98.4 <sup>a</sup>	296.6 ± 100.1 <sup>a</sup>	278.5 ± 69.0 <sup>a</sup>
Eggs/female (× 10 <sup>3</sup> )	865.8 ± 577.3 <sup>a</sup>	717.6 ± 467.4 <sup>b</sup>	371.4 ± 142.9 <sup>b</sup>
Egg fertilization (%)	71 ± 27 <sup>a</sup>	64 ± 24 <sup>ab</sup>	41 ± 16 <sup>b</sup>

\* This is the maturation frequency; observation of ovarian maturation stage 3 or 4 according to King (1948).

† Also referred to as rematuration; Statistical differences detected with  $\chi^2$  test.

during maturation. In addition, maturation and reproduction cause increased metabolic energy demands (Harrison 1990), which can be met by augmenting maternal nutrition (Clarke 1982, Bray et al. 1990). The results of the present study indicate that through the enrichment of *Artemia* more adequate lipid levels were obtained that could sustain optimal reproductive performance of *P. vannamei*.

Experiment 2 further confirms that the role of the enrichment product is not only energetic but also nutritional, all treatments were iso-caloric. Most studies on shrimp broodstock nutrition have focused on the use of various natural diets, but few attempted to elucidate specific nutritional requirements (Harrison 1990) and/or possible effects on maturation, spawning, or offspring quality. Studies on lipid requirements indicate the importance of polyunsaturated fatty acids (PUFA; Middleditch et al. 1980, Chamberlain 1988, Jeckel et al. 1989; Lytle et al. 1990; Teshima and Kanazawa 1983, Xu et al. 1994; Cahu et al. 1995) and cholesterol (Middleditch et al. 1980; Kanazawa et al. 1988). Others studied the role of vitamins in shrimp reproduction: ascorbic acid (Cahu et al. 1991, Alava et al. 1993a, Cahu et al. 1995), alpha-tocopherol (Chamberlain 1988, Alava et al. 1993b, Cahu et al. 1995), and

TABLE 5.

Mean percentage hatch, percent larval survival from nauplii 2 to zoea 1 (N2-Z1), number of zoea 1 per spawn and zoea 1 length for the three *Artemia* treatments (experiment 2).

	<i>Artemia</i> Supplements		
	LV	L	V
Hatch (%)	50.9 ± 33.6 <sup>a</sup>	44.4 ± 29.4 <sup>a</sup>	32.2 ± 24.6 <sup>a</sup>
Larval survival N2-Z1 (%)	62 ± 24 <sup>a</sup>	54 ± 30 <sup>a</sup>	47 ± 27 <sup>a</sup>
Zoea 1/spawn (× 10 <sup>3</sup> )*	87.2 ± 84.4 <sup>a</sup>	69.2 ± 75.5 <sup>ab</sup>	40.6 ± 42.2 <sup>b</sup>
Zoea 1 length (µm)	895.7 ± 116.3 <sup>a</sup>	862.6 ± 193.5 <sup>a</sup>	799.9 ± 264.9 <sup>a</sup>

\* Calculated data (number of nauplii per spawn × larval survival/100).

TABLE 6.

Spermatophore quality of *P. vannamei* male reproducers at the end of experiment 2 estimated by mean sperm count (number of sperm cells in both spermatophores) and mean spermatophore weight.

	<i>Artemia</i> Supplements		
	LV	L	V
Sperm count (10 <sup>6</sup> )	11.05 ± 4.24 <sup>ab</sup>	28.72 ± 2.40 <sup>a</sup>	12.37 ± 4.50 <sup>b</sup>
Spermatophore weight (g)	0.0956 ± 0.0172 <sup>a</sup>	0.0879 ± 0.0422 <sup>a</sup>	0.0836 ± 0.0175 <sup>a</sup>

vitamin A (Alava et al. 1993b, Dall 1995). Also, astaxanthin can be considered as a vitamin (it may serve as a vitamin A precursor), and several biochemical studies have detected considerable levels of this carotenoid in hepatopancreas and gonads of crustacean reproducers (Vincent et al. 1988, Dall 1995, Dall et al. 1995; Sagi et al. 1995, Mantiri et al. 1996). The second experiment does not allow determination of the optimum dietary levels of each enrichment component, but may give us an estimation of the importance and role of the vitamin and lipid fractions in penaeid reproduction. Best results were obtained with the treatment that received a supplement of *Artemia* biomass enriched with lipids and vitamins (LV). However, by reducing the concentration of PUFA and cholesterol in the enriched *Artemia*, a decline in egg fertilization, a lower incidence of repeated spawns, and a lower egg production per female was observed, which clearly demonstrates the importance of PUFA and cholesterol.

Obviously, good reproductive performance also depends on male testis maturation and spermatophore quality. Several authors evaluated spermatophore quality by monitoring such parameters as spermatophore regeneration, spermatophore weight and color, sperm count, and percentage of abnormal and dead sperm cells (Chamberlain 1988, Leung-Trujillo and Lawrence 1991, Alfaro and Lozano 1993). Chamberlain detected a dietary effect on spermatophore quality. Others adopted a unisex system to detect male diet effects on mating and fertilization but failed to do so (Naessens et al. 1997). In experiment 1 of the present study, no male effect was observed (Table 3), but in experiment 2, significant differences in mean sperm count were detected at the end of the experiment (Table 6). The sperm count in treatment L was twice that of the remaining treatments, but only significantly different from treatment V. The latter result suggests a negative effect of high vitamin levels on sperm production. The nonsignificant differences with the LV treatment might be explained by the fact that part of the vitamins were used as antioxidative agents protecting the high PUFA levels. In contrast, Chamberlain (1988) reported a positive effect of vitamin E on spermatophore quality; he detected lower percentages of abnormal and lysed sperm cells in a treatment that received a diet high in vitamin E (approximately 500 mg/kg ATA) and suggested that this is related to the membrane stabilizing properties of tocopherol. It is possible that the vitamin concentrations in the *Artemia* biomass of treatments LV and V were excessively high, causing oversaturation or hypervitaminosis, particularly of fat-soluble vitamins. This would also explain the observed treatment differences in frequencies of ovarian maturation of the female reproducers. However, vitamins and astaxanthin do seem to play a positive role on shrimp reproductive performance if they are provided together with high PUFA and cholesterol levels:



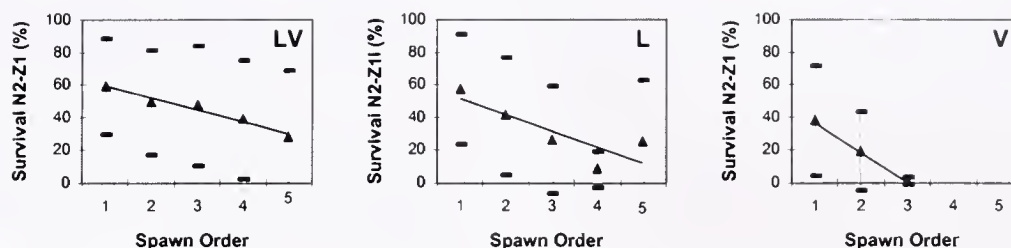


Figure 1. Mean percentage larval survival from Nauplii 2 to Zoea 1 according spawn order for the three dietary treatments of experiment 2. Best fitted lines and standard deviation bars are shown.

the best performing treatment in most aspects is the one that received the LV enriched *Artemia*. We assume that the positive role of high vitamin levels can be attributed to their antioxidant properties. Cowey et al. (1985) demonstrated that vitamins C and E are very efficient lipid antioxidants in eggs of salmon. Also Cahu et al. (1995) suggest that the beneficial action of vitamins C and E can be found in their antioxidant properties, through the protection of biological membranes from oxidation and degradation by free radicals. Further research on the role of vitamins and astaxanthin and their optimal diet inclusion levels would help to formulate performing broodstock diets.

It is interesting to note that between treatment EA of experiment 1 and treatment LV of experiment 2, which were fed similar diets, very distinct values were recorded for percentage hatch, larval survival, and zoea length. The wild reproducers used in this study were obtained at different times of the year (June to July 1996 and February to March 1997) and at different locations (Jama and San Pablo). Hansford and Marsden (1995) and Marsden et al. (1997) also reported high variability in reproductive performance between *P. monodon* prawns captured at different seasons and explained this by differences in age and environmental effects. In the present study, spawners of both experiments had similar weights and, therefore, presumably similar ages. As such, the observed differences are probably attributable to seasonal-environmental and/or geographical effects. For reproducers of experiment 1, a high mortality was recorded during the purchase as well as a high occurrence of necrosis. It seems that the adopted acclimation period (2–3 weeks) on natural diets did not help to overcome the initial poor condition and poor nutritional status of the spawners used in experiment 1.

Finally, it is observed that spawner exhaustion; that is, decreasing larval survival with successive spawns, was less pronounced in

treatments LV and L as in treatment V. In the latter treatment, no larval survival was obtained from spawns of the third order. This is a clear indication that rematuration and repeat performance can be promoted by providing adequate diets, a finding that is in agreement with the reported data for *P. monodon* by Marsden et al. (1997).

### CONCLUSION

Supplementing regular *Artemia* biomass to *P. vannamei* broodstock diets promotes maturation and spawning, and improves larval quality. Better results are obtained if animals are co-fed with *Artemia* biomass that is enriched with high levels of polyunsaturated fatty acids (PUFA) and cholesterol, vitamin C, vitamin E, and astaxanthin. The PUFA-cholesterol fraction of the booster emulsion plays the most important role, improving egg fertilization and promoting spawning. A positive role of the vitamin fraction can be obtained if combined with high levels of oxidative products (PUFA and cholesterol), but too high a vitamin level may cause oversaturation and, thus, negative effects. Therefore, including enriched *Artemia* biomass daily in the broodstock feeding regime at a rate of 6% of the tank biomass (WWB) or higher can be recommended.

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## SALINITY AND TEMPERATURE EFFECTS ON *HEMATODINIUM* SP. IN THE BLUE CRAB *CALLINECTES SAPIDUS*

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**ABSTRACT** The parasitic dinoflagellate *Hematodinium* sp. infects and causes mortalities in blue crabs *Callinectes sapidus* Rathbun 1896 from high salinity coastal embayments. The seasonal infection cycle and apparent salinity and temperature requirements for infections reported from wild crab populations indicate that environmental factors influence the parasite's ability to proliferate within crab hemolymph. A series of experiments held crabs at various water temperatures and salinities to assay infection intensity and crab survival. There was a significant increase in mean intensity in infected crabs held in ambient 15–9 °C seawater for 32 days; at temperatures below 9 °C, mean intensity diminished. Mean intensity decreased significantly in infected crabs held in 10‰ or 29‰ artificial seawater at 9 °C for 73 days; the decrease was significantly greater at 10‰ than at 29‰. Mean intensity increased in infected crabs held in 22‰ seawater at either 12 or 16 °C. Presumably uninfected crabs held at 22 °C presented infections after 14 days. No infections were transmitted by exposure of uninfected crabs to infected crabs after 85 days. Low water temperature and salinity appear to limit the proliferation of *Hematodinium* sp. in blue crab hemolymph. Apparently uninfected crabs from endemic areas can carry pre-patent or latent infections.

**KEY WORDS:** *Hematodinium* sp., *Callinectes sapidus*, salinity, temperature, parasite

### INTRODUCTION

Parasitic dinoflagellates have been reported in numerous crustaceans including copepods, amphipods, and decapods (Chatton and Poisson 1931, Chatton 1952, Manier et al. 1971, Shields 1994). *Hematodinium perezii* Chatton and Poisson 1931 was originally reported as a rare hemolymph-infecting parasitic dinoflagellate in European crabs *Carcinus maenas* (Linnaeus 1758) and *Liocarcinus depurator* (Linnaeus 1758) (Chatton and Poisson 1931). Crustaceans of commercial value reported with *Hematodinium* spp. infections include the Alaskan Tanner crab *Chionoecetes bairdi* Rathbun 1924 (Meyers et al. 1987), the Alaskan and Newfoundland snow crab *C. opilio* (Fabricius 1788) (Meyers et al. 1990, Taylor and Khan 1995), the Norway lobster *Nephrops norvegicus* (Linnaeus) (Field et al. 1992), the American blue crab *Callinectes sapidus* (Newman and Johnson 1975), rock crabs *Cancer irroratus* Say 1817 and *C. borealis* Stimpson 1859 (MacLean and Ruddell 1978), the Australian blue crab *Portunus pelagicus* Linnaeus (Shields 1992, Hudson and Shields 1994), Alaskan spot shrimp *Pandalus platyceros* Brandt 1851 (Bower et al. 1993, Meyers et al. 1994), and the velvet swimming crab *Necora (Liocarcinus) puber* (Linnaeus) (Wilhelm and Mialhe 1996). Epizootics in some economically important species have had detrimental effects on their fisheries. Heavy mortalities in blue crabs from coastal bays of Maryland and Virginia have been associated with *Hematodinium* sp. infections, with up to 100% infection prevalence during peak periods (Messick 1994).

Seasonality of *Hematodinium* sp. infections has been reported in numerous crustaceans including *C. bairdi* (Meyers et al. 1987, Eaton et al. 1991, Love et al. 1993), the cancer crab *Cancer pagurus* Linnaeus 1758 (Latrouite et al. 1988), *N. (L.) puber* (Wilhelm

and Boulo 1988; Wilhelm and Mialhe 1996), *N. norvegicus* (Field et al. 1992), and *C. sapidus* (Newman and Johnson 1975). Prevalence of *Hematodinium* sp. infections in blue crabs from coastal bays of Maryland and Virginia is highest in autumn and early winter, but is reduced to zero by spring (Messick 1994).

*Hematodinium* sp. infections are found only in blue crabs collected from waters with salinity higher than 11‰, although crabs from lower salinities have been sampled (Newman and Johnson 1975). The prevalence of this parasitic dinoflagellate in crustaceans has been attributed to several factors, including salinity, temperature, and molt cycle (Newman and Johnson 1975, Latrouite et al. 1988, Meyers et al. 1990, Eaton et al. 1991, Field et al. 1992). This paper reports results from a series of experiments conducted to assay salinity and temperature effects on *Hematodinium* sp. infections in blue crabs. Details on how salinity and temperature affect proliferation of this parasitic dinoflagellate within the hemolymph of its host will improve our understanding of its epizootiology and pathogenicity.

### METHODS

Juvenile and adult blue crabs from coastal bays of Maryland were collected with an otter trawl. Each crab was tagged or placed in a numbered compartment to assure individual identification. Carapace width (CW) was measured as the longest distance between epibranchial spines. Crabs were bled from the hemal sinus at the joint between the thorax and the 5th pleopod (swimmer fin) using a 1-cc insulin syringe equipped with a 0.5-in, 28-gauge needle. Expressed cells were allowed to adhere to an acid-cleaned, 0.1% w/v poly-L-lysine-coated microscope slide. Hemolymph preparations were fixed in Bouin's fluid and stained with Mayer's hematoxylin and eosin (H&E) (Luna 1968). To avoid puncture of

internal organs, some smaller crabs measuring less than 25 mm CW were induced to autotomize an appendage. Previous laboratory experiments have shown that mortality was low when blue crab chelae were induced to autotomize (Costlow 1963). Autotomized appendages were squashed, smearing hemolymph and muscle tissue onto slides and processed as above. Crabs were assayed to determine whether they had patent *Hematodinium* sp. infections before they were utilized in experiments. Infection intensity was expressed as a percentage, derived by counting at least 300 cells per hemolymph preparation, dividing the number of parasites by the total number of cells (parasites + hemocytes) counted and multiplying by 100.

Due to concerns that infections were misdiagnosed, a comparison was made to assay whether the parasite was undetected in hemolymph but present in other internal tissues. A sample of crabs was collected from a Maryland coastal bay in December 1997. In addition to hemolymph preparations, various tissues were dissected, placed in fixative, and processed for histology. Of the 24 crabs assayed, 16 (67%) had *Hematodinium* sp. parasites in the hemolymph, whereas 13 (54%) had parasites in other tissues. Based on this preliminary study, it was assumed that assaying hemolymph provided equivalent accuracy in diagnosis as other internal tissues, which require sacrificing animals and the additional expense of histologic processing.

#### Experiment 1. Environmental influences

Experiment 1 was designed to assay intensity of *Hematodinium* sp. infections and mortality in blue crabs exposed to ambient environmental fluctuations in water temperature. Adult and juvenile crabs were held in a shallow tank measuring 3.7-m long  $\times$  0.6-m wide  $\times$  0.25-m deep. Smaller crabs were placed in a 6.4<sup>2</sup>-mm mesh cage measuring 0.9-m long  $\times$  0.3-m wide  $\times$  0.2-m deep within the larger tank to avoid cannibalism by larger crabs. Raw, untreated coastal bay seawater flowed through tanks which were protected from direct sunlight and rain but exposed to ambient air temperature. Sixty-seven infected crabs, 43 males and 24 females, were placed in the experiment. Mean CW was 70 mm, range 23–130 mm, and standard deviation (SD) = 35.7 mm. Crabs were fed Atlantic ribbed mussels *Geukensia demissa* Dillwyn 1817. Hemolymph was assayed for infection intensity on days 1, 9, 12, 22, 32, 42, and 58 from 18 November 1993 to 11 January 1994. Over this period, temperature dropped from 15 to 1.1 °C; salinity ranged from 28–30‰.

#### Experiment 2. Transmission

Experiment 2 was designed to assay whether *Hematodinium* sp. could be transmitted to uninfected blue crabs via the water column, infected crab feces, or direct contact with infected crabs. Forty-three uninfected crabs were divided into 5 treatment groups: 3 uninfected crabs were exposed to raw seawater, 5 uninfected crabs were exposed to ultraviolet (UV) light-treated seawater, 10 uninfected crabs were downstream from 10 infected crabs; 28 uninfected crabs were in direct contact with 11 infected crabs; and 3 uninfected crabs were exposed to feces from 3 infected crabs. Infected and uninfected crabs were held in compartmentalized tanks measuring 15-cm long  $\times$  10-cm wide  $\times$  10-cm deep, open cages with 30-cm diameter  $\times$  46-cm high, or an open tank 3.7-m long  $\times$  0.61-m wide  $\times$  0.25-m deep. Tank compartments isolated crabs from each other yet allowed seawater to flow between compartments. Tanks were located in a boathouse and had flow-

through coastal bay seawater at ambient temperature and salinity. The mean CW of 22 male and 21 female uninfected crabs which were assayed for disease transmission was 73.5 mm (range 28–128 mm). Crabs were fed Atlantic ribbed mussels, *G. demissa*, ad libitum. The mean CW of 10 male and 11 female infected crabs which were analyzed for disease progression was 72 mm (range 28–140 mm). From days 1 to 85 (1 November 1994 to 24 January 1995) temperature dropped from 16 to 6 °C. Salinity ranged from 29–30‰. Crabs were assayed for infection intensity on days 1, 10, 21, 31, 43, 59, and 85.

#### Experiment 3. Salinity effects

Experiment 3 was designed to compare parasite intensity between infected crabs held in high or low salinity seawater. Crabs which were assayed and determined to be infected with *Hematodinium* sp. were placed in either 10 or 29‰ non-circulated artificial seawater maintained at 9 °C in a walk-in incubator at the University of Maryland's Center for Environmental Science Horn Point Laboratory near Cambridge, Maryland. Crabs were held in plastic boxes with 4-cm long  $\times$  5-cm wide  $\times$  5-cm deep watertight compartments which prevented water flow between sections. Seawater was changed approximately every 10 days and freshly hatched *Artemia* nauplii were fed to the crabs. Eighty-six infected crabs were divided into 2 groups: 41 crabs were held in salinity representative of the Maryland coastal bays (29‰), and 45 crabs were held in 10‰ which was lowered from 29‰ over a 2-day period. Mean CW of 47 female and 39 male crabs was 16 mm (range 10–30 mm). Hemolymph preparations were assayed blindly without knowledge of the salinity treatment. Due to lack of data from dead crabs, 71 of the original 86 crabs were statistically analyzed. Hemolymph samples were taken from crabs on days 0, 37, and 73. Crabs were held an additional 20 days after the last hemolymph assay to evaluate mortality.

#### Experiment 4. Temperature and water type

Experiment 4 compared the intensity of *Hematodinium* sp. infections in relation to temperature. Infection intensity was also compared between crabs held in either artificial or untreated raw seawater to investigate whether parasite proliferation is influenced by water type. Treatments included 22‰ artificial seawater at 16 and 12 °C, and 22‰ untreated, raw seawater at 16 and 12 °C. Crabs were isolated in plastic boxes with water-tight compartments which measured 4-cm long  $\times$  5-cm wide  $\times$  5-cm deep. The experiment was conducted in 2 walk-in incubators at the Horn Point Laboratory where temperatures were maintained at either 12 or 16 °C. Crabs were fed freshly hatched *Artemia* nauplii approximately every 20 days; seawater within compartments was intermittently replaced. Seventy-two crabs, 37 females and 35 males, mean CW 18 mm (range 8–62 mm), were held in this experiment. All crabs were not assayed on the same day due to malfunctioning of temperature control equipment and other factors. Hemolymph samples were taken on day 1 and either days 32, 42, or 56.

#### Experiment 5. Pre-patent infections

Experiment 5 was conducted to determine if apparently uninfected crabs carried pre-patent or latent infections which become patent at higher temperatures. Eighty-one apparently uninfected crabs were collected from Sinepuxent Bay, Maryland, in March when infection prevalence has historically been 0% (Messick 1994). Crab hemolymph was assayed to ensure no infections were apparent. Forty-two male and 39 female crabs, mean 28 mm CW (range 14–51 mm), apparently uninfected crabs were divided into

2 groups: 53 crabs were placed in raw seawater and 28 crabs were placed in artificial seawater at 22 °C to assay whether seawater type in addition to temperature affected patency of infections. Crabs were isolated in plastic boxes with compartments which measured 4-cm long × 5-cm wide × 5-cm deep. Crabs were fed freshly hatched *Artemia* nauplii on day 7. Seawater within compartments was replaced approximately every 5 days. Hemolymph samples were taken on days 1 and 14.

#### Statistical methods

Data from experiment 1 and experiments 3–5 were analyzed using a multivariate repeated measures analysis of variance (MANOVA) technique (SAS Institute Inc. 1988). The models tested the effects of class variables (sex, type of water, salinity, or temperature treatment) and a covariate (CW) on *Hematodinium* sp. infection intensity measured repeatedly on individual crabs. In addition to treating each repeated measure as a separate response variable, the method tested the effect of time on intensity, where each repeated measure represented a separate level of a derived class variable (time). Linear contrasts in the statistical models tested effects of time (= repeated measures) interactions (example: intensity at time 2 vs. intensity at time 4), and interactions of time (= repeated measures) with other variables (time × size, time × sex, time × salinity, etc.). Observations with missing measurements of intensity were omitted from the analysis. Effects were considered significant if MANOVA tests or contrasts generated probabilities  $\leq P = 0.05$ .

## RESULTS

### Experiment 1

Mean intensity of infection peaked on day 32 at 35%, then declined to 23% on day 58 (Fig. 1). Mean intensity decreased after water temperature fell below 8 °C (Fig. 1). Due to low survivorship beyond day 32, statistical analysis was limited to assays from days 1–32. There was a significant difference in intensity among the assays ( $P = 0.004$ ), although it could not be determined whether this difference was due to the effects of time, temperature, or repeated measures. Crab size ( $P = 0.06$ ) and sex ( $P = 0.29$ ) did not have significant effects on infection intensity. Mean intensity was significantly higher on days 22 ( $P = 0.04$ ) and 32 ( $P = 0.01$ ) than on day 1. Survivorship in infected crabs was 18% after 58 days (Fig. 1).

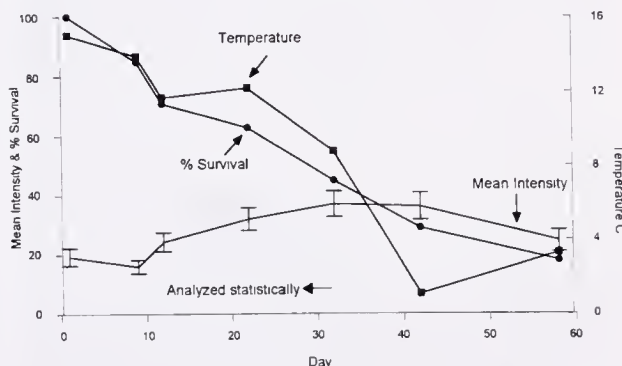


Figure 1. Experiment 1: environmental influences. Water temperature, percent survival, and mean intensity of *Hematodinium* sp. infections in crabs held in untreated coastal bay seawater exposed to environmental temperature and salinity. Error bar = standard error.

### Experiment 2

No disease transmission was detected after 85 days in uninfected crabs exposed to either raw seawater, UV-treated seawater, downstream water from infected crabs, direct contact with infected crabs, or vertical exposure to feces from infected crabs. A possible source of infective stage *Hematodinium* sp. from either the water column, infected crabs, or infected crab feces was not ascertained. To assay disease progression and survivorship, data from infected crabs were combined from the various treatment groups. Infection intensity increased slightly from days 1–85 although there was considerable fluctuation. Survivorship diminished to 10% after 85 days (Fig. 2).

### Experiment 3

Intensity declined over time in both treatment groups; crabs held at 10‰ had a greater reduction in intensity than crabs held at 29‰ (Fig. 3) ( $P = 0.03$ ). Crab size ( $P = 0.12$ ) and sex ( $P = 0.21$ ) did not have significant effects on mean intensity.

Crabs were held an additional 20 days after the last hemolymph assay to measure mortality. Each group had 41 infected crabs which survived 93 days. Crabs held in 10‰ had 51% survivorship; those held in 29‰ had 88% survivorship (Fig. 3). In addition, 4 uninfected crabs held in 10‰, 9 °C-seawater did not survive to day 93.

### Experiment 4

Crabs held at 16 °C had a higher infection intensity than those held at 12 °C (Fig. 4). The overall difference in infection intensity from the initial assay to the final assay was not significant ( $P = 0.17$ ) and the difference in intensity among crabs held at the 2 temperatures was not significant ( $P = 0.54$ ). Crabs held in artificial seawater had a decrease in mean infection intensity whereas crabs held in raw seawater had an increase in mean infection intensity; these differences were significant ( $P = 0.049$ ) (Fig. 5). No significant difference in mean infection intensity was found between crab sex ( $P = 0.77$ ) or crab sizes ( $P = 0.22$ ).

### Experiment 5

After 14 days at 22 °C, 68% ( $n = 55$ ) of the crabs survived; 13% ( $n = 7$ ) of these apparently uninfected crabs presented infections; and mean infection intensity was 11% (Fig. 6). The increase in infection prevalence was not significant ( $P = 0.38$ ). Crabs held in artificial seawater had a higher infection intensity

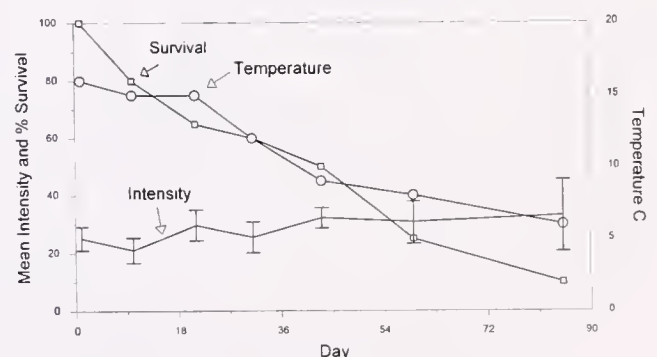


Figure 2. Experiment 2: disease transmission. Water temperature, percent survival, and mean intensity of *Hematodinium* sp. infections in crabs held in coastal bay seawater exposed to combined transmission treatments. Error bar = standard error.

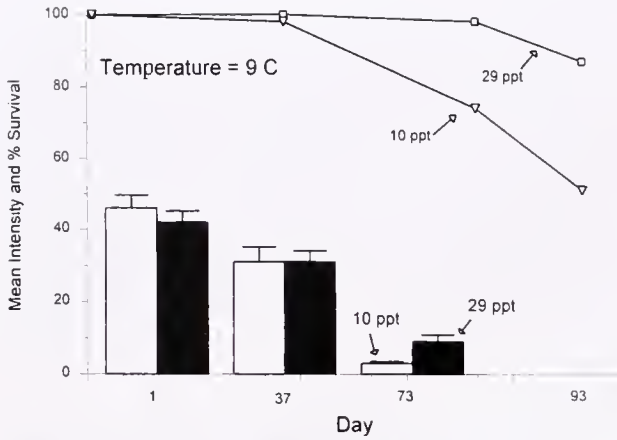


Figure 3. Experiment 3: salinity effects. Mean intensity (bars) and percent survival (lines) of *Hematodinium* sp. infections in crabs held at 9 °C in either 10 or 29‰ seawater. Error bar = standard error.

(19%) than those held in raw seawater (5%) but this difference was not significant ( $P = 0.93$ ), nor did the sex ( $P = 0.64$ ) or size ( $P = 0.21$ ) of the crab have significant effects on infection intensity.

**Assay techniques**

Potential problems with quantifying infection intensity during this study were identified. Hemolymph preparations from experiments 3 and 4 were examined twice with a difference in infection intensity each time preparations were examined from individual crabs, although the sample mean intensity did not vary between the two examinations ( $t = 0.80$ ,  $df = 124$ ). These results indicate there may be some variation in numbers of parasites present in different areas of hemolymph preparations. The second and third preparations of leg squashes from small crabs (< 15 mm CW) in experiment 3 contained fewer cells (hemocytes and parasites) than the first preparation.

**DISCUSSION**

Blue crabs infected with *Hematodinium* sp. which were held in seawater at or below 9 °C had a decrease in infection intensity; crabs held in ambient seawater at higher temperatures had an increase in infection intensity. Salinity of 10‰ induced a greater reduction in intensity than 29‰ at 9 °C, and presumed uninfected crabs from coastal bays of Maryland presented infections when

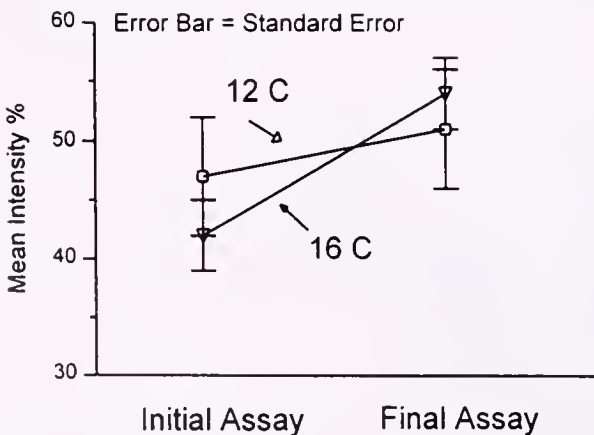


Figure 4. Experiment 4: temperature and water type. Mean intensity change over time of *Hematodinium* sp. infections in crabs held at 12 or 16 °C.

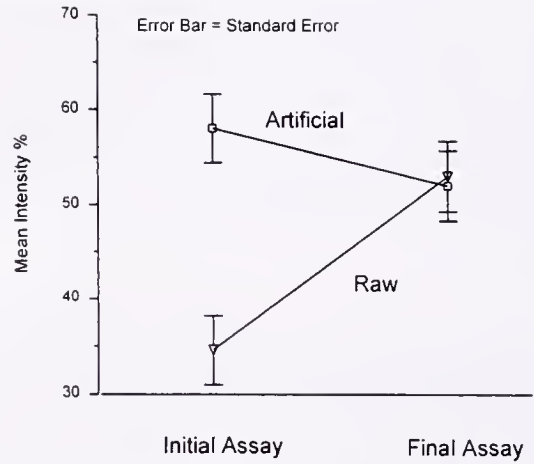


Figure 5. Experiment 4: temperature and water type. Mean intensity change over time of *Hematodinium* sp. infections in crabs held in artificial or raw seawater.

held at warmer temperatures. Studies indicate the prevalence and intensity of *Hematodinium* sp. in blue crabs is seasonal and peaks in late autumn and early winter (Newman and Johnson 1975). The apparent 0% prevalence from late winter through spring in coastal bays of Maryland and Virginia (Messick 1994) is likely caused by low water temperature reducing *Hematodinium* sp. numbers (Fig. 3) to pre-patent levels within the hemolymph. Winter temperatures appear to provide a refuge from infection for crabs overwintering in coastal bays of Maryland since crabs held at 9 °C have reduced infection intensity (Fig. 3), and water temperatures from December to March 1997 averaged 3.5–9.8 °C (Phillip Wirth, University of Maryland Eastern Shore, pers. comm.). Distinct seasonal peaks in *Hematodinium* sp. infections are apparent in other crustacean fisheries, in which some occur in relatively cold waters. These include *C. bairdi* in Alaska (Eaton et al. 1991, Love et al. 1993), *L. puber* in France (Latrouite et al. 1988, Wilhelm and Boulo 1988), *N. norvegicus* (Field et al. 1992), Alaskan spot shrimp *P. platyceros*, and pink shrimp *P. borealis* Kröyer (Meyers et al. 1994).

*Hematodinium* sp. infections in *C. bairdi* in Alaska have a long pre-patent period (55+ days) (Meyers et al. 1987), but infections in *P. pelagicus* have a relatively short pre-patent period (16+ days) (Hudson and Shields 1994). *N. norvegicus* infections are pre-patent from July to December, but infections were apparent in presumed pre-patent animals when tissues were assayed with a *Hematodinium*-specific polyclonal antibody (Appleton and Vickerman 1998). The *Hematodinium* sp. found in *N. norvegicus* is cultured at 6–10 °C and undergoes a series of developmental

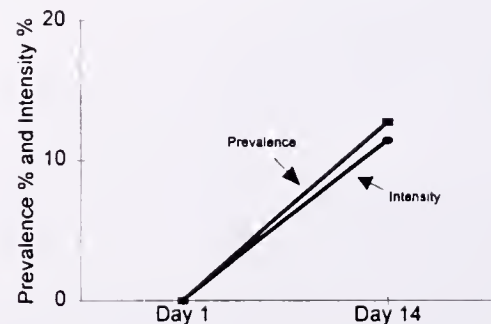


Figure 6. Experiment 5: pre-patent infections. Prevalence and mean intensity of *Hematodinium* sp. infections in presumed uninfected crabs held at 22 °C for 14 days.

changes at 8 °C (Appleton and Vickerman 1998). The *Hematodinium* sp. found in *C. bairdi* proliferates in culture at 4–6 °C. Meyers et al. (1987) suggest higher seawater temperatures initiate parasite sporulation, and lower temperatures retard sporulation in Tanner crabs. Results from our study indicate temperatures at or below 9 °C have a negative effect on proliferation of *Hematodinium* sp. in blue crabs but higher temperatures are associated with increased intensity and the presentation of infections in prepatent crabs after 2 weeks. Temperature appears to have a strong influence on the parasite's life cycle and may influence the period required for new infections to become patent. The apparent lack of disease transmission in experiment 2 may have been influenced by temperature effects. Since crabs were exposed to ambient temperature which decreased from 15 to 6 °C, the crabs were held within a temperature refuge for the latter portion of the experiment, which diminished the possibility of transmitted infections becoming patent in hemolymph smears.

Numerous blue crab fisheries, including those in Gulf of Mexico and Atlantic coastal states have reported reduced catch per unit effort in recent years (Evans 1998, Cole 1998, Guillory et al. 1998, Henry and McKenna 1998, Whitaker et al. 1998). *Hematodinium* sp. has been detected in crabs collected from various coastal areas (Newman and Johnson 1975, Couch and Martin 1982, Messick 1994). The effect of this parasite on crab populations in warm climates where water temperatures do not fall below 9 °C for extended periods is unknown. Bottom water temperatures in Texas averaged 10–16 °C during December to February 1975 to 1996 from mid-bay trawls along the mid-eastern coast (Tom Wagner, Texas Parks and Wildlife Department, pers. comm.). At these temperatures, *Hematodinium* sp. may continue to grow and proliferate within the hemolymph of infected crabs throughout the year. The apparent lack of seasonality in *Hematodinium australis* Hudson and Shields 1994 in *P. pelagicus* (Hudson and Shields 1994) may indicate that water temperatures are too high in Moreton Bay to cause a decrease in the foregoing low parasite prevalence, or perhaps that *H. australis* is not limited by reduced water temperature. We infer that infected crab populations in regions where water temperatures drop below 9 °C over the winter have reduced *Hematodinium* sp. infections, but in regions where water temperatures do not drop below 9 °C for extended periods, crab populations may be more heavily impacted by the parasite due to lack of a temperature refuge.

Experimental results from this study on salinity effects corroborate previous reports that the parasite is not found in blue crabs collected from salinity below 11‰ (Newman and Johnson 1975). Blue crabs are euryhaline hyperosmoregulators that maintain hyperosmotic hemolymph in low salinity water, but follow the osmolality of the external environment in more saline waters (Péqueux 1995). *Hematodinium* sp. had a greater reduction in infection intensity at 10‰ than at 29‰ despite the relatively high osmolality in blue crab hemolymph. Crabs have limited regulation capabilities when salinity decreases to certain limits (Péqueux 1995), which may indirectly affect the proliferation of *Hematodinium* sp. in the host's hemolymph.

Although survivorship in experiment 3 indicated that infected crabs die at low salinity, it should be noted that uninfected crabs not included in the results shown in Figure 3 died before day 93 (n = 4). Since all uninfected crabs died in 10‰ but few infected crabs died in 29‰, this suggests that low salinity, apart from other factors, reduces survival of crabs when held at low (9 °C) temperature. Osmoregulation in crustaceans is affected by temperature (Péqueux 1995) and lower salinity may cause higher mortalities in

crabs overwintering in cold temperatures (Van Engel 1982). Additionally, low temperature may alter crab physiology and induce cellular defense mechanisms to manifest in tissues (Messick 1998). Salinity appears to cause a decreasing gradient of antibacterial activity in blue crabs from oceanic to riverine areas (Noga et al. 1994) and hemocyanin, a metalloprotein respiratory pigment necessary for growth and survival, has reduced levels in the hemolymph of crabs overwintering in cold water (6 °C) (Engel and Brouwer 1987). The physiologic mechanisms which decrease hemocyanin levels and antibiotic activity may also influence the reduction in *Hematodinium* sp. intensity at lower salinities and temperatures. Additionally, based on inconclusive results from this study, it is uncertain whether there are components in either raw or artificial seawater which affect proliferation of *Hematodinium* sp. in crab hemolymph.

Numerous crustacean pathogens are regulated by salinity or temperature. The prevalence of some parasites reflects the host's biological requirement for certain salinities or temperatures; other parasites are regulated directly by salinity and temperature, which can control the parasite's dispersal or ability to parasitize the host. Research into the elemental and nutritional requirements and direct effects of salinity and temperature on *Hematodinium* sp. are needed to better understand the interactions among the host, parasite, and environment.

In summary, water salinity and temperature are two environmental parameters which influence the intensity of *Hematodinium* sp. infections within the hemolymph of blue crabs. A conceptual model illustrates the potential increase in parasite intensity in crabs at different salinity and temperature regimes (Fig. 7). In this model an infection refuge exists when ambient salinity remains below 11‰ and water temperature remains at or below 9 °C for an unspecified time period. The presentation of infections in apparently uninfected crabs from endemic areas indicates crabs carry latent infections through late winter and spring which become evident when ambient water temperatures exceed the temperature refuge. Additionally, the apparent lack of disease transmission in this study suggests the parasite is not transmitted via the various pathways tested at low temperatures. The temperature and salinity refuge observed in this study may be due to physiological characteristics of the host, the parasite, or synergistic processes. The development of better assay tools such as gene probes, immunoas-

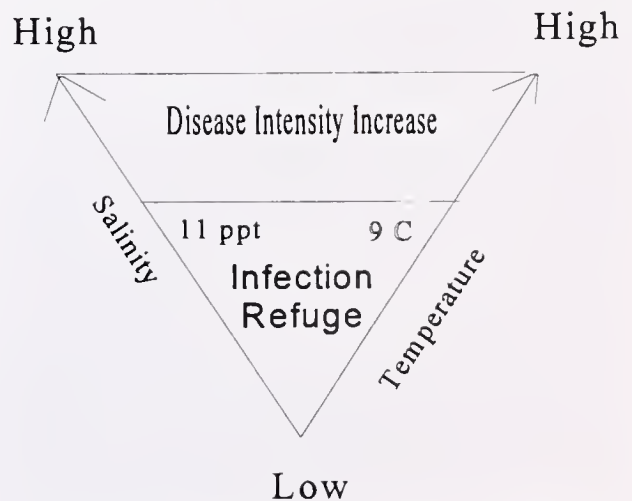


Figure 7. Conceptual model illustrating the infection refuge and area of potential increase in intensity of *Hematodinium* sp. in hemolymph of infected blue crabs held at different salinity and temperature regimes.

says, and monocultures can help answer some basic questions concerning the epizootiology of *Hematodinium* sp. in blue crabs.

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## SIZE AS INDICATOR OF SWIMMING SPEED IN CRAB MEGALOPAE

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**ABSTRACT** In this work, we evaluated the relationship between swimming speed and size of megalopae of the crabs *Uca uruguayensis*, *Chasmagnathus granulata*, *Cyrtograpsus angulatus* and *C. altimanus* in a flume and compared the results with the available information for other crab megalopae. Megalopa swimming speeds ranged from 1.4 cm s<sup>-1</sup> to 13.2 cm s<sup>-1</sup> in *C. granulata*, 1.02 cm s<sup>-1</sup> to 14.9 cm s<sup>-1</sup> in *C. angulatus*, 1.2 to 6.9 in *U. uruguayensis*, and 3.3 to 20.8 in *C. altimanus*. The results of our experiments together with the available information show that, contrary to what is stated in the current literature for larvae of similar size, size is a good indicator of swimming speed for crab megalopae. Furthermore, our results suggest that general models describing the relationship between size and swimming speed largely underestimate the average and maximum swimming speed of megalopae.

**KEY WORDS:** swimming speed, megalopae, estuarine crabs

### INTRODUCTION

The relationship between body size and swimming speed has been reviewed for invertebrate marine larvae (Chia et al. 1984) and more recently for aquatic animals in general (Peters et al. 1994). Although the idea that big animals swim faster than small ones is generally accepted, proportional relationships between size and swimming velocities have been reported only for particular taxa and certain ranges of size. Only animals with muscular locomotion and in the range from 1 mm to 1 m of maximum length (Chia et al. 1984; Peters et al. 1994) showed a proportional increase of swimming speed in relation to size. Among invertebrate larvae, decapods showed the highest swimming speeds (Chia et al. 1984; Rooney and Cobb 1991; Luckenbach and Orth 1992; Fernandez et al. 1994). On the basis of their size and muscular locomotion, we might expect a proportional relationship between size and swimming velocities for decapod larvae. However, the information available for crab zoeae does not support such an idea (Chia et al. 1984), and the information available on the swimming performance of crab megalopae under realistic flow conditions is very scarce (*Callinectes sapidus*: Luckenbach and Orth 1992; *Cancer magister*: Fernandez et al. 1994) and insufficient to evaluate this prediction. Furthermore, a strictly comparative approach has not been possible given the variety of methodologies used, many of which were not appropriate to evaluate these questions (see Chia et al. 1984).

Four species of crabs live in Mar Chiquita Lagoon (Argentina, 37°32'S and 57°19'W): *Uca uruguayensis* Nobili 1901, *Chasmagnathus granulata* Dana 1851, *Cyrtograpsus angulatus* Dana 1851, and *Cyrtograpsus altimanus* Rathbun 1914. The first two species are semiterrestrial burrowing crabs, restricted to estuarine areas; whereas, *C. angulatus* also inhabits rocky seashores (Boschi 1964). *C. altimanus* is a marine species mostly restricted to the mouth of the lagoon. These species export larvae to the ocean, and re-invade the estuary as megalopae (Anger et al. 1994). Megalopae and juveniles are found mainly in burrows of conspecifics (*C. granulata*), in reefs of the polychaete *Ficopomatus enigmaticus* and under rocks (*C. angulatus* and *C. altimanus*) (Spivak et al.

1994). Megalopae of *U. uruguayensis* are found only sporadically and in individual burrows.

Their coexistence and differences in megalopal size provide an interesting possibility to evaluate the relationship between swimming speed and size of megalopa. With this purpose in mind, we evaluated the swimming capabilities of megalopae of these crabs under different flow conditions and reviewed the information available for other species.

### MATERIALS AND METHODS

The study was performed at the Mar Chiquita Coastal Lagoon (37°32'S and 57°19'W). The study site is a body of brackish water (46 km<sup>2</sup>) affected by low amplitude (< 1 m) tides and characterized by mudflats and a large surrounding cordgrass (*Spartina densiflora*) area (Spivak et al. 1994). Megalopae were collected at approximately 1 km inside from the lagoon entrance during the summer of 1996 to 1997. Megalopae were identified before experimentation with a 16X binocular microscope following larval descriptions of Boschi et al. (1967) (*Chasmagnathus granulata*), Scelzo and Lichtschein (1978) (*Cyrtograpsus altimanus*), Boschi (1981) (*Cyrtograpsus angulatus*), and Rieger (1996) (*Uca uruguayensis*). Moreover, a subsample was followed until megalopae molted to crab-1 to confirm species identification. Megalopae of *Cyrtograpsus angulatus* and *C. altimanus* were collected with artificial collectors standing 50 cm from the bottom. Each collector was constructed of a plastic bag filled with 10 pieces of 20 by 20 cm window screens (0.2 cm size mesh). Collectors were inspected daily, and megalopae were hand picked and placed in containers with 23‰ salinity water. Megalopae of *Chasmagnathus granulata* were collected by hand from conspecific burrows and *Uca uruguayensis* were collected from individual burrows in the mud. Megalopae were held in an aquarium under ambient light (approximately 15 h daylight) and temperature conditions (20–23 °C). The water used in holding tanks and experiments was collected from the open sea, filtered through a 20 µm size mesh and then adjusted to 23‰ salinity with addition of freshwater. Only intact megalopae were used in this work.

Maximum length (from tip of rostral spine to the tip of the

telson) of 40 *Chasmagnathus granulata*, 40 *Cyrtograpsus angulatus*, 10 *Cyrtograpsus altimanus*, and 10 *Uca uruguayensis* megalopae were measured to the nearest 0.01 mm. Analysis of variance (ANOVA) (Zar 1984) followed by a posteriori analyses (Tukey test) were used to evaluate the null hypotheses of no differences in megalopa mean size between species.

Swimming speeds of megalopae were evaluated under different flow regimes using a recirculating flow tank. This tank is made of transparent Plexiglas with specifications similar to those used by Fernandez et al. (1994), which followed the suggestions of Nowell and Jumars (1987). The working section of the artificial channel measured 58 cm long, 14 cm wide, and 16 cm deep. Flow was generated by a plastic propeller attached to a 450 W electric engine. Drinking straw panels were used as flow straighteners to create near laminar flow. Observations of dye movements (rose Bengal) were taken to assess the water flow along the channel before running the experiments. Measures of flow velocity were taken by timing small particles in suspension in the centerline of the flume in successive 10-cm sections between the first 5 cm and the last 55 cm of the channel. Flow velocity was adjusted before each experimental run with a rheostat and controlled over the course of each trial. The coefficient of variability in the measurements of flow velocities was always < 4 %. All trials were run in a partially shadowed room with no direct light over the flume.

Swimming speeds of megalopae were evaluated under three different flow regimes: still water, 1 cm/s<sup>-1</sup>, and 3 cm/s<sup>-1</sup>. Single ANOVAs were performed for each flow velocity (Zar 1984) followed by a posteriori analyses (Tukey test) to evaluate the null hypotheses of no differences in swimming speeds mean size between species.

There were at least 15 replicates at each flow velocity for *Chasmagnathus granulata* and *Cyrtograpsus angulatus*. However, the episodic presence and scarcity of megalopae of *Cyrtograpsus altimanus* and *Uca uruguayensis* allow us to test them only under still water and other additional flow velocity. Because *Uca uruguayensis* showed at still water low average swimming speeds (near 3 cm/s<sup>-1</sup>), testing this species at 3 cm/s<sup>-1</sup> would have been unproductive because of the criteria used in measurements (see above). In contrast, *Cyrtograpsus altimanus* showed a high average swimming speed in still water (near 8 cm/s<sup>-1</sup>), and so we decided to test it at the higher flow velocity available in our flume (3 cm/s<sup>-1</sup>). Therefore, *U. Uruguayensis* was tested at still water (6 replicates) and 1 cm/s<sup>-1</sup> (3 replicates), and *Cyrtograpsus altimanus* was tested at still water (15 replicates) and at 3 cm/s<sup>-1</sup> (15 replicates).

Megalopae were tested individually, and only in a single flow velocity. In each trial, a single megalopa was deployed using a 4-mm diameter pipette in the center of the water column in the test channel. Then, after a minute given for acclimation, velocity and relative position in the channel were measured every time the megalopa swam against the current in an approximately straight direction and using the center of the flume channel. Runs with steep swimming angles or close to the walls or the bottom were discarded to avoid confounding effects. Distance swum was measured using a ruler attached to the backside of the flume and recorded on a hand-held tape recorder. Net swimming velocity for each megalopa was calculated as the sum of the recorded velocity and the current speed. At least three independent runs of more than 5-cm length were timed for each megalopa to assess the coefficients of variability (Zar 1984) of our measurements (it was always <

10%). For subsequent analyses, we used only the mean swimming speed of each megalopa.

The results of the above experiments were compared with information on maximum and average swimming speeds available for megalopae of the crabs *Varuna litterata* (Ryan and Choy 1990), *Callinectes sapidus* (Luckenbach and Orth 1992), *Cancer magister* (Fernandez et al. 1994), *Pachygrapsus crassipes* (Shanks 1985), and *Cancer oregonensis* (unpublished data). For our comparisons, maximum speed is the speed that can be maintained for distances between 10 cm and 1 m (it varies with authors), and average speed is that under still water conditions (the most common reported information). Only average swimming speed was reported for *P. crassipes*; whereas, it was not available for *C. oregonensis*. The data were fit to linear models (following Netter et al. 1990) to obtain the simplest expressions between size and speed for both average and maximum speeds. Studentized residuals were analyzed to search for outliers (following Netter et al. 1990).

## RESULTS AND DISCUSSION

The megalopae of the species studied in this work had significant differences in maximum length (ANOVA  $F = 1623$ ,  $df = 3$ ,  $96$ ,  $P < .001$ ; Tukey a posteriori test, all differences  $P < .001$ ; Fig. 1A). The maximum sustained swimming speed of megalopae over distances greater than 10 cm was 6.9 cm/s<sup>-1</sup> (34.4 body lengths/s<sup>-1</sup>) for *Uca uruguayensis*, 13.2 cm/s<sup>-1</sup> (49.3 body lengths/s<sup>-1</sup>) for *Chasmagnathus granulata*, 14.9 cm/s<sup>-1</sup> (49.4 body lengths/s<sup>-1</sup>) for *Cyrtograpsus angulatus* and 20.8 cm/s<sup>-1</sup> (60.7 body lengths/s<sup>-1</sup>) for *Cyrtograpsus altimanus*. Average swimming speed was significantly different among species at all current speeds tested (ANOVA: still water:  $F = 25.50$ ,  $df = 3$ ,  $52$ ; 1 cm/s<sup>-1</sup>:  $F = 16.18$ ,  $df = 2$ ,  $31$  and 3 cm/s<sup>-1</sup>:  $F = 10.96$ ,  $df = 2$ ,  $42$ ;  $P < .001$  in all cases). The relationship between average swimming speed and species (i.e., size of megalopa) was different at still water, 1 and 3 cm/s<sup>-1</sup>. In still water, the middle-sized *Chasmagnathus granulata* and *Cyrtograpsus angulatus* showed similar average swimming speeds ( $P > .05$ ), which were higher than the smaller *Uca uruguayensis* ( $P < .05$ ) and lower than the larger *Cyrtograpsus altimanus* ( $P < .05$ ; Fig. 1B). In 1 cm/s<sup>-1</sup>, *U. uruguayensis* was still the slowest swimmer, but *Chasmagnathus granulata* was faster in terms of average speed (17 %,  $P < .05$ ; Fig. 1C) than the larger *Cyrtograpsus angulatus*. In 3 cm/s<sup>-1</sup> *C. granulata* was still faster than *C. angulatus* (25 %,  $P < .05$ ; Fig. 1D), but the speed was not different from that of *Cyrtograpsus altimanus* ( $P > .05$ ; Fig. 1D). Average swimming speed of *C. altimanus* did not differ between still water and 3 cm/s<sup>-1</sup>; whereas, the other species increase speed when increasing flow velocity. These differences may be related to the ecology of the species. Directional flow, a typical estuarine stimulus, is not expected to affect a mainly marine crab such as *C. altimanus* to the extent that affect the other three strictly estuarine species studied here. Among these, *C. granulata* is restricted to estuarine habitats; whereas, *C. angulatus* also inhabits seashores. Thus, it is possible that a megalopa that is restricted to estuaries during settlement may be more sensitive to particular factors (e.g., directional flow) of such habitat than a megalopa that could settle in other habitats.

Comparing our results with the information available for other species, we found that maximum length of megalopa explained over 50% of the variance of the model for average speed and over 90% of the model for maximum speed (Fig. 2). The model of

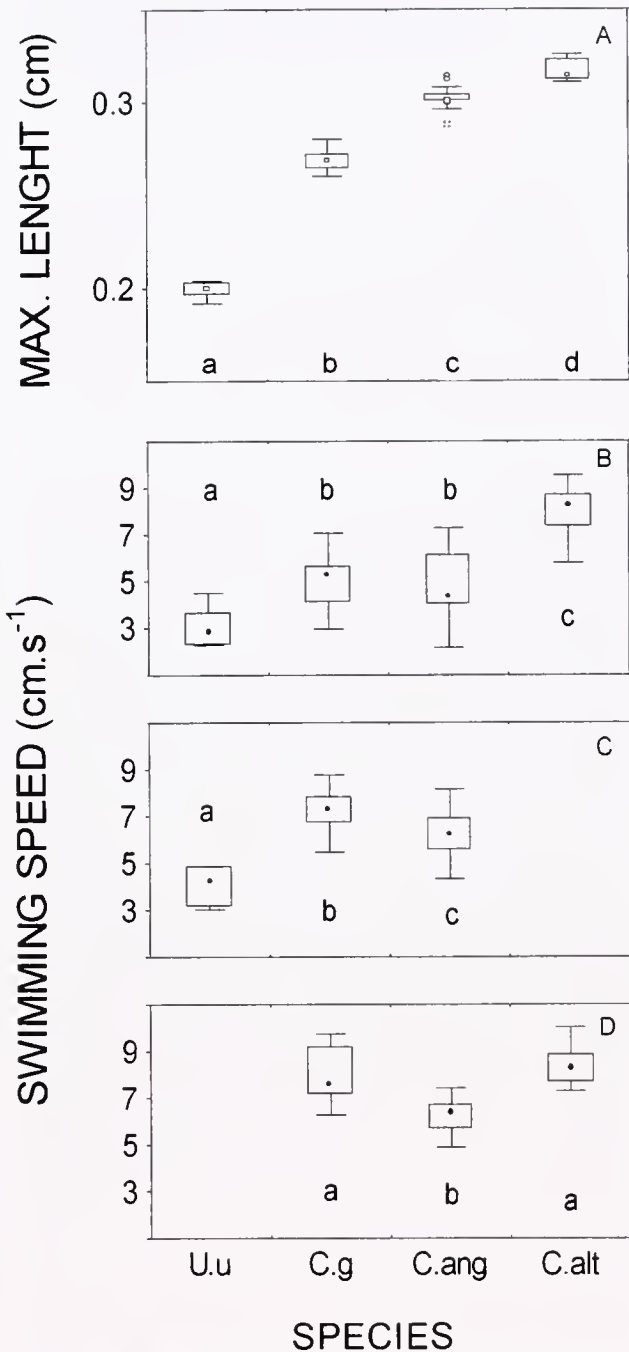


Figure 1. Maximum length of megalopae (A) and average swimming speed of *Uca uruguayensis* (U.u), *Chasmagnathus granulata* (C.g), *Cyrtograpsus angulatus* (C.ang), and *Cyrtograpsus altimanus* (C.alt) in still water (B), 1 cm/s<sup>-1</sup> (C) and 3 cm/s<sup>-1</sup> (D). Different lowercase letters indicate significant differences ( $P < .05$ ). Box plots show median values (black points), quartiles (edges of central box), and non-outlier ranges (whiskers).

average speed is strongly influenced by the departure of *Varuna litterata* (Studentized residual: 4.05,  $P < .01$ ). This may be explained by differences in the biology of this species. *Varuna litterata* undertakes long distance up-river migrations, including swimming against substantial currents and even climbing perpendicular waterfalls (Ryan and Choy 1990). Thus, it is expected that this species will have a higher relative average swimming speed

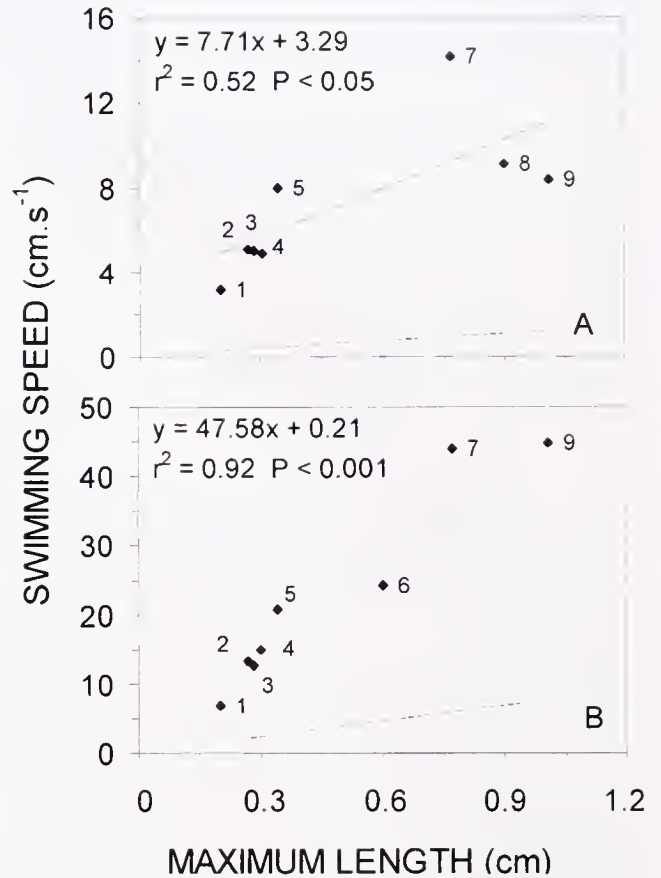


Figure 2. Relationship between body length of megalopae and their average (A) and maximum swimming speed (B). Numbers indicate species: (1) *Uca uruguayensis*, (2) *Chasmagnathus granulata*, (3) *Callinectes sapidus*, (4) *Cyrtograpsus angulatus*, (5) *Cyrtograpsus altimanus*, (6) *Cancer areganensis*, (7) *Varuna litterata*, (8) *Pachygrapsus crassipes*, and (9) *Cancer magister*. Data of 1, 2, 4, and 5 are from our work. Other information was obtained from (3) Luckenbach and Orth (1992), (6) O. Iribarne (unpublished), (7) Ryan and Choy (1990), (8) Shanks (1985) and (9) Fernandez et al. (1994). Dashed lines indicate the prediction of Peters et al. (1994).

than the other studied species that do not face such extreme conditions.

The relationship between size and swimming speed depends upon the range of size (Peters et al. 1994) and type of locomotion (Chia et al. 1984). In the range of size for crab megalopae, positive associations have been described between size and swimming speed for other marine invertebrate larvae (e.g., ascidian tadpoles: Chia et al. 1984). The model of Peters et al. (1994) fits the maximum swimming speed of ascidian tadpoles (mean error = 42%; data reconstructed from Fig. 10 of Chia et al. 1984) but largely underestimates both average (mean error = 1061 %) and maximum swimming speeds (mean error = 486 %) of crab megalopae (Fig. 2). Bias of such magnitude may have important implications in the interpretation of the role of swimming in settlement (Luckenbach and Orth 1992; Fernandez et al. 1994) and dispersal of megalopae (Shanks 1995).

General models describing the relationship between size and swimming speed may be useful in the analysis of the ecological and physiological general patterns (e.g., Peters et al. 1994). Despite this, caution should be made in the use of such models in

particular taxa. Distinctive characteristics of singular taxa may account for marked departures from the predictions of the general models and lead to biased conclusions.

The results of our experiments together with the available information show that, contrary to what is reported for most taxa of similar range of sizes, maximum length is a good indicator of swimming speeds of crab megalopae. We found departures from this relationship in our treatments of running water and an increase in the unexplained variance in the linear model of average swimming speed. Some departures may be explained by ecological differences among the species. Thus, the ecology of each species may be important in determining the average swimming speed of the species; whereas, maximum swimming speed seems to be more dependent upon physical constraints. Structural and physiognomic

differences between species of similar size (Alexander and Chen 1990) and even intraspecific differences in swimming strategy (Rooney and Cobb 1991) or age (Valero et al. unpublished) may explain the variation within the models.

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## EVALUATION OF HARVEST STRATEGIES FOR TANNER CRAB STOCKS THAT EXHIBIT PERIODIC RECRUITMENT

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**ABSTRACT** Recruitment to most Tanner crab (*Chionoecetes bairdi*) stocks in Alaska is periodic, causing wide fluctuations in population abundance. We evaluated alternative management approaches for such Tanner crab stocks with a size-based computer simulation model. Our study focused on Bristol Bay Tanner crab, the largest Tanner crab stock in Alaska, for which a stock–recruitment relationship with recruitment periodicity has been estimated. Alternative management approaches include a 40% harvest rate on legal males (status quo), variable harvest rates based on reproductive biomass, and strategies based on mature abundance, gear selectivity, and shell condition. Under the apparent recruitment periodicity of 10–18 years, maximum mean yield is achieved with a legal harvest rate >60% with large variation in yield and a high probability of fishery closure. Because of weak density dependence, the yield curve is relatively insensitive to high harvest rates. No harvest strategies can prevent stock collapse when recruitment has long periodicity and high amplitude, although a conservative strategy reduces the probability of stock collapse. We propose a harvest strategy for Bristol Bay Tanner crab that is 0, 10%, or 20% of molting mature males when effective reproductive biomass is <7,030 t,  $\geq 7,030$  and <15,400 t, or  $\geq 15,400$  t, respectively, with a 50% cap on harvest rate for exploitable legal crabs. The proposed strategy adjusts legal harvest rates according to changes in stock productivity indexed by recruitment strength: high legal harvest rates during the upward recruitment cycle and low rates that protect large-size crabs and reproductive potential during the downward recruitment cycle. As compared to the status quo harvest strategy, the new approach is easily implemented, has similar tradeoffs between high mean yield and relatively low variation in yield, while reducing shortages of mates for mature females and increasing fishing opportunities.

**KEY WORDS:** Tanner crab, *Chionoecetes bairdi*, periodic recruitment, harvest strategies, fisheries management, Alaska

### INTRODUCTION

Tanner crab (*Chionoecetes bairdi*) are widely distributed in the waters off Alaska, extending as far north as Norton Sound and as far south as Southeast Alaska. The stocks used to support some of the most important fisheries in Alaska. The fisheries have followed a boom and bust cycle. In the eastern Bering Sea, Tanner crab were first targeted by Japanese and Russian fleets in 1965. The eastern Bering Sea fishery expanded quickly in the late 1960s, and the catch reached 24,000 t in 1968. Foreign fishing for Tanner crab has been prohibited under the Magnuson Fisheries Conservation and Management Act since 1980. Directed fisheries for eastern Bering Sea Tanner crab by the U.S. fleet began in 1974. Catch peaked in 1978 at 31,300 t (Otto 1990). The population collapsed in the mid-1980s, and no fishing was allowed in 1986 and 1987. During 1990 to 1993, catches averaged 15,000 t and annual ex-vessel values averaged US\$46 million. Catches dropped sharply after 1993, and the eastern Bering Sea fishery has been closed since 1997 because of the depressed stock condition. Most other Tanner crab fisheries in Alaska collapsed in the early to mid-1990s, and none of the depressed stocks have recovered.

Wide fluctuations in catches are caused by fluctuations in population abundance for which highly variable recruitment dynamics are responsible. Like many fish stocks (Koslow 1989), recruitment to most Tanner crab stocks in Alaska is periodic and strongly autocorrelated (Zheng and Kruse in press). Recruitment to the Bristol Bay stock was strong in the mid-1970s and late and early 1990s and weak during the mid-1980s and mid- and late 1990s; recruitment to the northern Gulf of Alaska stocks was strong in the mid-1970s and has been weak since the early 1990s (Zheng and Kruse in press). Although recruitment is likely to result from a combination of density-dependent and density-independent factors, it is difficult to separate the effects of density-dependent

reproductive stock and autocorrelated environmental factors, as is typically the case (Deriso et al. 1986; Walters and Collie 1988). To date, stock–recruitment (S–R) relationships have been estimated only for Bristol Bay Tanner crab in the eastern Bering Sea (Zheng and Kruse 1998). For this stock, reproductive biomass explained only a small portion of recruitment variability, and residuals from the fitted S–R curve showed a strong cyclic trend (Zheng and Kruse 1998).

Currently, Tanner crab fisheries in Alaska are managed by a size/sex/season approach; that is, harvest of only large males and no fishing during spring molting and mating periods. The size/sex/season approach is based on economic consideration of market value and meat yield, protection of females for reproduction, and allowance of at least one mating season for males. In addition, commercial removals from assessed populations are based on a constant harvest rate strategy when abundance estimates are available. For example, for the eastern Bering Sea stock, a harvest rate of 40% is applied to the abundance of legal-sized male crabs (>137 mm carapace width, CW). Optimal harvest rates have not formally been evaluated for any Tanner crab stock in Alaska. Fishery thresholds have not been established and evaluated either, despite the fact that many Tanner fisheries in Alaska are currently closed because of the depressed stocks. In 1999, the U.S. Secretary of Commerce ruled that Tanner crab were overfished in the eastern Bering Sea.

National Standard 1 of the Magnuson–Stevens Fishery Conservation and Management Act requires that “conservation and management measures shall prevent overfishing while achieving, on a continuous basis, the optimal yield from each fishery . . .” (NMFS 1996). For a Tanner crab stock exhibiting a strong periodic and autocorrelated recruitment pattern, what is the optimal harvest strategy to produce relatively high yield, low variation in yield, and minimum chance of stock collapses? Although harvest strat-

egies for fish stocks with such recruitment patterns have been evaluated (e.g., Koslow 1989; Parma 1990; Walters and Parma 1995). No such studies have been conducted for Tanner crab stocks. In this study, we constructed a size-based model, based on crab CW, to facilitate a computer simulation analysis of alternative harvest strategies for Tanner crab stocks that exhibit periodic recruitment. Our study focused on Bristol Bay Tanner crab, the largest Tanner crab stock in Alaska. Alternative harvest strategies include a 40% harvest rate on legal males (status quo), variable harvest rates based on reproductive biomass, and strategies based on mature abundance, gear selectivity, and shell condition.

## METHODS

### *Population Model and Parameters*

The size-based population model constructed by Zheng et al. (1998) for Bristol Bay Tanner crab was used in this study and is summarized in the Appendix. We set the minimum CW at 93 mm for males and 70 mm for females and simulated crab abundance using width class intervals of 5 mm. The last width class included males  $\geq 163$  mm CW and females  $\geq 115$  mm CW. Population parameters from Zheng et al. (1998) were updated using data from 1975 to 1997 and are summarized in Table 1. Population abundances were simulated for June each year, after crabs have generally completed annual molting and mating. Because fishing usually occurred during November each year since 1993, we used a lag of 0.4 year between the abundance assessment and the November fishery in our simulations.

A constant natural mortality was used in our simulations. Handling mortality from the other crab fisheries was part of natural mortality. Handling mortality from the directed Tanner crab fishery and bycatch mortality from all nonpot fisheries were separated from natural mortality. Therefore, natural mortalities for both males and females were lower than those estimated by the size-based model of Zheng et al. (1998), in which all handling mortality was included in the estimates of natural mortality. To examine sensitivity of the alternative strategies to levels of natural mortality, we compared evaluation criteria for low natural mortality and high natural mortality represented by 62.5% and 137.5% of the baseline natural mortalities (Table 1).

The level of handling mortality from the directed pot fishery was determined by gear selectivities of sublegal males and females and handling mortality rate. We estimated the gear selectivities of sublegal male and mature female crabs from the observer data from 1990 to 1996 (Table 1) and assumed a 20% handling mortality rate for those crabs that are caught and returned to the sea (Zheng et al. 1998). To investigate sensitivity of results to handling mortality rate, we also simulated scenarios with 0% and 50% handling mortality rates that bracket the range of likely values.

Bycatches were estimated for two kinds of nonpot fisheries: scallop and groundfish. Annual Tanner crab bycatch from the eastern Bering Sea scallop fishery was assumed to equal the modeled population abundance times the current bycatch limitation rate of 0.1354% (J. Barnhart, Alaska Dept. of Fish and Game, Kodiak, Alaska, pers. comm.). The current limit of Tanner crabs in the eastern Bering Sea groundfish fisheries was a step function of total Tanner crab abundance estimated from the survey and was separately set for two zones (Witherell 1997). Zone 1 and part of Zone 2 are in Bristol Bay. The abundance of the modeled Bristol Bay population was about 40% of the total surveyed abundance of the

eastern Bering Sea from 1988 to 1997; so, all bycatch limits for the groundfish fisheries were multiplied by 0.4 in the simulations. In addition, all bycatch limits from Zone 2 were also multiplied by 0.89, because an average of 89% of the observed bycatch in Zone 2 came from Bristol Bay from 1993 to 1997. The bycatch in Zone 2 rarely exceeded the limits; therefore, we set the maximum bycatch limit for the modeled population from the groundfish fisheries in the Bristol Bay area of Zone 2 as 0.748 million of crabs ( $2.1 \times 0.4 \times 0.89$ ) (Witherell 1997). Mortality rates for Tanner crab bycatches from the scallop fishery and groundfish fisheries were assumed as 40% and 80%, respectively (NPFMC 1996).

Survey measurement error was assumed to follow a lognormal distribution. Simulated "true" values of effective reproductive biomass and crab abundance were multiplied by a measurement error to mimic the survey estimation process for each year. Effective reproductive biomass was defined as biomass of females  $>79$  mm CW that can be mated by mature males (Zheng and Kruse 1998). The lognormal measurement errors were simulated with a standard deviation of 0.2 and a mean of zero. To prevent extremely large errors in estimated values of abundance, both ends of the measurement-error distribution were truncated to fall within its 98% confidence limits.

S-R data for Bristol Bay Tanner crab were fitted to a normal Ricker model by Zheng and Kruse (1998), and this S-R relationship with cyclic residuals from a sine function was used to conduct our simulations (Fig. 1). Sensitivity of the harvest strategies to depensation was also examined by using a depensatory Ricker S-R curve with cyclic residuals. Sex ratio of recruits was assumed to be 55% males and 45% females based on the average ratio of recruitment estimates from 1976 to 1997 (Zheng et al. 1998). A lower proportion of female recruits is likely caused by a lower catchability by the trawl survey gear. The period length of recruitment cycles was randomly set from 10 to 18 years. Sensitivities to cycle period length and amplitude were investigated by varying cycle period length from 4 to 30 years and cycle amplitude from 0.4 to 2.5.

Molting probabilities for males and maturity probabilities for females varied over time (Zheng et al. 1998). Although these probabilities were not strongly correlated with recruitment strengths, periods with higher molting probabilities for males and lower maturity probabilities for a given size for females generally occurred during good recruitment periods. To incorporate this dynamic feature into the simulation model, we used two molting probability functions for males and two maturity functions for females based on the updated results by Zheng et al. (1998). The high molting probability function was used during periods with upward recruitment cycles whereas the low molting probability function was used during periods with downward recruitment cycles. Only a few years occurred when the 50% maturity for females were at large sizes (Zheng and Kruse 1998); thus, the low maturity probability function (becoming mature at large size) was used only during periods with the highest 50% of upward cycles. The high maturity probability function was used during the rest of a recruitment cycle.

### *Alternative Strategies*

In this study, we examined three kinds of alternative harvest strategies to set guideline harvest levels (GHL; i.e., annual catch quotas). These approaches ranged from a simple approach to a

TABLE 1.

Population parameters for a size-based model of Bristol Bay Tanner crab updated from Zheng et al. (1998), "New" and "Old" refer to shell condition of crabs.

Male Crabs									
Mid-CW (mm)	Weight (Kg)	Initial Abund.		Molting Probability				Selectivity	
		New	Old	New		Old		New	Old
		(million)		High	Low	High	Low		
95.5	0.260	1.202	0.613	0.944	0.827	0.136	0.000	0.104	0.057
100.5	0.304	1.449	0.975	0.922	0.777	0.136	0.000	0.103	0.056
105.5	0.354	1.371	1.201	0.893	0.717	0.136	0.000	0.164	0.057
110.5	0.408	1.070	1.295	0.855	0.648	0.136	0.000	0.311	0.099
115.5	0.468	0.780	1.643	0.805	0.572	0.136	0.000	0.333	0.123
120.5	0.534	0.554	2.100	0.745	0.491	0.136	0.000	0.488	0.126
125.5	0.606	0.366	2.233	0.673	0.410	0.136	0.000	0.706	0.139
130.5	0.684	0.175	1.994	0.592	0.332	0.136	0.000	0.958	0.201
135.5	0.768	0.118	1.893	0.506	0.261	0.136	0.000	1.000	0.220
140.5	0.860	0.071	0.884	0.420	0.200	0.136	0.000	1.000	0.317
145.5	0.958	0.039	0.590	0.338	0.150	0.136	0.000	1.000	0.317
150.5	1.064	0.019	0.398	0.265	0.111	0.136	0.000	1.000	0.317
155.5	1.177	0.008	0.242	0.203	0.080	0.136	0.000	1.000	0.317
160.5	1.298	0.003	0.145	0.152	0.058	0.136	0.000	1.000	0.317
165.5	1.428	0.000	0.204	0.113	0.041	0.136	0.000	1.000	0.317

Female Crabs							
Mid-CW (mm)	Weight (Kg)	Initial Abund.		Mature Probability		Selectivity	
		New	Old	Low	High	New	Old
72.5	0.215	1.359	2.158	0.024	0.368	0.007	0.030
77.5	0.256	1.520	2.816	0.056	0.488	0.024	0.042
82.5	0.300	1.255	2.893	0.126	0.632	0.059	0.080
87.5	0.349	0.842	2.822	0.260	0.768	0.170	0.102
92.5	0.402	0.476	2.409	0.462	0.867	0.323	0.142
97.5	0.461	0.227	1.624	0.677	0.928	0.333	0.205
102.5	0.524	0.091	0.873	0.837	0.961	0.333	0.359
107.5	0.592	0.021	0.335	0.926	0.978	0.333	0.359
112.5	0.665	0.007	0.115	0.968	0.987	0.333	0.359
117.5	0.743	0.003	0.043	0.987	0.992	0.333	0.359

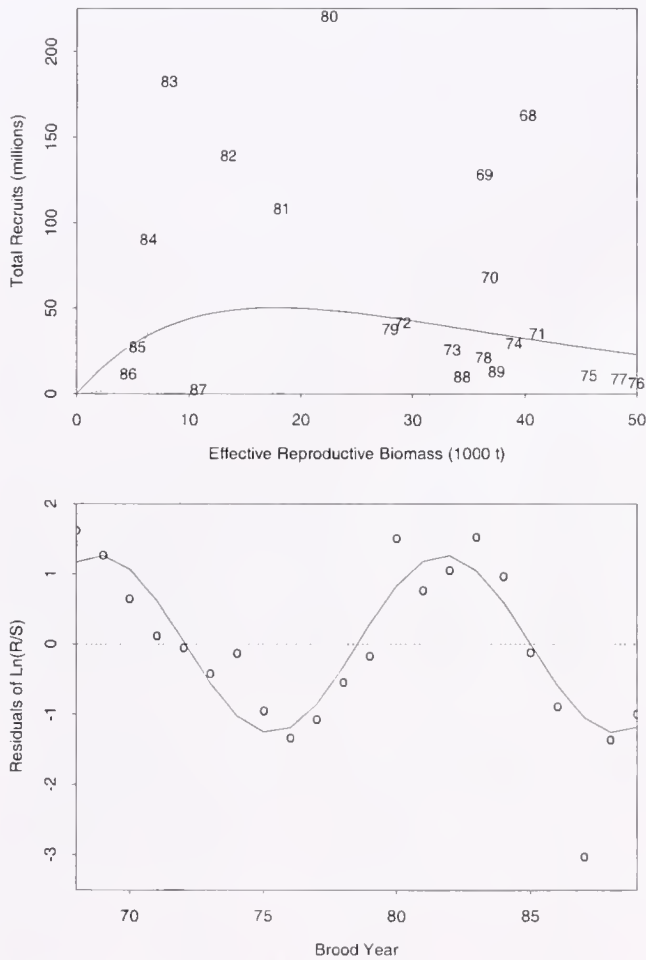
  

Growth		Natural Mortality			S-R Models				Prop. by Width			
Para	Male	Female	Level	Male	Female	Para	Normal	Para	Depensa	Para	Male	Female
a	15.75	25.60	mean	0.40	0.43	$\alpha$	2.0402	$\kappa$	0.2031	$\alpha_r$	100.0	80.67
b	0.070	-0.134	low	0.25	0.27	$\beta$	0.0563	$\theta$	2.8031	$\beta_r$	1.023	0.955
$\beta$	0.746	1.000	high	0.55	0.59	A	1.2676					
						P	10-18 yrs					
						$\sigma$	0.4570					

more complex approach incorporating gear selectivity and shell condition. Under the first harvest strategy, the status quo, GHL was set by legal harvest rate multiplied by legal male crab abundance. The current legal harvest rate is 40%, but we also evaluated nine other rates ranging from 10% to 60%. Under the second alternative, GHL was set by legal harvest rate multiplied by "exploitable" legal male crab abundance. Because the fishery disproportionately harvests new-shell crabs over old-shell crabs, we defined exploitable legal males based on fishery selectivity parameters. We estimated 100% selectivity for new-shell crabs and 32% selectivity for old-shell crabs based on comparison of catch and survey data from 1975 to 1997. Ten alternative harvest rates for

exploitable legal males ranging from 15% to 65% were evaluated. Under the third approach, GHL was set by mature harvest rate multiplied by "molting mature males," but only legal males were allowed to be harvested with a catch cap of 50% of exploitable legal male abundance. In other words, the legal harvest rate is equal to the mature harvest rate multiplied by "molting mature male" abundance divided by legal male abundance. "Molting mature males" were defined as 100% of new-shell males and 15% of old-shell males >112 mm CW. These mature males have a high probability of molting within a year. Ten alternative mature harvest rates ranging from 10% to 35% were evaluated.

Because the S-R relationship is weakly density dependent, we



**Figure 1.** Relationships between effective reproductive biomass ( $S$ ) and total recruits at age 7 (i.e., 8-year time lag, upper plot) and residuals of logarithm of recruits per  $S$  from a normal Ricker curve (lower plot) for Bristol Bay Tanner crab. In the upper plot, numerical labels are brood year, solid line is a normal Ricker curve without autocorrelated component, and dotted line is an exponential S-R curve (depensatory curve). In the lower plot, open circles represent residuals estimated from deviations of S-R datapoints from the normal Ricker curve, and solid line represents residuals fitted to a sine function.

did not attempt to estimate an optimal threshold in our simulations. Rather, we set a threshold based partly on past fishery management practice and partly on the S-R relationship. In the past, the effective reproductive biomass was always below 7,030.0 t in the years when the fishery was closed. This level of effective reproductive biomass is slightly above the smallest effective reproductive biomass with an above average recruitment level (Fig. 1).

A stair-step harvest rate schedule similar to that employed for the Bristol Bay red king crab (*Paralithodes camtschaticus*) fishery (Zheng et al. 1997) was also evaluated for each approach. We used 22,000 t of effective reproductive biomass as a base level, which is the average of simulated effective reproductive biomass under the status quo constant 40% legal harvest rate. When effective reproductive biomass was at or below 50%, 60%, or 70% of this base level, harvest rates would decrease by 50% or 40%. A combination of three levels of effective reproductive biomass and two levels of reduced harvest rates resulted in six alternative stair-step harvest rate schedules. We evaluated each stair-step schedule in combination with each of the three harvest strategy approaches.

### Simulations

The alternative harvest strategies were evaluated by simulating the Bristol Bay Tanner crab stock and fishery with the population dynamic model and a standard set of population parameters. The simulation model was initialized with effective reproductive biomass from 1990 to 1997 (Zheng and Kruse 1998) and population abundance in 1997 (Table 1) so that year 1 corresponded to 1998. The simulated time horizon was set at 100 years. Each scenario was replicated 1000 times to ensure relative stability of statistics. Identical seeds for random number generators were used for all scenarios to compare different strategies under identical environmental conditions.

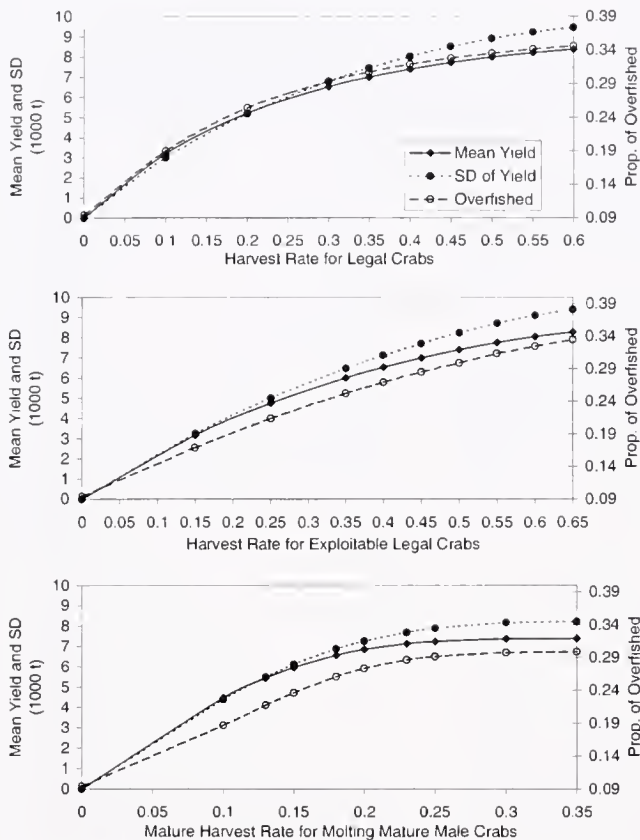
We examined sensitivity of each strategy to changes in natural mortality, handling mortality, and S-R curve. The standard set of population parameters was used in each sensitivity analysis, except that both a normal Ricker S-R curve and depensatory S-R curve were used and that the parameter under consideration was assigned one of two opposite and extreme values. For sensitivity studies on recruitment cycles, we used 200 replicates, each for 1000 years. A longer simulated time horizon was needed to examine cycle period length.

To evaluate the strategies, statistics were collected on effective reproductive biomass, probabilities of fishery closure, probabilities that the stock is below the overfished reference point as defined in the fishery management plan (NPFMC 1998), and yield. Probabilities of fishery closure are denoted as the proportions of replicates with estimated effective reproductive biomass below threshold so that the fishery is prohibited for a given year. The overfished level is defined for Tanner crab in the entire eastern Bering Sea, not just Bristol Bay. Based on the survey data from 1983 to 1997, we approximated the equivalent overfished level for Bristol Bay Tanner crab as 26,600 t of total mature male and female biomass. Results were averaged over the simulated time horizon and over all replicates. To assess optimality, an equal tradeoff value between increase in mean yield and decrease in standard deviation of yield was computed as  $0.5 \cdot \text{yield} - 0.5 \cdot \text{standard deviation}$  (Zheng et al. 1997) for each alternative strategy.

### RESULTS

The tradeoff between mean yield and standard deviation of yield as a function of constant harvest rate (i.e., without the stair-step) was similar among the three approaches (Fig. 2). Mean yield, standard deviation of yield, and proportion of years that mature population abundance was below the overfished reference point increased as a function of harvest rate, but the standard deviation increased at a faster rate than mean yield. The rate of increase in mean yield generally slowed down as harvest rate increased, especially with legal harvest rate >40%, exploitable harvest rate >45%, and mature harvest rate >20%. Variations in yield, indexed by standard deviations of yield, were very high for all three approaches. This is a direct result of the periodic recruitment feature of Tanner crab population dynamics. Even without a fishery, reproductive biomass fell below the overfished reference point in 9.4% of years. The legal harvest rate of 40% (status quo) is equivalent to an exploitable legal harvest rate of 45% and a mature harvest rate of 20%. Under equivalent harvest rates, both legal harvest rate and exploitable legal harvest rate approaches had similar mean yield and standard deviation of yield, but the proportion of years at overfished levels was lower for the exploitable harvest rate approach than the harvest rate approach. Mean yield, standard





**Figure 2.** Mean yield (solid lines), standard deviation of yield (dotted lines), and probability of being at overfished levels (dashed lines) as a function of constant harvest rate for Bristol Bay Tanner crab under the normal S-R curve and a 20% handling mortality rate. In the top, middle, and lower plots, harvest rates apply to total legal crabs, exploitable legal crabs, and molting mature male crabs, respectively.

deviation of yield, and proportion of years at overfished levels with the 20% mature harvest rate approach were the lowest among the three equivalent approaches. The 50% cap on exploitable harvest rate for the mature harvest rate approach resulted in relatively flat curves of mean yield, standard deviation, and proportions of years at overfished levels when mature harvest rates were high (Fig. 2).

Alternative stair-step functions of harvest rate generally did not change the results very much (Table 2). Because standard deviation of yield increased much faster than mean yield at legal harvest rate >40%, exploitable legal harvest rate >45%, and mature harvest rate >20% (Fig. 2), we used these harvest rates as the high harvest rate levels in the stair-step functions. The legal harvest rate of 40% also happens to be the status quo harvest rate. For each approach, a decrease from 70 to 60 to 50% in cut-off levels of effective reproductive biomass or an increase in low harvest rates from 50 to 60% resulted in slightly higher trade-off values between increase in mean yield and decrease in standard deviation of yield but caused slightly higher percentages of years with fishery closure and with mature biomass being below the overfished reference point (Table 2). Overall, the mature harvest rate approach had slightly higher trade-off values between increase in mean yield and decrease in standard deviation of yield compared to the other two approaches. It also had slightly lower percentages of years with fishery closure and fewer years being overfished. The harvest strategy with a high mature harvest rate of 20% and a low rate of 10%

with a cut-off of 15,400 t of effective reproductive biomass had the lowest percentages of years with fishery closure and at overfished levels among all the alternatives (Table 2). The trade-off value between increase in mean yield and decrease in standard deviation of yield was intermediate among the range in values among all harvest strategies (Table 2). In the context of National Standard 1, we considered this strategy as the most attractive alternative to the status quo strategy.

Sensitivity analyses of natural mortality, handling mortality, S-R curve, and recruitment cycle were conducted on the proposed new strategy and the status quo strategy. As expected, higher natural mortality or handling mortality rate resulted in much lower catch and higher percentages of years with fishery closure and at overfished levels for all alternative strategies (Table 3), and vice versa for lower natural mortality or handling mortality rate. The depensatory S-R curve had a minor effect on the results of simulations except when depensation was combined with high natural mortality, which resulted in extremely low population abundances and few fishing opportunities (Table 3). Effective reproductive biomass rarely fell into the depensatory range under other circumstances.

Under the same conditions, the status quo harvest strategy had slightly higher mean yield, lower standard deviation of yield, higher percentages of years with fishery closure and at overfished levels than when the status quo harvest strategy included stair-step harvest rates (Table 3). The status quo harvest strategy also had higher mean yields than those for the proposed new strategy under the same conditions, but its standard deviations of yield and its percentages of years at overfished levels were much higher (Table 3).

With the normal S-R curve, the status quo and proposed harvest strategies were very sensitive to period length and amplitude of recruitment cycle, especially for a long period length and high amplitude (Fig. 3). Coefficient of variation of yield and proportions of years of fishery closure and at overfished levels increased substantially as period length and amplitude of recruitment cycle increased. For a given combination of period length and amplitude of recruitment cycle, the proposed harvest strategy resulted in only a minor improvement on coefficient of variation of yield and proportion of years of fishery closure over the status quo strategy (Fig. 3). The proposed harvest strategy reduced proportions of years at overfished levels considerably when the recruitment cycle period length was 18 years or less.

The sensitivities of the status quo and suggested harvest strategies to the depensatory S-R curve depended on period length and amplitude of recruitment cycle (Fig. 4). For a period length  $\leq 10$  years or an amplitude  $\leq 1.0$ , effective reproductive biomass rarely fell below the depensatory range; thus, the simulation results between the normal S-R curve and the depensatory S-R curve were almost identical for this region of parameter values (Figs. 3, 4). For combinations of period lengths  $\geq 15$  years and amplitudes  $\geq 1.5$ , coefficients of variation of yield and proportions of years of fishery closure and years at overfished levels were much higher with the depensatory S-R curve than with the normal S-R curve (Figs. 3, 4). For combinations of extremely long period length and high amplitude, effective reproductive biomass with the depensatory S-R curve was always below the threshold level (Fig. 4). Under likely ranges of 10–18 years of period length and amplitudes of 1.0–1.4, the depensatory S-R curve did not have a major impact on the simulation results. Similar to the results with the normal S-R curve, the proposed new harvest strategy reduced proportions of

TABLE 2.

Comparisons of mean yield, standard deviation of yield (SD), equal trade-off between increase in mean yield and decrease in standard deviation of yield, mean effective reproductive biomass (SP), mean total mature biomass (TMB), percentage of years without fishing (Closure), and percentage of years below the overfished reference point (Overfished) for alternative harvest strategies.

Cut-off (1000 t)	High HR	Low HR	Yield (1000 t)	SD (1000 t)	Tradeoff	SP (1000 t)	TMB (1000 t)	Closure (%)	Overfished (%)
<b>Harvest Rates Applied to Total Legal Crabs</b>									
<u>7.030</u>	<u>0.400</u>	<u>0.000</u>	<u>7.377</u>	<u>7.999</u>	<u>-0.311</u>	<u>21.660</u>	<u>62.133</u>	<u>14.521</u>	<u>31.847</u>
11.000	0.400	0.200	7.313	8.095	-0.391	21.812	62.676	14.034	30.917
13.200	0.400	0.200	7.267	8.138	-0.435	21.872	62.957	13.848	30.162
15.400	0.400	0.200	7.211	8.170	-0.480	21.923	63.245	13.721	29.385
11.000	0.400	0.240	7.328	8.071	-0.371	21.778	62.552	14.150	31.146
13.200	0.400	0.240	7.294	8.101	-0.403	21.824	62.763	14.018	30.579
15.400	0.400	0.240	7.253	8.122	-0.435	21.862	62.978	13.919	30.004
<b>Harvest Rates Applied to Exploitable Legal Crabs</b>									
7.030	0.450	0.000	6.988	7.702	-0.357	21.971	63.944	13.598	28.480
11.000	0.450	0.225	6.912	7.801	-0.444	22.088	64.436	13.291	27.483
13.200	0.450	0.225	6.862	7.851	-0.494	22.139	64.701	13.167	26.772
15.400	0.450	0.225	6.800	7.895	-0.548	22.185	64.989	13.059	26.019
11.000	0.450	0.270	6.928	7.780	-0.426	22.065	64.335	13.351	27.706
13.200	0.450	0.270	6.889	7.818	-0.464	22.106	64.545	13.251	27.122
15.400	0.450	0.270	6.841	7.850	-0.505	22.143	64.772	13.158	26.524
<b>Mature Harvest Rates Applied to Molting Mature Male Crabs</b>									
7.030	0.200	0.000	6.844	7.258	-0.207	21.964	64.078	13.366	27.333
11.000	0.200	0.100	6.778	7.339	-0.281	22.049	64.462	13.121	26.464
13.200	0.200	0.100	6.732	7.381	-0.324	22.089	64.682	13.000	25.754
<b>15.400</b>	<b>0.200</b>	<b>0.100</b>	<b>6.676</b>	<b>7.418</b>	<b>-0.371</b>	<b>22.125</b>	<b>64.922</b>	<b>12.875</b>	<b>24.986</b>
11.000	0.200	0.120	6.796	7.316	-0.260	22.025	64.355	13.188	26.714
13.200	0.200	0.120	6.761	7.346	-0.292	22.054	64.520	13.087	26.192
15.400	0.200	0.120	6.718	7.372	-0.327	22.082	64.703	12.994	25.620
<b>Estimated Historical Averages from 1975 to 1997</b>									
			6.813	6.846	-0.016	23.586	65.205	13.043	26.087

"Cut-off" is a level of SP below which the low harvest rate (HR) is used and at or above which the high harvest rate is used. The status quo strategy is underlined and the proposed new strategy is shown in **bold**. Historical data were included for comparison.

years of fishery closure and at overfished levels considerably when period length of recruitment cycle was 18 years or less and amplitude was 1.8 or less (Fig. 4).

Although the status quo harvest strategy is a constant legal harvest rate of 40%, legal harvest rates actually implemented during the last 23 years were quite different from this level and varied greatly over time (Fig. 5). Realized legal harvest rates were higher than 40% during 1977 to 1980 and 1989 to 1992 and much lower during 1983 to 1988 and 1994 to 1997. It seems that it is difficult to implement a constant legal harvest rate strategy. Preseason GHs were generally slightly higher than actual yields for most years but much higher than actual yields when the GHs were low (Fig. 5). The proposed harvest strategy leads to higher legal harvest rates than the historical rates of the status quo strategy when population abundance is increasing and to lower rates when population abundance is decreasing. Historical harvest rates more closely match the proposed new harvest strategy than the status quo "constant" harvest rate strategy (Fig. 5).

## DISCUSSION

Changing environments pose great challenges to fishery managers. Environmental shifts cause large changes in growth, mortality, and recruitment, making it difficult to design, evaluate, and implement optimal harvest strategies that are robust to wide swings in productivity. When trends in environmental effects on recruitment can be predicted, harvest strategies can be adjusted to maximize expected discounted yield. Escapement goals (Parma 1990) or harvest rates (Criddle et al. 1998) can be raised when

favorable conditions are anticipated and lowered when poor conditions are expected. Even lacking knowledge of environmental effects, a constant harvest rate strategy still produces a long-term harvest close to the theoretical optimum for stocks with periodic or autocorrelated recruitment (Walters and Parma 1995). However, neither constant harvest rate nor escapement goal strategies can prevent collapse of stocks with high-amplitude, low-frequency recruitment variability, although a constant escapement strategy minimizes the risk (Koslow 1989).

Harvest strategies for Tanner crab differ from those for many fish stocks, because they take into account differences in biology. Female Tanner crabs can store sperm for more than 1 year, and stored sperm from multiple matings may fertilize clutches for the subsequent 2 years (Paul 1984). A mature male Tanner crab can mate with a maximum of 8–10 females in a laboratory setting during a breeding season (Paul 1984), although the number of females a male can mate in the field may be less than this maximum number because of low density or discrete spatial distributions of the sexes and a limited mating window. Conceivably, the size/sex/season approach coupled with a suitable harvest rate on legal crabs of size one or two molts larger than mature males could adequately protect reproductive potential of Tanner crab. However, molting probabilities of male crabs decrease sharply when they attain large claws (unpublished data), and periods of poor recruitment lead to depressed populations predominated by old "skip molt" or "terminal molt" crabs. Applying a constant harvest rate to total legal abundance when the abundance is low could result in a high discard rate of old-shell crabs and a very high

TABLE 3.

Comparisons of mean yield, standard deviation of yield (SD), mean effective reproductive biomass (SP), mean total mature biomass (TMB), percentage of years without fishing (Closure), and percentage of years below the overfished reference point (Overfished) for three harvest strategies under low and high natural mortality (M), three levels of handling mortality (HM) and two S-R curves for the status quo, modified status quo, and proposed new strategy (see Table 2).

Harvest Strategy	M	HM	Yield (1000 t)	SD (1000 t)	SP (1000 t)	TMB (1000 t)	Closure (%)	Overfished (%)
<b>Normal S-R Curve and Harvest Rates Applied to Total Legal Crabs</b>								
Status quo	Low	0.2	11.080	12.157	33.494	89.759	2.26	13.92
Status quo	High	0.2	4.226	4.837	13.709	41.418	33.55	43.45
Status quo	Normal	0.0	8.213	8.933	23.675	67.118	11.81	28.84
Status quo	Normal	0.5	6.172	6.687	19.000	55.236	18.43	35.65
Modif. status quo	Low	0.2	11.046	12.227	33.673	90.272	2.15	12.44
Modif. status quo	High	0.2	3.857	4.841	13.923	42.721	32.88	40.90
Modif. status quo	Normal	0.0	8.004	9.067	23.682	67.632	11.78	27.05
Modif. status quo	Normal	0.5	6.077	6.895	19.614	57.198	16.44	32.48
<b>Normal S-R Curve and Harvest Rates Applied to Molting Mature Male Crabs</b>								
New proposed	Low	0.2	9.404	9.865	34.374	97.521	1.94	4.07
New proposed	High	0.2	3.868	4.753	13.872	42.442	32.78	40.37
New proposed	Normal	0.0	7.267	8.085	23.694	69.373	11.75	23.01
New proposed	Normal	0.5	5.757	6.396	19.886	58.441	14.79	28.38
<b>Depensatory S-R Curve and Harvest Rates Applied to Total Legal Crabs</b>								
Status quo	Low	0.2	10.998	12.094	33.240	89.113	2.26	13.78
Status quo	High	0.2	1.942	3.401	7.039	22.962	63.97	69.56
Status quo	Normal	0.0	8.013	8.787	23.098	65.573	11.82	29.16
Status quo	Normal	0.5	5.847	6.427	18.003	52.570	18.76	36.51
Modif. status quo	Low	0.2	10.962	12.165	33.420	89.640	2.16	12.25
Modif. status quo	High	0.2	1.756	3.263	7.686	25.569	60.41	64.24
Modif. status quo	Normal	0.0	7.790	8.924	23.109	66.134	11.79	27.30
Modif. status quo	Normal	0.5	5.803	6.697	18.834	55.173	16.60	32.89
<b>Depensatory S-R Curve and Harvest Rates Applied to Molting Mature Male Crabs</b>								
New proposed	Low	0.2	9.334	9.812	34.117	96.845	1.94	4.05
New proposed	High	0.2	1.765	3.178	7.655	25.373	60.43	64.09
New proposed	Normal	0.0	7.060	7.942	23.123	67.870	11.77	23.19
New proposed	Normal	0.5	5.524	6.221	19.228	56.738	14.921	28.83

The modified status quo strategy is represented by a cut-off SP of 15,400 t, high harvest rate of 0.4 and low harvest rate of 0.2. "Cut-off" is a level of SP below which the low harvest rate is used and at or above which the high harvest rate is used.

harvest rate on new-shell, relatively young crabs because of fishery selectivity to meet market demands.

The proposed harvest strategy takes into account the relationship between shell condition and productivity levels of Tanner crab stocks. Strong year classes are dominated by new-shell crabs. Simulation results show that the proposed new strategy adjusts legal harvest rates according to recruitment strength, which is indexed by changes in shell condition. Contrary to the current harvest strategy based on legal male abundance only, use of mature crab abundance and shell condition gives the proposed new strategy a forward-looking feature. When an increase in future legal crab abundance is expected because of increased recruitment to the mature segment of the stock, legal harvest rates are increased. Conversely, during a downward recruitment cycle, reduced legal harvest rates will forestall the decline of large, old-shell males that are most virile (Stevens et al. 1993; Paul et al. 1995).

As a comparison to the status quo harvest strategy, the new approach had similar trade-off values between mean yield and variation in yield, but it led to fewer shortages of mates for mature females and reduced probability that population abundance falls below the overfished reference point over a long term. If reproduction can be limited because of a shortage of mature males, it is most likely during periods of low population abundance. As abun-

dance declines, spatial distribution becomes more patchy, thereby potentially reducing mating encounters. By incorporating a fishery threshold and stair-step harvest rates, the proposed new harvest strategy embodies a precautionary approach to fishery management (Restrepo et al. 1998). These features reduce mature harvest rates to protect reproductive potential during periods of low abundance when risks of overfishing or falling below the overfished reference point are high because of uncertainties in abundance estimates and population dynamics (i.e., depensation vs. compensation).

Although we did not explicitly evaluate economic impacts of the management alternatives, the proposed new strategy compares favorably to the current strategy. Slightly greater mean yield implies higher average gross revenues under the status quo as compared to the proposed strategy. However, the proposed strategy results in greater fishery stability, as indicated by lower variability in yield and more fishing opportunities because of fewer fishery closures. At low population abundance, catch expectations (pre-season GHY) are much more indicative of actual harvests under the proposed new strategy than the status quo. Under the status quo harvest strategy, pre-season GHY is set as 40% of legal male abundance, despite the fact that old-shell males predominate the population and that industry targets new-shell males, leading to more

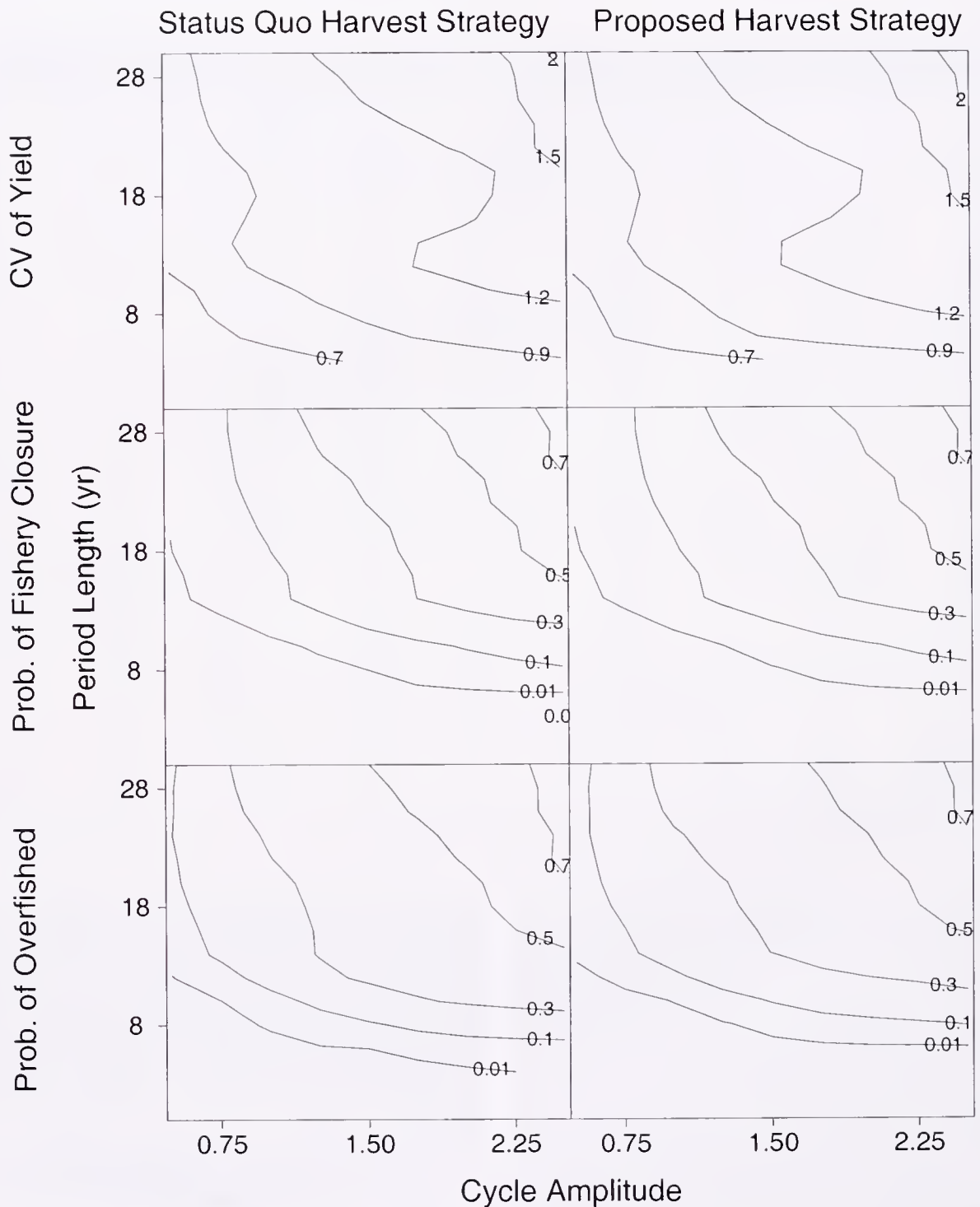


Figure 3. Contour plots of CV of yield, probability of fishery closure, and probability of total mature biomass below the overfished reference point by cycle amplitude and period length of the recruitment dynamics under the normal S-R curve for Bristol Bay Tanner crab. The plots are classified by the status quo harvest strategy and the proposed new strategy based on a 0, 10%, and 20% stair-step harvest rates of molting mature males.

grounds prospecting and catch sorting. As a result, low in-season catch-per-unit-effort triggers fishery closures short of the GHF as a conservation measure. Not only do inflated catch expectations depress prices, they may attract more fishery participants, thus reducing average revenues and increasing aggregate costs.

In our analysis of alternative harvest rate strategies, we attempted to consider total fishing mortality as the aggregate of landed catch, handling mortality of discards in the directed fishery, and bycatch mortality in ground fish and scallop fisheries. Landings are documented on transaction receipts between processors

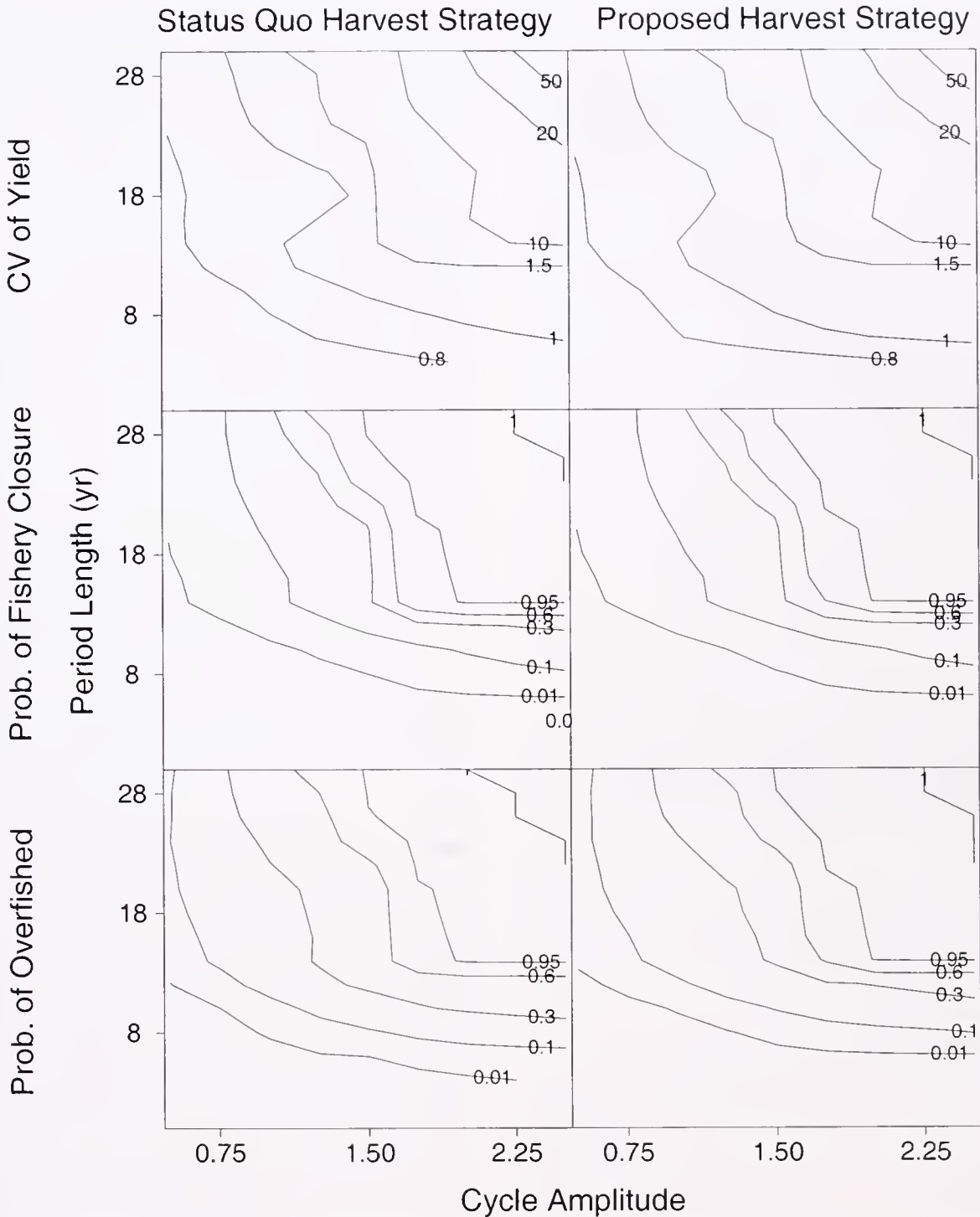
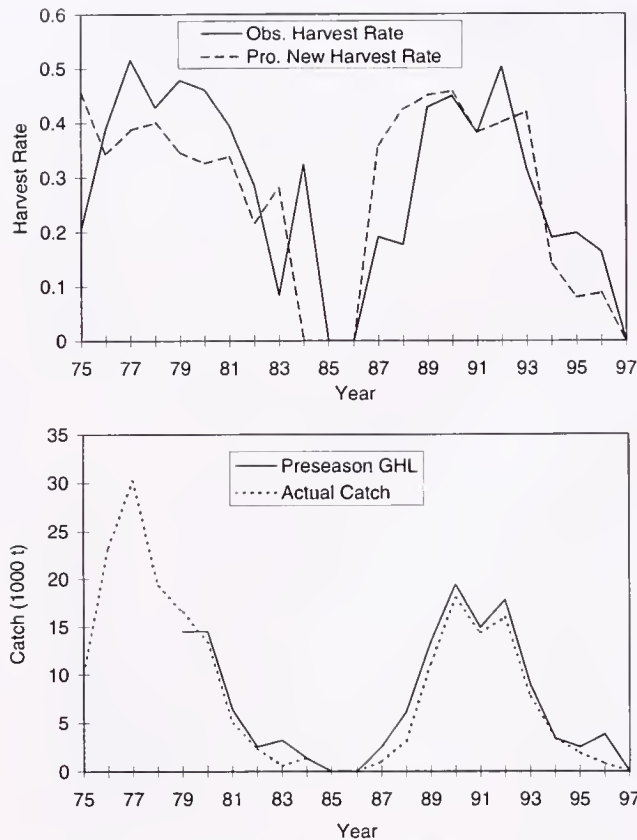


Figure 4. Contour plots of CV of yield, probability of fishery closure, and probability of total mature biomass below the overfished reference point by cycle amplitude and period length of the recruitment dynamics under the depensatory S-R curve for Bristol Bay Tanner crab. The plots are classified by the status quo harvest strategy and the proposed new strategy based on a 0, 10%, and 20% stair-step harvest rates of molting mature males.

and fishers called "fish tickets." At-sea observers monitor bycatch aboard vessels fishing for other species. Typically, total bycatch of Tanner crabs by ground fish and scallop fisheries is a small percentage of total crab abundance in the eastern Bering Sea. Whether a significant proportion of the Tanner crab population is adversely

impacted by dredges and trawls, but not caught and observed, remains a matter of speculation. Large numbers of Tanner crabs are handled and discarded during crab fisheries because of restrictions on size, sex, season, and target species. In our study of the red king crab fishery in Bristol Bay, increased handling mortality in



**Figure 5.** Comparison of the historical harvest rates (solid line) and the harvest rates derived from the proposed new harvest strategy (dotted line) as a proportion of total legal crab abundance (upper plot) for Bristol Bay Tanner crab and comparison of preseason guideline harvest level (GHL) and actual yield for eastern Bering Sea Tanner crab (lower plot) from 1975 to 1997.

our model resulted in lower optimal harvest rates and higher optimal threshold levels (Zheng et al. 1997). For the Bristol Bay Tanner crab fishery, we found that handling mortality had similar, but less pronounced, effects. Female Tanner crabs have much lower catchability during the fishery than legal-sized males. Thus, the impact of handling mortality on female Tanner crabs is smaller than on sublegal male Tanner crabs or female red king crabs. In our sensitivity analysis, we bracketed handling mortality rate at 0 and 50% to span low rates from a study that attempted to emulate the fishing process (MacIntosh et al. 1996) and high rates from a laboratory study (Carls and O'Clair 1995) that considered extremely cold air temperatures during winter fisheries. An extensive bibliography of capture and handling effects was compiled by Murphy and Kruse (1995) and reviewed in some detail by Zheng et al. (1997). Additional research is needed to assess handling mortality rates experienced by Tanner crabs accurately during commercial fisheries in the Bering Sea. Results from ongoing studies of cold wind chill effects (Kruse 1998) may significantly affect our estimates of handling mortality rate during winter fisheries. As this research is completed, the implications on crab fishery management need to be analyzed.

Recruitment cycles are the most striking feature of the population dynamics of Bristol Bay Tanner crab. Recruitment cycles are common to many fish and crab populations with typical periodicity of 10 to 26 years (Koslow 1989; Zheng and Kruse in press).

At the short end of periodicity, strong year classes occurred every four years from 1976 to 1988 for Pacific herring (*Clupea pallasii*) stocks in Prince William Sound and Sitka Sound, Alaska (Zheng 1996). The recruitment periodicity of snow crab (*Chionoecetes opilio*) in the northwest Gulf of Saint Lawrence is 8 years (Sainte-Marie et al. 1996); whereas, periodicity for Dungeness crab (*Cancer magister*) off Northern California is about 10 years (Higgins et al. 1997). At the long end of periodicity, some fish stock sizes had periodicity as long as 150 years (Koslow 1989). Although the time series is too short to estimate periodicity of the recruitment cycle by time series methods for Bristol Bay Tanner crab, it seems that the span between strong recruitment periods was about 13–14 years during the past 25 years. Because of the brevity of the available time series, the period length, and even the existence of repeatable cycles are not well established. The strong recruitment cycles may also be caused in part by age-class overlap in recruitment estimated by the size-based model. Nevertheless, despite uncertainty about the details, recruitment of Bristol Bay Tanner crab, as well as other Tanner crab stocks in Alaska, seems to be at least quasiperiodic. In our simulations for Bristol Bay Tanner crab, we set recruitment periodicity randomly from 10 to 18 years. We also examined the sensitivity of harvest strategies to recruitment cycles ranging from 4 to 30 years. We reached the same conclusion for Bristol Bay Tanner crab as Koslow (1989) did for fish stocks: no harvest strategies can protect a stock from collapse if the recruitment cycle is long. This is intuitive from crab biology. Because Tanner crabs mature at about age 6 and few live longer than 12 years (Donaldson et al. 1981), significant numbers of mature Tanner crabs cannot be "banked" for more than 6 years for future reproduction. However, reducing harvest rates and saving some mature crabs for future reproduction when recruitment is in the downward cycle will reduce the chance of prolonged stock collapse.

Cyclic or periodic recruitment of Tanner crab in Bristol Bay results in a weak density-dependent S-R relationship. So, recruitment strength depends partly on reproductive biomass but mostly on cycle phase. This weak density-dependent S-R relationship has important implications on Tanner crab harvest strategies. Our simulations showed that yield is maximized at legal harvest rates >60%. Because mature male Tanner crabs can annually mate with multiple females, and females can store sperm for future fertilization, harvesting large males does not have a proportional reduction on the reproductive stock. Because of this feature, our results are similar to Somerton's (1981) yield per recruit analysis, which showed that yield is maximized at legal harvest rates >70%. However, this "catching them before they die or are too old" strategy may have problems, such as causing insufficient males for mating, leading to recruitment overfishing, or depleting the reproductive stock to such a low level that depensation may occur. In the Gulf of Alaska, many depressed crab stocks have had extended periods of poor recruitment—red king stocks for >20 years and Tanner crab stocks for >10 years (Zheng and Kruse in press). The Alaska Board of Fisheries (a regulatory body making fisheries management policies for the State of Alaska) policy on king and Tanner crab management does not strive to maximize yield (ADF&G 1998). Instead, other objectives are considered, such as maintaining multiple size classes in the stock, maintaining sustained and reliable yields, and minimizing risks of irreversible adverse effects on reproductive potential. For Bristol Bay Tanner crab, the yield curve is relatively flat at high harvest rates, but much lower harvest rates can attain just slightly lower mean yields.

Compared to other alternative strategies we considered, our proposed new harvest strategy produces slightly lower mean yield, significantly lower variation in yield, it adjusts harvest rates according to stock productivity and creates more fishing opportunities while affording greater protection when the stock abundance is low. These features seem more consistent with the Board policy and provide a precautionary approach to fishery management.

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## APPENDIX. POPULATION MODELS

## Male Population Model

The abundances by carapace width (CW) and shell condition in any one year result from abundances the previous year minus catch and bycatch, handling, and natural mortality, plus recruitment and additions to or losses from each width class attributable to growth.

$$\begin{aligned} N_{m,l+1,t+1} &= \sum_{l'=l+1} \{P_{m,l',l+1}[(N_{m,l',t}e^{-M_m} - CN_{m,l',t}e^{(y-1)M_m})mm_{l',t} \\ &\quad + (O_{m,l',t}e^{-M_m} - CO_{m,l',t}e^{(y-1)M_m})mo_{l'}]\} + R_{m,l+1,t+1} \\ O_{m,l+1,t+1} &= (N_{m,l',t}e^{-M_m} - CN_{m,l',t}e^{(y-1)M_m})(1 - mm_{l',t}) \\ &\quad + (O_{m,l',t}e^{-M_m} - CO_{m,l',t}e^{(y-1)M_m})(1 - mo_{l'}) \quad (A1) \end{aligned}$$

where  $N_{m,l,t}$  and  $O_{m,l,t}$  are new- and old-shell male ( $m$ ) crab abundances in width class  $l$  and year  $t$ ,  $M_m$  is instantaneous natural mortality for male crabs,  $mm_{l,t}$  and  $mo_{l'}$  are molting probabilities for new-shell and old-shell crabs,  $R_{m,l,t}$  is recruitment,  $y$  is lag in years between abundance assessment and the fishery, and  $P_{m,l',t}$  is proportion of molting crabs growing from width  $l'$  to width  $l$  after one molt.  $CN_{m,l,t}$  and  $CO_{m,l,t}$  are combinations of bycatch mortality and catch (legal males) or bycatch and handling mortality (sublegal males) for new-shell and old-shell male crabs. Recruitment is defined as recruitment to the model and survey gear rather than recruitment to the fishery. We modeled male crabs  $\geq 93$  mm CW.  $P_{m,l',t}$  is computed as follows.

Mean growth increment per molt is assumed to be a linear function of pre-molt width.

$$G_l = a + b\iota, \quad (A2)$$

where  $a$  and  $b$  are constants. Growth increment per molt is assumed to follow a gamma distribution.

$$g(x|\alpha,\beta) = x^{\alpha-1}e^{-x/\beta}/(\beta^\alpha\Gamma(\alpha)), \quad (A3)$$

The expected proportion of molting individuals growing from width class  $l_1$  to width class  $l_2$  after one molt is equal to the sum of probabilities with width range  $[l_1, l_2]$  of the receiving width class  $l_2$  at the beginning of next year; that is,

$$P_{m,l_1,l_2} = \int_{l_1-1}^{l_2-1} g(x|\alpha,\beta)dx \quad (A4)$$

where  $\iota$  is the mid-width of width class  $l_1$ .

## Female Population Model

Major differences between the male and female population models are molting probability and fishing mortality. Upon maturity, female crabs stop molting and growing. Female crabs are not allowed to be retained in catch and are returned to the sea. The growth of immature females was modeled by functions similar to males [Eqs. (A2–4)]. Because females mature at smaller sizes than males, we modeled female crabs  $\geq 70$  mm CW.

New-shell females are either immature or primiparous (first-time spawners), and their abundance results from a combination of recruitment, growth, handling mortality, bycatch mortality, and natural mortality. Old-shell mature females are survivors of the mature females from the previous year:

$$\begin{aligned} N_{f,l+1,t+1} &= \sum_{l'=1}^{l+1} [P_{f,l',l+1}(N_{f,l',t}e^{-M_f} - CN_{f,l',t}e^{(y-1)M_f})(1 - m_{l',t})] \\ &\quad + R_{f,l+1,t+1} \end{aligned}$$

$$\begin{aligned} O_{f,l+1,t+1} &= (N_{f,l+1,t}e^{-M_f} - CN_{f,l+1,t}e^{(y-1)M_f})m_{l+1,t} + (O_{f,l+1,t}e^{-M_f} \\ &\quad - CO_{f,l+1,t}e^{(y-1)M_f}) \quad (A5) \end{aligned}$$

where  $M_f$  is instantaneous natural mortality for female ( $f$ ) crabs,  $m_{l,t}$  is maturity probability for width class  $l$  and year,  $R_{f,l,t}$  is recruitment,  $CN_{f,l,t}$  and  $CO_{f,l,t}$  are combinations of bycatch and handling mortality for new-shell and old-shell female crabs.

## Catch, Bycatch Mortality, Handling Mortality, and Recruitment

Effective reproductive (or spawning) biomass was described in detail by Zheng and Kruse (1998). Annual effective reproductive biomass,  $SP_r$ , was estimated as

$$SP_r = \sum_l [(N_{f,l,t}nr_t + O_{f,l,t}or_t)W_l], \quad \iota \geq 80 \text{ mm CW} \quad (A6)$$

where  $N_{f,l,t}$  and  $O_{f,l,t}$  is new-shell and old-shell female abundances in width class  $l$  and year  $t$ ,  $W_l$  is mean weight of female crabs in width class  $l$ ,  $\iota$  is the mid-width of width class  $l$ , and  $nr_t$  or  $or_t$  are the ratios of male reproductive potentials  $TNM_t$  and  $TOM_t$  to new-shell and old-shell mature female abundances  $TNF_t$  and  $TOF_t$  ( $\geq 80$  mm CW) in year  $t$  or year  $t-1$ , respectively; that is,

$$nr_t = TNM_t/TNF_t$$

$$or_t = \max[TOM_{t-1}/TOF_{t-1}, \quad TOM_t/TOF_t] \quad (A7)$$

Because female Tanner crabs can store sperm for subsequent fertilization, the ratios in year  $t-1$  are also used. If  $nr_t$  or  $or_t > 1$ , we set them equal to 1; that is, there are sufficient mature males to mate with all mature females, and so the number of reproductive females is equal to the number of mature females. The male reproductive potentials for new-shell and old-shell mature females were defined as

$$TNM_t = \sum_l [(0.3N_{m,l,t} + O_{m,l,t})mm_t], \quad 113 \text{ mm} \leq \iota \leq 137 \text{ mm CW}$$

$$TOM_t = \sum_l [(0.1N_{m,l,t} + O_{m,l,t})on_t], \quad \iota \geq 113 \text{ mm CW} \quad (A8)$$

where  $N_{m,l,t}$  and  $O_{m,l,t}$  are mature male crab abundances in width class  $l$  and year  $t$  with new-shell and old-shell conditions, respectively, and  $mm_t$  and  $on_t$  are the maximum average number of new-shell and old-shell females mated by a matable male (old-shell mature males: 100%; new-shell mature males: 30% for primiparous females or 10% for multiparous females) in year  $t$  and are computed as follows.

$$mm_t = i + j(TNF_t - a1)/(a2 - a1) \text{ and } i \leq mm_t \leq i + j$$

$$on_t = i + j(TOF_t - b1)/(b2 - b1) \text{ and } i \leq on_t \leq i + j \quad (A9)$$

where  $a1$  and  $a2$  are the lowest and highest estimated mature new-shell female abundances (1.2 and 78.5 millions of crabs) from 1975 to 1997,  $b1$  and  $b2$  are the lowest and highest estimated old-shell mature female abundance (5.7 and 60.8 millions of crabs) during the same period (Zheng and Kruse 1998), and  $i$  and  $i+j$  are the maximum average mates per matable male at the low and high female abundances. We assumed  $i$  to be 1 for both new-shell and old-shell females and  $j$  to be 4 for new-shell females and 2 for old-shell females (Zheng and Kruse 1998).

Annual effective reproductive biomass,  $SP_r$ , was used to determine whether the population is above threshold,  $T$ . If  $SP_r \leq T$ , then



no fishing is allowed; otherwise, the legal male harvest rate applied to exploitable legal crabs ( $\iota \geq 138$  mm CW),  $EL_p$  is

$$\begin{aligned} H_t &= H L_t / EL_t && \text{for 1st approach,} \\ H_t &= H && \text{for 2nd approach,} \\ H_t &= \min[E(NM_t / EL_t) MH] && \text{for 3rd approach,} \end{aligned} \quad (A10)$$

where  $H$  is legal harvest rate,  $L_t$  is total legal crab abundance,  $E$  is mature male harvest rate applied to  $NM_t$ , molting mature male abundance ( $\iota \geq 113$  mm CW, 100% of new-shell crabs and 15% of old-shell crabs), and  $MH$  is the maximum allowable legal male harvest rate (50%). Catch by width from the directed fishery is equal to the product of exploitable legal harvest rate, legal male abundance, and selectivity ( $sn_{m,t}$  for new-shell and  $so_{m,t}$  for old-shell crabs),

$$\begin{aligned} CN'_{m,t} &= H_t N_{m,t} sn_{m,t} && \iota \geq 138 \text{ mm CW,} \\ CO'_{m,t} &= H_t O_{m,t} so_{m,t} && \iota \geq 138 \text{ mm CW,} \end{aligned} \quad (A11)$$

and total yield,  $TC_t$ , is obtained by multiplying by the corresponding weight and summing over all widths

$$TC_t = \sum_l [(CN'_{m,t} + CO'_{m,t}) W_l], \quad \iota \geq 138 \text{ mm CW} \quad (A12)$$

Handling mortality is incorporated in the size-based model for female and sublegal male crabs. The number of deaths from handling mortality is a function of harvest rate, gear selectivity, and handling mortality rate ( $HM$ ). Handling mortality for sublegal males is

$$\begin{aligned} PN_{m,t} &= H_t N_{m,t} sn_{m,t} HM, && 93 \leq \iota \leq 137 \text{ mm CW} \\ PO_{m,t} &= H_t O_{m,t} so_{m,t} HM, && 93 \leq \iota \leq 137 \text{ mm CW} \end{aligned} \quad (A13)$$

and handling mortality for females is

$$\begin{aligned} PN_{f,t} &= H_t N_{f,t} sn_{f,t} HM, && \iota \geq 70 \text{ mm CW} \\ PO_{f,t} &= H_t O_{f,t} so_{f,t} HM, && \iota \geq 70 \text{ mm CW} \end{aligned} \quad (A14)$$

To account for handling mortality of female crabs, effective reproductive biomass is updated after fishing by modifying Equation (A6) to deduct handling mortality from female abundance.

$$SP_t = \sum_l \{[(N_{f,t} - PN_{f,t}) nr_t + (O_{f,t} - PO_{f,t}) or_t] W_l\}, \quad \iota \geq 80 \text{ mm CW} \quad (A15)$$

Catch from the directed pot fishery, male handling mortality, and male bycatch mortality are combined for  $CN_{m,t}$  and  $CO_{m,t}$  in Equation (A1), and female handling mortality and bycatch mortality are summed for  $CN_{f,t}$  and  $CO_{f,t}$  in Equation (A5).

Recruitment is separated into a time-dependent variable,  $R_t$ , and size-dependent variables,  $U_{m,t}$  and  $U_{f,t}$ , representing the proportion of male and female recruits belonging to each width class.

$$\begin{aligned} R_{m,t} &= 0.55 R_t U_{m,t} \\ R_{f,t} &= 0.45 R_t U_{f,t} \end{aligned} \quad (A16)$$

where  $U_{m,t}$  and  $U_{f,t}$  are described by gamma distributions similar to Equations (A3) and (A4) with two sets of parameters  $\alpha_r$  and  $\beta_r$ . Annual recruitment is described by a normal Ricker S-R model.

$$R_t = SP_{t-k} e^{\alpha - \beta SP_{t-k} + v_t} \quad (A17)$$

where  $k$  is recruitment age (8 years for males and 7 years for females),  $\alpha$  and  $\beta$  are constants, and  $v_t = \delta_t + A \sin(2\pi t/P)$  as environmental noise.  $\delta_t$  was assumed as a  $N(0, \sigma)$ . We also assumed a depensatory S-R model as follows.

$$\begin{aligned} R_t &= \kappa SP_{t-k}^\theta e^{v_t}, && \text{when } SP_{t-k} \leq 6395t \\ R_t &= SP_{t-k}^\theta e^{\alpha - \beta SP_{t-k} + v_t}, && \text{when } SP_{t-k} > 6395t \end{aligned} \quad (A18)$$

where  $\kappa$  and  $\theta$  are parameters.



## EVIDENCE OF DIARRHETIC SHELLFISH POISONING ALONG THE COAST OF MAINE

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**ABSTRACT** Following the occurrence of several unexplained incidents of shellfish-related gastroenteritis, field studies were conducted to determine if diarrhetic shellfish poisoning (DSP) toxins are present in Maine coastal waters. A protein phosphatase inhibition assay for DSP toxins revealed the presence of low levels of okadaic acid-like activity in blue mussels (*Mytilus edulis*) at sampling sites in the Frenchman Bay-Eastern Bay region. All other sites along the Maine coast were negative. Phytoplankton populations from this area were dominated by *Dinophysis norvegica*, a known toxic species. Two additional known toxic species of *Dinophysis* were also found: *Dinophysis acuminata* and *D. rotunda*. However, all plankton samples were negative for okadaic acid-like activity. Examination of the epiphytic communities from areas where mussels showed okadaic acid-like activity revealed the presence of the toxic dinoflagellate *Prorocentrum lima* in association with the brown alga, *Ectocarpus* sp. Epiphytic samples rich in *P. lima* were active in the phosphatase inhibition assay. Subsequent analysis of these samples using LC-MS/MS identified the presence of dinophysin toxin-1 (DTX-1). Empty *P. lima* thecae identified in the digestive tract of mussels from these areas indicate that *P. lima* is consumed by mussels. This is the first confirmation of *P. lima* in northern United States coastal waters and identifies DSP as a potential public health issue.

### INTRODUCTION

Diarrhetic Shellfish Poisoning (DSP) is one of several classes of seafood poisonings caused by naturally occurring marine microalgae, primarily dinoflagellates. The state of Maine has annual blooms of the paralytic shellfish poisoning (PSP) producing dinoflagellate, *Alexandrium tumarensis*, and has a comprehensive phytoplankton monitoring and shellfish testing program for PSP which successfully protects the public health (Shumway et al. 1988). Nonetheless, the occasional incidence of unexplained shellfish associated gastroenteritis, as well as the rejection of a single lot of shellfish tested for DSP for import to the Netherlands, has raised the question of whether DSP is also an issue of public health significance for Maine.

DSP causes severe cramping, nausea, vomiting and diarrhea. The syndrome is distinguishable from microbial food poisoning in its rapid onset (as early as 30 min) and generally lasts 2–3 days. The toxins responsible for DSP are a suite of polyethers containing transfused or cyclic ether rings (Wright and Cembella 1998), including okadaic acid and the dinophysin toxins (DTX1–4). The first incidence of human shellfish-related illness identified as DSP occurred by Japan in the late 1970's (Yasumoto et al. 1978), where the dinoflagellate *Dinophysis fortii* was identified as the causative organism, and the toxin identified as the causative agent was termed dinophysin toxin (DTX-1). Retrospective analysis of similar disease outbreaks in the Netherlands (Kat 1985) and Scandi-

navia (Kumagai et al. 1986) confirmed that these were also associated with *Dinophysis*. DSP is now a frequently encountered problem in Europe and Japan, where it significantly impacts extensive aquaculture industries. In 1990, the first confirmed outbreak of DSP in North America occurred in Nova Scotia, Canada (Quilliam et al. 1990). DSP outbreaks have also been reported from South America, South Africa, and Australia.

Two species of *Dinophysis* present in Maine coastal waters, *D. acuminata* and *D. norvegica*, are found seasonally in high numbers. Phytoplankton data collected by the Maine volunteer phytoplankton-monitoring network in 1998 found peaks in the abundance of *Dinophysis* spp. in June and September (L. Bean pers. comm.). An anecdotal report from the PSP testing station in La Moine, ME (J. McGowan, pers. comm.) indicated that *Dinophysis* was present in Salsbury Cove, ME in July 1998 at sufficient concentrations to discolor the water, prompting a "red tide" report from a local citizen. However, causative organism responsible for the DSP outbreak in Canada was not *Dinophysis* but an unrelated, benthic dinoflagellate, *Prorocentrum lima* (Marr et al. 1992).

In this study, we surveyed the 31 sites along the Maine coast for okadaic acid-like activity in *Mytilus* using a rapid protein phosphatase inhibition assay for DSP toxins. We next monitored for DSP producing dinoflagellates of the genus *Dinophysis* and *Prorocentrum* and tested for toxin production. DSP toxicity was found only in association with *P. lima* containing samples, which was identified as DTX-1 by LC-MS/MS.

## MATERIALS AND METHODS

### Sample Collection

Blue mussels (*Mytilus edulis*) were collected during low tide at 31 sites along the Maine coast (Fig. 1). The mussels were transported to the laboratory and the digestive gland dissected. Approximately 20 to 50 g of digestive gland were harvested from each location for toxin analysis. From select locations, the digestive gland from three individuals was preserved in 2% glutaraldehyde for gut content analysis.

Discrete water (500 ml) and net tow (35  $\mu\text{m}$  mesh, 3 min) samples were collected at both low tide and high tide at each of the sampling sites. Discrete water samples were concentrated using a 8  $\mu\text{m}$  filter and preserved in 1% glutaraldehyde for cell counts. From each net tow, a 25 mL aliquot was collected and preserved in 1% glutaraldehyde for species identification. The remainder of the plankton tow was concentrated by centrifugation for toxin analysis. During sample periods of low tide, the dominant macroalgae were collected at each site using the methods of Morton and Faust (1997).

Phytoplankton populations of the discrete water samples and epiphytic dinoflagellate populations were estimated from counts using a 0.1 mL Palmer-Maloney Counting chamber. Each sample was counted at least four times. Because of the large errors associated with macroalgal surface area determination, dinoflagellate

abundance in the epiphytic samples was expressed as cells per gram wet weight of host macrophyte. For positive identification of dinoflagellates, cells were examined using a Zeiss Axiovert S100 inverted microscope fitted with a Spot digital camera (Diagnostic Instruments, Inc.).

### Toxin Analysis

#### Mussel and Algae Extraction

For each mussel sample, 1 g of digestive gland was homogenized in 4 mL 80% methanol for 2 min using a Polytron. The resulting homogenate was centrifuged and the supernatant was washed twice with 4 ml hexane. A 2 mL aliquot of the methanol extract was retained and analyzed directly or stored frozen at  $-20^\circ\text{C}$ . Algal samples were size fractionated by filtration through a 100  $\mu\text{m}$  and then a 20  $\mu\text{m}$  screens. The 20–100  $\mu\text{m}$  fraction collected and concentrated by centrifugation. The cell pellet was extracted with 100% methanol. All extracts were stored at  $-20^\circ\text{C}$  until analyzed.

#### Colorimetric Phosphatase Inhibition Assay

The protein phosphatase inhibition assay was carried out in a 96 well format using the procedure of Tubaro et al. (1996). The assay tests the ability of okadaic acid (OA) standard or unknown sample to inhibit activity of purified protein phosphatase 2A



Figure 1. Sample sites along the coast of Maine.

against a colorimetric substrate, p-nitrophenyl phosphate (pNPP). All samples were diluted in reaction buffer (40 mM Tris HCl pH 8.4, 34 mM MgCl<sub>2</sub>, 4 mM EDTA, 4 mM DTT) at least 4-fold to reduce the methanol concentration to  $\leq 5\%$  in the assay in order to eliminate inhibition of the enzyme by methanol. For the assay, 50  $\mu$ l samples and standards (0.1–1000 nM OA; LC Laboratories, Cambridge, MA), 100  $\mu$ l reaction buffer and 50  $\mu$ l purified PP2A enzyme (final concentration 0.1 U/mL; Upstate Biotechnology, Lake Placid, NY) were added to duplicate wells of a 96 well plate (Costar, Corning, NY). To start the reaction, 50  $\mu$ l pNPP (50 mM; Sigma, St. Louis, MO) was then added to each well and the reaction allowed to proceed for 1 h at room temperature. Protein phosphatase activity was determined by color development (405 nm) in the wells using a plate reader (Titertek Multiscan Plus, Huttsville, AL). OA-like activity in the sample was quantified relative to the standard curve. The detection limit of the colorimetric phosphatase assay was approximately  $1 \times 10^{-10}$  M okadaic acid equivalents.

#### LC-MS/MS

Samples that displayed protein phosphatase inhibition activity were analyzed by LC-MS/MS using a Finnigan LCQ mass spectrometer. The methanolic extracts were injected on a C18 column (Zorbax 2.1  $\times$  150 mm) and eluted with a gradient of 50–95% methanol/water containing 0.1% TFA at a flow rate of 0.2 mL/min.

A splitter device was used to direct 10% of the column effluent to the electrospray source. The mass spectrometer was operated in positive ion mode. Analysis of the toxins was achieved by trapping  $[M + Na]^+$  species for each toxin and conducting selected ion monitoring experiments for distinctive fragment ions from the collisionally activated dissociation of the trapped parent ions. Chromatographic traces were acquired for the detection of the fragment ions as well as undissociated parent ions. Limits of detection for LC-MS/MS of okadaic acid and DTX-1 were approximately  $1 \times 10^{-8}$  M.

#### RESULTS

From the 31 sites where *Mytilus edulis* was collected, 4 sites from Eastern Bay and Frenchman Bay displayed protein phosphatase inhibition activity in the colorimetric assay for DSP toxins (Fig. 2). In all cases, the activity detected (6–20 ng/g) was close to the limit of detection of the assay (0.1 nM in-well or 1.6 ng/g hepatopancreas). All other sites from Maine were consistently negative. Subsequent analysis of the positive samples by LC-MS/MS did not indicate the presence of okadaic acid or DTX-1. Given the low concentrations determined in the phosphatase inhibition assay, the absence of these toxins by LC-MS/MS may reflect differences in the respective detection limits of these methods of detection. Alternatively, it may indicate that the inhibitory activity



Figure 2. Sample sites from the Frenchman Bay-Eastern Bay sites where *Mytilus edulis* was found to have okadaic acid-like activity.

in these samples was due in part or entirely to the presence of DSP toxin congeners other than OA or DTX-1.

*Dinophysis* species, primarily dominated by *D. norvegica*, were observed throughout the sampling period from the Rockland, ME to Bar Harbor, ME areas. Two additional species, *D. acuminata* and *D. rotundia*, were also observed in low numbers. Highest concentrations of *Dinophysis norvegica* were routinely observed at Salisbury Cove, ME. All plankton tows that were dominated by *Dinophysis*, were negative for phosphatase inhibition activity and displayed no OA or DTX-1 when analyzed by the LC-MS/MS.

Sites where *Mytilus* tested positive for okadaic acid-like activity were therefore further studied in order to identify a causative organism. At all sites where *Mytilus* tested positive, from the Lamoine-Bar Harbor area and the Prospect Harbor area, the dominant macrophyte found was the brown alga, *Ectocarpus* sp. At these locations *Ectocarpus* had an epiphytic community that included the toxic dinoflagellate, *Prorocentrum lima* (Fig. 3). This dinoflagellate was not found at any other locations. A large sample (535 g wet weight) of the epiphytic microalgae associated with *Ectocarpus* was collected from the Lamoine Airport site, concentrated, and extracted in methanol. Density of *P. lima* from this sample was approximately 200 cells/g wet weight. This extract displayed protein phosphatase inhibitory activity using the colorimetric assay. Subsequent analysis by LC-MS/MS showed the production of DTX-1 by the wild population of *P. lima*; however no OA was detected (Fig. 4).

Microscopic analysis of the digestive gland from *Mytilus* collected from the Lamoine Airport site and Bar Harbor site showed empty thecae, consistent with the morphology of *P. lima* (Fig. 5). Intact cells of *P. lima* were not observed within the digestive gland of *Mytilus*.

#### DISCUSSION

Analysis of *Mytilus* digestive gland from 31 sites along the Maine coast resulted in one region, Eastern Bay and Frenchman Bay, located east of Mount Desert Island, that consistently showed low levels of okadaic acid-like protein phosphatase inhibition activity, indicative of DSP toxins. The range of okadaic acid-like activity found (6.4–20 ng/g) was at least 100-fold below the European and Canadian regulatory limits of 2 µg/g digestive gland. This indicates that although DSP-like toxins were present in the Frenchman Bay-Eastern Bay region, they were not present at levels that represent a significant public health issue. No DSP-like toxin activity was found in *Mytilus* along any of Maine's peninsulas, which are commonly sampled for PSP.

Subsequent LC-MS/MS analysis of *Mytilus* samples that tested positive for OA-like activity did not identify the presence of OA or DTX-1, the two most common toxins associated with DSP. However, the toxin concentrations found in the colorimetric assay were at or below the detection limits of the LC-MS/MS method for these two congeners. Samples were not analyzed for other DSP toxin congeners due to the lack of standards. Thus, it remains unknown if the phosphatase inhibitory activity present may be due in part to different DTX metabolites. For example, DTX-3 has been found to be a direct metabolite of DTX-1 in scallops (*Patinopecten yessoensis*) (Suzuki et al. 1999). This metabolite has also been found in Irish and Spanish mussels (Marr et al. 1992; Fernandez et al. 1996). Thus, an okadaic acid metabolite such as DTX-3 could also be present, leading more phosphatase inhibition than can be attributed to OA or DTX-1.



Figure 3. Light micrograph of *P. lima* collected from *Ectocarpus* sp.

During the field-sampling period, several plankton tows displayed large populations of *Dinophysis*, primarily *D. norvegica*. Other species of known toxic *Dinophysis*, *D. rotunda* and *D. acuminata*, were also observed but in low abundance. The Gulf of Maine Phytoplankton Monitoring Network has found *Dinophysis* species along the entire coast of Maine between June through September (W. Norden, pers comm). However, in the present study, *Dinophysis* rich samples were consistently negative in both the protein phosphatase inhibition assay as well as LC-MS/MS. In some European and Canadian outbreaks of DSP, *Dinophysis* abundance has not always been correlated to these events.

The locations where *Mytilus* tested positive for OA-like activity correlated with locations where the epiphytic dinoflagellate, *P. lima*, was found. This is the first study to show this toxic dinoflagellate in northern United States coastal waters. Outbreaks of DSP from the Atlantic coast of Canada have been attributed to *P. lima*, rather than *Dinophysis* (Marr et al. 1992; Lawrence et al. 1998). *P. lima* is one of the most wide spread toxic dinoflagellates, being found in both tropical areas as well as cold temperate areas of the Atlantic, Pacific, and Indian Oceans. However, its presence has not been documented in the intervening latitudes along the eastern seaboard of the United States. The cell densities found for *P. lima* in this current study are lower than that generally found in tropical areas (Bomber 1985; Carlson and Tindall 1985, Morton and Faust 1997).

Collections of microalgae associated with the filamentous macrophyte, *Ectocarpus*, rich with *P. lima*, were found to possess protein phosphatase inhibitory activity. Extracts of these field collected *P. lima* contained DTX-1, but OA was not detected. DTX-1 was also found to be the primary toxin produced by *P. lima* in Nova Scotia, Canada, with approximately 10-fold lower amounts of OA than DTX-1 (Marr et al. 1992). Environmental conditions such as temperature, light intensity, salinity, and N:P ratio have been shown to have a direct effect on the growth rates and toxin

OA092999a-002/Extract

DTX-002 - 9/29/99 - 11:18 AM  
PL2

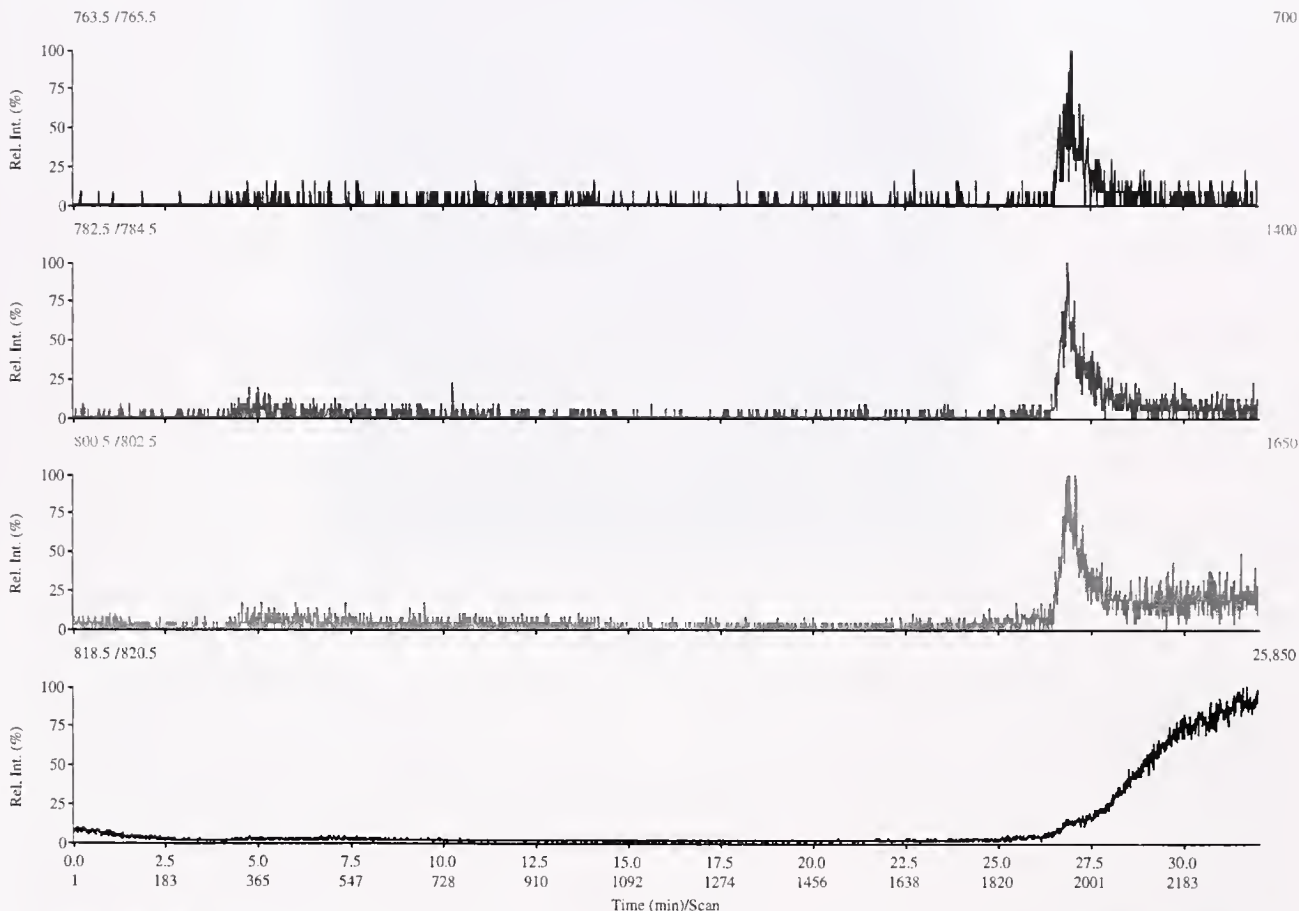


Figure 4. LC-MS/MS chromatograph of dinophysis toxin-1 from *P. lima*. The traces reflect selected ion monitoring of distinctive fragments ions at M/Z of 765, 783, and 801 as well as the parent ion of 819.

ratios of *P. lima* and other closely related toxic species of *P. lima* (Morton and Norris 1990, McLachlan et al. 1994, Morton et al. 1994). Clonal isolates of *P. lima* from field collections are currently being grown in large scale laboratory cultures for further study on toxin production to identify which DSP-toxins are produced.

The results of this study identify DSP-like activity in Maine coastal waters. *P. lima* and DSP toxins were found only in the Frenchman Bay-Eastern Bay region. In the current study, levels of OA-like activity found in mussels were not at levels that present a public health issue. However, the presence of DSP as a potential problem in Maine coastal waters must be considered in future management decisions regarding shellfish aquaculture. Maine's shellfish industry is based upon a publicly owned resource with limited aquaculture lease sites for the bottom culture of mussels and scallops. DSP became a problem in Canadian maritime waters when the practice of raft culture was introduced, creating a suitable substrate for the growth of *Pilayella littoralis*. This filamentous brown macrophyte species commonly fouls aquaculture lines in that region and serves as a host for the epiphytic dinoflagellate, *P. lima* (Lawrence et al. 1998). Thus, as aquaculture activity in Maine expands, the addition of DSP to the state shellfish-monitoring

program may become important for protection of public health. The sampling sites where both *P. lima* and OA-like activity in *Mytilus* were protected embayments with muddy to gravel substrates which supported the growth of *Ectocarpus*. Other sites along the Maine coast with a similar substrate may also support the growth of *Ectocarpus* or other filamentous brown macrophytes, which may serve as a host for *P. lima*. Clearly, further ecological studies are required to determine the extent of *P. lima* along the coast of Maine. These sites differ substantially in character and location from the primary monitoring sites used by the State of Maine's PSP monitoring program. This, in addition to the benthic behavior of the causative organism, *P. lima*, indicates that a different sampling strategy than that currently employed by the state for the long established PSP monitoring program maybe required to monitor for occurrences of DSP.

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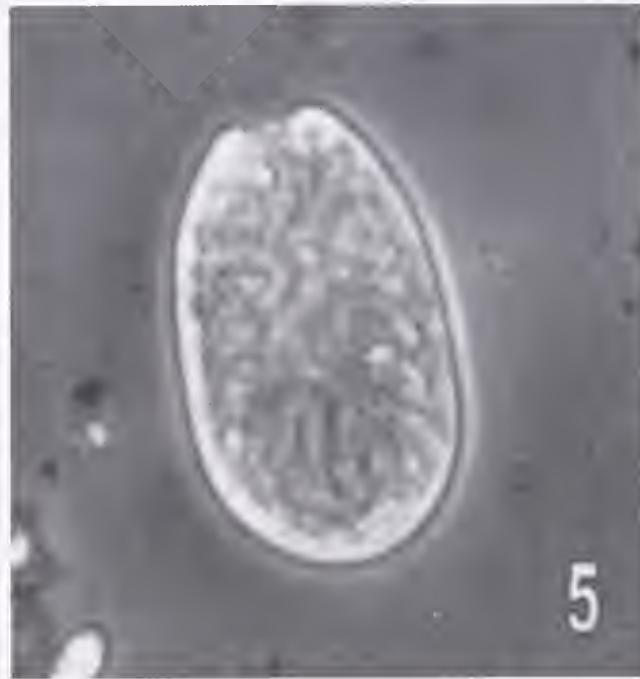


Figure 5. Empty theca of *P. lima* from the digestive gland of *Mytilus edulis*.

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## ABSTRACTS OF TECHNICAL PAPERS

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**THE INFLUENCE OF SPATIAL HETEROGENEITY ON THE FORAGING OF LITTORINES.** T. C. Addy and L. E. Johnson, GIROQ, Université Laval, Local 2056, Poste 2266, Pavillon, Vachon, Ste-Foy, Québec G1K 7P4 Canada.

The abundance and distribution of littorines often depends on the availability of topographic irregularities in the rock surface (e.g., holes and crevices). Presumably, the littorines use these microhabitats as shelters during periods of unfavorable conditions (e.g., wave action, desiccation) which restricts their foraging activities to areas near shelters. This study examined several environmental factors that might influence the use of shelters by the littorine *Littorina saxatilis* along a gradient of wave exposure at Pointe Métis, Québec (Canada). Specifically, we measured the percentage of animals inside and outside of both natural and artificial irregularities (crevices and drilled holes, respectively) under a variety of climatic conditions experienced during low tide. The number of littorines outside of shelters was strongly correlated with wave exposure with only 5–10% outside of crevices in the most exposed locations compared with >70% outside at more protected locations. Littorines were found outside of shelters mostly during nighttime low tides when the rocks remained wet for longer periods. During the daytime, higher proportions were found outside during sunny conditions (the rock surface remained wet longer than under cloudy conditions, apparently due to the higher winds associated with cloudy days). Surprisingly, rainfall during the low tide had little influence suggesting that osmotic problems might negate any advantages of wetting the rock surface. Parallel laboratory experiments generally support these field patterns and together suggest that littorine foraging during low tide is largely determined by the climatic conditions.

**GENETIC ANALYSIS OF BRAZILIAN POPULATIONS OF NODILITTORINA LINEOLATA (PROSOBRANCHIA: GASTROPODA).** S. C. S. Andrade, V. N. Solferini, Depto. de Genética e Evolução, IB, UNICAMP and C. A. Magalhães, Depto. de Zoologia, IB, UNICAMP, Campinas, SP, Brazil.

There are controversies about the number of species of the "Ziczac Complex" that inhabits Brazilian coast. Isozymic analyses (18 loci) were carried out on 13 different populations of those periwinkles to attempt to clarify the taxonomic question, and also to verify if there is correlation between their spatial distribution and variability patterns. *Nodilittorina* were sampled on one beach on the South coast (SC), 7 beaches on the Southeast coast (SP, RJ), 2 on the Northeast (PE) and 3 on the North coast of Brazil, covering a distance of about 6000 km. Genetic analysis showed that there is only one species in our samples, identified as *Nodilittorina lineolata* (D'Orbigny, 1840). The samples presented high variability ( $H_m = 0.17$ ) and exhibited low genetic differentiation among them ( $F_{st} = 0.065$ ). This is probably due to the fact that this species has long-living pelagic larvae. All samples presented

heterozygote deficiency on at least 2 loci. These results suggest that these samples of Brazilian *N. lineolata* may be subject to strong selective pressures. The heterozygote deficiency and the high genetic variability could be related to high microhabitat heterogeneity on the rocky shore, but new experiments are necessary to test this hypothesis.

**CONTRASTING LIFE HISTORIES AND DEMOGRAPHIES OF EIGHT SPECIES OF LITTORINES AT NINGALOO REEF, WESTERN AUSTRALIA.** Robert Black and Michael S. Johnson, Department of Zoology, University of Western Australia, Nedlands, Western Australia, Australia 6907.

This study considers four species of littorines from rocky shores, *Nodilittorina australis*, *N. millegrana*, *N. trochoides*, and *Littoraria undulata*, and four species of littorines from mangroves, *Littoraria cingulata*, *L. filosa*, *L. scabra*, and *L. sulcalosa* over the period from July 1989 to June 1999. We conducted periodical censuses of the same replicate areas at four rocky shore sites spread over 50 km of shoreline, and at the same replicate mangrove trees at two sites at the opposite ends of a bay, about 2 km apart. This design allowed us to partition the variability in abundance of each species of snails into components associated with the sites, areas or trees within sites, years, areas  $\times$  years, and the residual. The littorines of the rocky shores had greatest variability associated with differences among the four sites for the total populations and among sites and year  $\times$  site for recruits, while the littorines of the mangroves showed greatest variability associated with years, and sites  $\times$  years.

We also conducted shorter term mark and recapture studies to provide direct evidence about growth and survivorship of these snails. All three species of littorines from mangroves with sufficient recaptures showed rapid growth, reaching half their maximum size in 0.94, 0.35, and 0.75 years for *L. cingulata*, *L. filosa*, and *L. scabra*, respectively, and attaining maximum lengths of 22 to 27 mm. The littorines of the rocky shores all had slower growth rates than the snails from the mangroves, and were all smaller in maximum size, from 10 to 17 mm. With the exception of *N. millegrana*, which took only 0.50 years to reach half maximum size, the snails on the rocky shores took much longer to reach half their maximal lengths than the littorines in the mangroves (1.23, 2.87, and 1.28 years for *N. australis*, *N. trochoides*, and *L. undulata*, respectively). Patterns in survival parallel the estimates of times to reach half maximal size. We never recaptured *N. millegrana* over intervals as long as 1 year, but some *N. trochoides* marked in 1988 were still alive in 1995. Between these extremes, the littorines of the mangroves seemed shorter lived than the rest of the littorines from the rocky shores.

This long-term study of eight similar species in a single geographic area highlights the variability in life histories and demography of littorines.

**REGULATION OF WAVE-SHELTERED *LITTORINA SITKANA* POPULATIONS BY PILE PERCH, *RHACOCHILUS VACCA*.** Elizabeth G. Boulding, Department of Zoology, University of Guelph, Guelph, ON, N1G 2W1, Canada.

The pile perch, *Rhacochilus vacca* (Embiotocidae) is abundant on the Pacific Coast of North America and is known to crush littorinid gastropods and other hard shelled prey with its heavy pharyngeal teeth. I investigated whether this predator had the potential to regulate the population density of *Littorina sitkana*, a direct-developing littorinid, on wave-sheltered shores. Laboratory feeding experiments at Bamfield Marine Station, B.C., Canada showed that that pile perch were powerful and efficient predators on these snails. The fish required an average of only 19.1 (s.e. = 5.61, N = 20) seconds to crush and swallow a large snail and their laboratory consumption rates averaged 33.29 (s.e. = 6.27, N = 4) large snails per day. Even small adult fish (fork length: 21 cm) could crush the largest *L. sitkana* available (>11 mm shell width). Indeed, some fish showed a significant preference for large snails (shell width >6.3 mm) over small snails (3.35 mm < shell width <4.0 mm). Our observations with SCUBA during daytime high tides showed that the density of pile perch foraging in the intertidal averaged only 0.0592 (s.e. = 0.010, N = 20) fish per square metre (estimated fork lengths 5–40 cm). However, the intertidal distribution of fish which were actually consuming prey was highly aggregated. The fish swam parallel to shore and quickly located and consumed any high density patches of *L. sitkana* but were slower to locate and consume low density patches. This behaviour resulted in strongly density-dependent predation on the snails we deployed. I conclude that this predatory fish has the capability to regulate *L. sitkana* populations at least on this wave-sheltered shore.

**AN INTRODUCTION TO JAMAICAN LITTORINIDS AND LITTORINID HABITATS.** Joseph C. Britton, Department of Biology, Box 298930, Texas Christian University, Fort Worth, Texas 76129, U.S.A.

Jamaican rocky shores include natural, mobile, boulder, and cobble beaches, man-made boulder breakwaters, natural vertical sea cliffs, man-made concrete walls or ramps, and, especially, uplifted limestone platforms (microkarsted ironshore), all of which may harbor one to several species of littorinids, including *Cenchritis muricatus*, *Tectarius antonii*, and at least five species of *Nodilittorina*. Littorinids dominate the upper rocky shore and share tidepools and the midlittoral with at least four species of nerites, two species of planaxids, two polyplacophorans, and at least one patellid, fissurellid, potamid, muricid and pulmonate. Unlike most temperate rocky shores, those of Jamaica, and the tropical Caribbean in general, are noticeably deficient of chthamalid and balanoid barnacles.

Jamaican mangroves are comprised of four distinctively zoned trees, the red mangrove, *Rhizophora mangle*, with prop roots usually rising from the sea, the black mangrove, *Avicennia germinans*,

with pneumatophores which are frequently inundated by tides, the white mangrove, *Laguncularia racemosa*, occasionally touched by tidal water and the buttonwood, *Conocarpus erectus*, occupying the highest position on the mangrove shore. There is only one species of littorinid, *Littoraria angulifera*, commonly found in the mangal, usually upon *Rhizophora* branches. The mangrove floor, however, is occupied by several species of ellobiids and melampids.

**ESTIMATING GENETIC VARIANCES FROM WILD DATA IN *LITTORINA SAXATILIS*.** M. Carballo, E. Rolán-Alvarez, Facultad de Ciencias, Universidad de Vigo, 36200 Vigo, Spain, and C. García, Facultad de Biología, Universidad de Santiago de Compostela, 15706, Santiago de Compostela, Spain.

On exposed Galician rocky shores, two ecotypes of *Littorina saxatilis* (RB versus SU) can be found at different shore levels. Parental ecotypes and intermediate individuals can be found in sympatry at a narrow mid-shore zone. These ecotypes differ in many morphological traits associated to different habitats, but the genetic basis of those differences are unknown. We assessed the inheritance of the morphological variability in these ecotypes by taking pregnant females from wild populations, and using offspring-mother regression and correlation between full-sibs methods. We sampled five levels of the shore in two localities (20 RB females from upper shore, 20 RB from upper mid-shore, 20 RB, 20 SU and 11–13 intermediate forms from mid-shore, 20 SU from lower mid-shore and 20 SU from lower shore). We took 11 shell measurements in every female and in three embryos per female.

Genetic variances could be evidenced for some traits and samples. Genetic variance estimates were usually similar between full-sibs correlation and offspring-mother regression methods. Furthermore, the coefficients of genetic variance were similar, but somewhat smaller (range = 0.5–7%), to those published in the literature. However, correlation between full-sibs rendered more frequent significant estimates than offspring-mother regression, and there was a pattern which related the level of genetic variance with shore level. These results suggest possible bias in the estimates. The present methods may be a valuable tool for estimating genetic components from wild data, but some caution is needed during the interpretation of the results.

**TRACING DIETS OF THREE SPECIES OF *LITTORARIA* USING STABLE ISOTOPES.** J. T. Christensen, P. D. Jensen, Dept. of Marine Ecology, Institute of Biological Sciences, University of Aarhus, Finlandsgade 14, DK-8200 Aarhus N, Denmark, and P.-G. Sauriau, P. Richard, Centre de Recherche en Ecologie Marine et Aquaculture de L'Houmeau, CREMA (CNRS-IFREMER UMR 10), BP 5, F-17137 L'Houmeau, France.

Analyses of carbon and nitrogen stable isotopes were used in an attempt to trace food sources of three *Littoraria* species in *Rhizophora apiculata* mangrove in Thailand. When occurring together

*Littoraria scabra*, *L. intermedia*, and *L. pallescens* exhibit a vertical zonation with *L. scabra* on prop roots nearest the water, *L. intermedia* on roots and branches, and *L. pallescens* primarily on mangrove leaves. Ranges do, however, overlap and the animals move with the tide. *Rhizophora* leaves, scrapings from both leaf and prop-root surfaces and local particulate organic matter (POM) were well separated on the basis of their  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values. In contrast, tissue from the three species of *Littoraria* showed considerable overlap and scatter in isotope ratio values suggesting that they are opportunistic feeders sharing similar food resources. The wide range of  $\delta^{13}\text{C}$  values ( $-17.3\text{‰}$  to  $-26.3\text{‰}$ ) suggest carbon assimilation from multiple sources (epiphytes from leaves and prop roots, suspended POM and *Rhizophora* detritus). *L. intermedia* and *L. pallescens*, the smallest species, had identical mean  $\delta^{13}\text{C}$  values while *L. scabra* was significantly more  $^{13}\text{C}$  depleted. A diet of microalgae and cork cells from prop roots could explain this pattern with *L. scabra*, being larger, consuming relatively more cork cells. However, all three species were on average more  $^{15}\text{N}$  depleted than these food sources. Slightly more than one third of the *L. scabra* and *L. intermedia* individuals had  $\delta^{15}\text{N}$  values consistent with such a diet while the remaining and all *L. pallescens* were too depleted. *L. pallescens* had a significantly lower mean  $\delta^{15}\text{N}$  value than the other two species and must derive a significant amount of its food from a strongly  $^{15}\text{N}$  depleted source. Such a source was present in scrapings from leaf surfaces ( $\delta^{15}\text{N} = 0.3 \pm 0.05\text{‰}$ ,  $n = 2$ ) and could represent  $\text{N}_2$ -fixing microorganisms. Due to the wide scatter of *Littoraria*  $\delta^{15}\text{N}$  values from 6.1 down to  $-7\text{‰}$ , it is hypothesised that all three species assimilate microorganisms such as cyanobacteria utilizing ammonia as substrate, bacteria involved in denitrification and fungi. These findings were compared with microscopic analysis of gut contents.

**AN ASSESSMENT OF THE POTENTIAL FOR THE SUSTAINABLE DEVELOPMENT OF THE EDIBLE PERIWINKLE (*LITTORINA LITTOREA* L.) INDUSTRY IN IRELAND.** S. Coughlan, J. Mercer, Shellfish Research Laboratory, Carna, Co. Galway, Ireland, O. McClean, N. Connolly, Coastal Resources Centre, National University of Ireland, Cork, Ireland, and G. Burnell, Dept. of Zoology, National University of Ireland, Cork, Ireland.

A study covering biological and socio-economic aspects of the edible periwinkle industry in Ireland was undertaken as a reaction to widespread reports of a decline in periwinkles of marketable size. This research includes an investigation of the distribution, biomass and age/size frequency of periwinkles. Sites selected were those thought to hold exploitable quantities of winkles. Samples were taken from each site and retained for measurement to determine biomass and to investigate geographic variation in morphology. It is hoped this information together with results of extensive interviews with those involved in the industry, will be used to formulate a management strategy for the species. Consultations

with wholesalers and harvesters suggest that a summer closed season would be the most widely supported of the proposed regulations. So far, seventy five sites have been visited; it appears that greater densities occur on the south coast on moderately exposed shores. In line with reports by other authors, morphometric ratios (i.e., ratios between shell height, aperture height, shell width and aperture width) appear to be influenced by physical factors such as exposure.

**BETWEEN-MORPHS COMPARISONS OF ADAPTIVE SURFACES IN A HYBRID ZONE OF *LITTORINA SAXATILIS*.** Raquel Cruz and Carlos García, Departamento de Biología Fundamental, Facultad de Biología, Universidade de Santiago de Compostela. 15706 A Coruña, Galicia, Spain.

Littorinid snails having direct development, such as *Littorina saxatilis*, can be very useful in experimental studies of evolutionary problems, due to their very limited dispersal ability, their relatively high population densities, and to the fact that individuals are easy to find in the field. This species shows also many polymorphisms of different kinds, which makes it an interesting subject to study the role of genetic variability in natural populations.

We studied one of these polymorphic populations found on the exposed rocky shores of Galicia, NW Spain, where there is one morph adapted to the upper shore, and another adapted to the more wave-exposed lower shore. We sampled 1158 adult females on all shore levels, and counted the number of normal embryos in their embryo pouches, as an estimate of their fecundity, along with measurements of 26 morphological characters and nine environmental variables. We calculated two discriminant functions to separate the two pure morphs. The first was based on the morphological measurements, and the second, on the environmental ones. Then we used the fecundity measurements to adjust a fitness surface to the bidimensional space defined by these two discriminant functions, to study the possible role of natural selection (acting through the variation in fecundity) in the differentiation observed between the pure morphs, and that between the pure morphs and their hybrids. We found some evidence for a fecundity depression in a section of the hybrid's surface, situated between the areas of the bidimensional space corresponding to each pure morph. The depression was not detected when the average fecundity of all hybrids was compared with those of the pure morphs, which would indicate that this average, as it is usually calculated, is not enough to describe the adaptive situation in a hybrid zone, because it does not take into account the position of the sampled individuals along the axis of differentiation between the pure morphs, and as our study shows, this axis does not need to coincide with the mere physical distance on the shore. The study of the interplay between a fecundity depression of hybrids, which would act to keep the pure morphs separate, and the gene flow between them, tending to join them in a single population, could perhaps improve our understanding of the processes of parapatric speciation. In addition,

our results indicate that the variation in fecundity is not enough to explain the maintenance of this hybrid zone, because the sampled individuals were not distributed around the fecundity optimums expected for their morphs. Other fitness components, as viability, would be also important for this maintenance.

**WINKLE NUTRITION—THE ROLE OF TRAIL-FOLLOWING.** Mark S. Davies and Peter Beckwith, Ecology Centre, University of Sunderland, Sunderland, SR1 3SD. U.K.

Gastropod locomotion typically involves deposition of a mucus trail which is energetically costly. Post-deposition function of the trail would defray costs. We aimed to assess the role in nutrition of the mucus trail of *Littorina littorea*. Mucus trails adhered more microalgal cells from suspension than did a glass substratum; *Amphora coffeaeformis* (pennate diatom) adhered in greater densities than did *Tetraselmis suecica* (flagellate prasinophyte). Trails containing microalgae of both species (50–100 cells mm<sup>-2</sup>, similar to *in situ*) induced more trail-following than bare trails. Conspecific trails were followed for longer than self-laid trails. Winkles moved significantly slower on bare trails (mean = 0.35 mm s<sup>-1</sup>) than on glass (0.68 mm s<sup>-1</sup>), though the addition of microalgae to trails increased the speed. The radular rasp rate was significantly increased on trails containing *A. coffeaeformis* (mean = 17.8 bites min<sup>-1</sup>) and *T. suecica* (12.9) in comparison to control trails (4.3). Microalgae embedded in mucus entered the mouth. Following the passage of a winkle, *A. coffeaeformis* density was reduced by 38% and *T. suecica* by 43%. Winkles can clearly exploit food in trails and in doing so modify trail-following and feeding behaviour. Trail-following seems inextricably linked to nutrition. Since the intertidal is likely to be covered with mucus, or its degradation products, experiments on trail-following using clean substrata may have been erroneous. Distribution patterns of both *L. littorea* and benthic microalgae might be shaped by the ability of trails to adhere microalgae and by their subsequent exploitation by the grazer.

**POSSIBLE EFFECTS OF ANTHROPOGENIC STRESS ON THE GENETIC AND MORPHOMETRIC POPULATION STRUCTURE OF *LITTORINA LITTOREA*.** Hans de Wolf, Ronny Blust, Machteld De Wit, Marcel Selens, University of Antwerp (RUC) Ecophysiology & Biochemistry, Groenenborgerlaan 171, B-2020 Antwerp, Belgium, and Thierry Backeljau, Royal Belgian Institute of Natural Sciences, Vautierstraat 29, B-1000 Brussels, Belgium.

Anthropogenic stressors introduced into the marine system, are primarily concentrated in coastal areas, from industrial and agricultural inputs and runoff from land. Recent ecogenetic studies have shown that anthropogenic stressors may affect the genetic constitution of a population, decreasing its variability and making it more vulnerable for extinction.

In the present preliminary study we analysed the correlations of heavy metal pollution on the genetic and morphometric population structure of *Littorina littorea*, collected at seven sites along the highly polluted Western Scheldt estuary.

Heavy metal levels (Ag, Al, As, Ca, Cd, Co, Cr, Cu, Fe, Hg, Mn, Ni, Pb, Sr and Zn) were determined in both the shells and the soft body parts of *L. littorea*, using Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) and Inductively Coupled Plasma Mass Spectrometry (ICP-MS). Esterase variation was analysed using isoelectric focusing (IEF), while the morphometric population structure was determined by simple shell measurements using calipers.

None of the analysed metals seemed to accumulate in the specimen's shell matrix, but some metals accumulated in the soft body parts (i.e., Fe, Cd, Mn, Zn, and Cu), showing a clinal concentration gradient towards the North Sea. Shell size and esterase variability increased with decreasing heavy metal pollution (i.e., increasing salinity).

While the current results indicate a structuring of the genetic and morphometric variability in populations of *L. littorea* collected at the Western Scheldt, they do not indicate whether this structuring is caused by the existing heavy metal and/or the salinity gradient.

**ELLOBIIDAE: LOST BETWEEN LAND AND SEA.** Antonio M. De Frias Martins, Departamento de Biologia, Universidade dos Açores, Ponta Delgada, São Miguel, Açores, Portugal.

The Ellobiidae are a diverse group of archaeopulmonate snails living mostly near the sea, except for the Carychiinae which live in mountain forests and inside caves. The halophile ellobiids are commonly associated with the supratidal fringe of mangroves and salt marshes; they are, also, important components of the supratidal biota of the mobile rocky shore, namely under piles of rubble and shells. Their ecological requirements range from the muddy bottoms of stagnant mangrove embayments to the wave-swept crevices of exposed cobble shores, and they could be very important elements in the mineralization of the organic mud or algal detritus in such habitats.

The various species of ellobiids occupy different portions of the shore, their distribution being loosely related to their taxonomic affiliation. The pythiinians (*Pythia* and, to a lesser extent, *Myosotella*) venture farther inland and may live in a nearly terrestrial environment, whereas the Pedipedinae prefer the upper intertidal. Within the Melampinae, some species of *Melampus* are found usually in mangroves and marshes, whereas others and *Tralia* prefer cobble shores. In rocky habitats, in addition to horizontal zonation, the ellobiids also partition their vertical distribution.

The Ellobiidae range in size from barely 2 mm (*Leuconopsis*) to over 100 mm (*Ellobium*); the shell is variable in shape and can not be used as a reliable character for subfamilial allocation. The anatomy is also variable, but presents more reliable diagnostic



characters. Six structural types of reproductive system set the boundaries for the subfamilial division, which is supported by the existence of three structural types of nervous system. The internal morphology of the penial complex has proven to be a helpful associated character in the subfamilial delimitation.

**WHEN TO MOVE AND WHERE TO GO: MOVEMENT BEHAVIOR OF THE CARIBBEAN LITTORINID, *CENCHRITIS MURICATUS*.** Deborah J. Gochfeld<sup>1</sup> and Dwayne T. Minton,<sup>2</sup> Hofstra University Marine Laboratory, Priory, Jamaica, West Indies.

Intertidal molluscs display a variety of movement behaviors, and the type of behavior expressed by a species is related to the intensity of physical and biological stresses to which it is exposed. Although littorinids are believed to be non-homing and to display random movement patterns when foraging, few studies have examined their behavior in detail. Tropical littorinids are exposed to extreme environmental conditions, and movement at night or on a tidal cycle may alleviate desiccation and heat stress, as would returning to a sheltered location following foraging.

We examined the movement behavior of the Caribbean littorinid, *Cenchritis muricatus*, for cyclic migrations, homing, and non-random movement. Individual snails were marked and their locations on an uplifted limestone bench were monitored daily for 30 days. On six days selected from different parts of the lunar cycle, snail locations were monitored at three-hour intervals. Movement did not occur on a diurnal or tidal cycle, however, we did observe a mass down-shore migration in June and July. Although *C. muricatus* preferentially rested in crevices, there was no evidence of homing behavior. Snails resting on exposed rock surfaces were four times more likely to move than snails resting in more sheltered microhabitats. In general, movement in *C. muricatus* appears to be a direct response to wetting: >87% of movements occurred within 12 hours after rainfall or heavy dew, but movement was elicited by both fresh and salt water. We believe this behavior is primarily a response to desiccation stress, but it may also facilitate foraging.

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**GRAZER BIOMASS AND BIOFILM STANDING STOCKS IN THE HIGH INTERTIDAL ON HONG KONG ROCKY SHORES.** Catriona Hassey, Ecology Centre, University of Sunderland, Chester Road, Sunderland, SR1 3SD, U.K.

Bottom-up processes and resultant net primary production rates are known to influence grazer biomass and subsequent community structure on rocky shores. Variation in abiotic conditions (e.g., coastal water nutrient levels, rock type, etc.) have also been shown

to influence algal production rates and, therefore, consumer biomass. High intertidal, splash zones worldwide support large densities of Littorinidae although these grazers often appear to be existing on bare rock. These zones do, however, support an epilithic biofilm which forms the base of the benthic food chain and maintains populations on the high shore. Refuge habitats are also known to be important for these snails in this physically harsh environment. The present study investigates the relationship between grazer biomass and biofilm standing stock in the high intertidal/splash zone on Hong Kong rocky shores. The sampling zone (2.0–3.0 m above C.D.) was defined according to the immersion:emersion curve to lie above the 10% immersion line and to extend to the upper limit of the littorinids. Two littorinid species were common in this zone, *Nodilittorina trochoides* (Gray 1939) and *N. radiata* (Eydoux and Souleyet, 1852) which reached densities in excess of 500/m<sup>2</sup> and 300/m<sup>2</sup>, respectively. These species are microalgal grazers feeding on the epilithic biofilm, composed primarily of cyanobacterial species such as *Gloeocapsa* and *Dermocarpa*. Chlorophyll *a* extraction of rock chips was used as an estimate of standing crop and the fractal dimension of the rock surface was used to estimate habitat complexity. These factors were correlated with littorinid biomass and abundance at a number of different spatial scales (both within and between shores) to investigate whether they had any predictive value and their spatial generality.

**DIFFERENTIATING SIBLING SPECIES: *LITTORINA SCUTULATA* AND *L. PLENA*.** Paul A. Hohenlohe, Friday Harbor Laboratories, University of Washington, Friday Harbor WA 98250, U.S.A., and Elizabeth G. Boulding, Department of Zoology, University of Guelph, Guelph, ON N1G 2W1, Canada.

The sibling species *Littorina scutulata* and *L. plena* are difficult to distinguish nondestructively in the field. Here we present a new molecular tool and an analysis of the quantitative and qualitative characters that have been proposed as diagnostic. We collected 385 snails from 11 sites in Washington state and scored three qualitative shell characters (presence of basal ridge, presence of basal hand, and size of tessellation pattern) as well as tentacle coloration and male penis morphology. Specimens were identified to species by amplification and restriction enzyme digestion of a 480 base pair fragment of the mitochondrial cytochrome *b* gene. Of the qualitative characters, penis morphology was infallible for differentiating the males and tentacle coloration was most effective for both sexes. The qualitative shell characters also showed significant differences between the species but are not likely to be effective for differentiation because of frequent damage to the shells from abrasion and fungus.

We also took eight quantitative measurements of shell shape. *L. scutulata* had significantly larger shells than *L. plena*. After correcting for size, *L. scutulata* had significantly taller, narrower apertures and spire angles, but the species did not differ in aperture

angle. Discriminant function analysis of these measures correctly assigned only 72.5% and 64.8% of the two species, respectively.

Contrary to previous reports of habitat differences, only *L. plena* was found at exposed outer-coast rocky shores, while both species co-occurred at low and medium exposure rocky shores and cobble beaches within Puget Sound.

**PARALLEL EVOLUTION—CHALLENGING TAXONOMY, CLADISTICS AND SPECIATION THEORY.** Kerstin Johannesson, Department of Marine Ecology, Tjärnö Marine Biological Laboratory, SE-452 96, Strömstad, Sweden.

Under parallel evolution, similar traits arise independently in different areas of a species. They do so because they have the same evolutionary background and they respond to similar local selection pressures. However, in heterogeneous environments this will result in parallel ecotypes among which phenotypic similarity and phylogenetic ancestry are incongruent. That is, we find common phenotypic traits in populations that live in similar but geographically separated habitats, despite these populations being genetically less related than phenotypically disparate populations living in adjacent but dissimilar habitats.

Over the last two decades a multitude of illustrative examples of parallel evolution have been documented in, for example, diatoms, higher plants, mollusks, fishes, and salamanders. In some of these cases, the originally related populations have diverged to such an extent as morphological or behavioural barriers to reproduction have developed. However, because of phenotypic similarity, these barriers do not exist between the phenotypically similar populations evolved in parallel (coined "parallel speciation"). Parallel species and parallel evolution raises conceptual problems of phylogenetic analysis, species definitions and theory of speciation. More problematic, however, may be the dramatic consequences this type of evolution may have on traditional taxonomy and systematics based on external morphological characters.

In this paper I will discuss these problems using data from species of *Littorina*, and from some other taxa, as examples.

**DNA-ALLOZYME COUPLING AND SHELL DIMORPHISM OVER MICROGEOGRAPHIC SCALE—FURTHER EVIDENCES OF A COMPLEX HABITAT-RELATED SUBSTRUCTURING IN THE MARINE SNAIL *LITTORINA FABALIS*.** K. Johannesson, M. Lejhall, Department of Marine Ecology, Tjärnö Marine Biological Laboratory, SE-452 96, Strömstad, Sweden, and N. Mikhailova, Institute of Cytology RAS, St. Petersburg 194064, Russia.

Genetic substructuring of species is probably a quite important factor in evolution but still we have too fuzzy data to test this hypothesis in most species. Obviously, we expect genetic substructuring primarily in species of poor dispersal, for example, marine benthic invertebrates having direct development. Mostly we ex-

pect substructuring to be due to isolation by distance, but this is not always the case.

In Wales, *Littorina fabalis* is dimorphic for size, colour and shell microstructure between exposed and protected habitats. We assessed the differences in Swedish populations, and found that these were less pronounced, except for size. However, earlier studies reveal strong allozyme differences in one locus. In this study we report DNA differences as well. Furthermore, the DNA and allozyme differences are strongly correlated which might be explained by a chromosomal inversion involving both markers. The genetic substructuring of *Littorina fabalis* is also strongly habitat linked, but it remains to be tested if there are adaptive differences between different genotypes. An alternative may be two independent evolutionary lineages.

**FEEDING ECOLOGY OF THE HONG KONG MANGROVE LITTORINIDS, *LITTORARIA ARDOUINIANA* AND *L. MELANOSTOMA*.** Olive H. K. Lee, Department of Ecology and Biodiversity, The University of Hong Kong, Pokfulam Road, Hong Kong.

*Littoraria ardouiniana* and *L. melanostoma* are commonly found in Hong Kong mangroves where they occur on the leaves and trunks of mangroves such as *Kandelia candel*, *Aegiceras corniculatum*, and *Avicennia marina*. Examination of the stomach and faecal contents showed that these two littorinid species mainly feed on the epidermal cells of leaves, while bark cells, fungal fragments and spores were also found. In contrast to studies from Thailand, fungal hyphae and spores were sparse in the diet. Monthly investigation revealed no seasonal variation in the diet nor in the availability of food items (particularly fungi) on the leaves. Laboratory and field-based observations showed that these littorinids were generally inactive when dry in the daytime but active at night and/or in rain. Activity could be stimulated by spraying with water. *L. ardouiniana* and *L. melanostoma* appear to be generalists in their diet, grazing on the leaf and bark surfaces of mangroves at night. Feeding activity can also, however, be triggered by environmental cues (e.g., rain), suggesting that these species are opportunists, feeding whenever conditions are suitable.

**AGGREGATION PATTERNS OF THREE SPECIES OF PERIWINKLES IN THE SOUTHEASTERN COAST OF SÃO PAULO, BRAZIL.** C. A. Magalhães, Depto. de Zoologia, IB, UNICAMP, Campinas, SP, Brazil.

Periwinkles tend to aggregate and remain inactive under different stressful conditions for grazing. In this work I studied the frequency and duration of aggregation behavior, the size of the aggregations, the fidelity to a specific aggregation, and aggregation arrangement in *Nodilittorina lineolata*, *Littoraria flava*, and *Littoraria angulifera*. *N. lineolata* aggregates in great numbers and for periods of until 25 days, usually associated to dry conditions of

the rock surface or with excessive freshwater seeps during the rainy season. Aggregation is fortuitous in this species and there is no special arrangement of the individuals in the cluster. *L. flava* exhibits a tidal aggregation behavior, forming small groups composed of 10 to 30 snails, usually associated with depressions or crevices on the rocky shore. Aggregation is due to dry conditions, and some snails do present fidelity to the aggregation site, following back their mucus tracks back to their aggregation site after a foraging excursion. The smaller individuals occupy the central area of the cluster, surrounded by the large ones. *L. angulifera*, a mangrove inhabitant, presents an aggregation behavior similar, but less frequent and ubiquitous than *L. flava*. It aggregates over mangrove tree's stems, close to the low water tidal line during extreme spring low tides, or high in the trees on high tides. Snails of this species search for depressions or fissures to begin aggregates, always on shadowed spots. Aggregation of individuals is random, with no apparent mucus following clues.

#### HEAT COMA TEMPERATURE AND SALINITY RESPONSE RELATIVE TO ZONATION PATTERN IN JAMAICAN INTERTIDAL GASTROPODS AND CHITONS.

**Robert F. McMahon**, Department of Biology, Box 19498, The University of Texas at Arlington, Arlington, Texas 76019, U.S.A.

Intertidal mollusc vertical distributions are assumed to be related to species tolerances of temperature and desiccation. However, species physiologic resistances exceed intertidal environmental extremes, suggesting that they have little influence on zonation patterns. In contrast, day-to-day maintenance of zonation could be influenced by salinity variation which increases with shore height due to evaporation and to precipitation during periods of tidal emersion. The relationships between zonation, heat coma temperature (HCT) and behavioral response to varying salinity were tested among molluscs from a rocky shore near Priory, Jamaica. HCT was determined as temperature of loss of pedal attachment on warming at  $1\text{ }^{\circ}\text{C } 5\text{ min}^{-1}$  while response to salinity was assessed by pedal re-attachment 1 hour after transfer into 0, 5, 10, 20, 30, 40, 50, 70, 100, 120, 160, 180, 200, or 220‰ seawater. Of 15 examined species, two low eulittoral chitons, *Chiton squamosus* and *Acanthopleura granulata*, had the lowest HTC's at  $35.14\text{ }^{\circ}\text{C}$  (s.e. = 0.35) and  $37.03\text{ }^{\circ}\text{C}$  (0.48), respectively. Among five low to mid-eulittoral nerites, HCT ranged from  $41.5^{\circ}$  (0.27) to  $45.7\text{ }^{\circ}\text{C}$  (0.15) and was not correlated with vertical distribution. *Batillaria minima*, a mid-eulittoral tide pool cerithiid had a similar HCT of  $43.04\text{ }^{\circ}\text{C}$  (0.14). Among seven littorinid species, HCT ranged from  $42.1\text{ }^{\circ}\text{C}$  (0.41) in the maritime, *Cenchritis muricatus*, to  $47.0\text{ }^{\circ}\text{C}$  (0.52) in the mid-eulittoral, *Notilittorina ziczac*. Thus, thermal tolerance was not correlated with zonation level among the rock dwelling species tested. In contrast, among six littorinid, five nerite and 1 cerithiid species, salinity tolerance was correlated with zonation and microhabitat. Among tide pool species, the highest zoned, *Purperita pupa*, pedally re-attached over

the greatest salinity range. Among eight species inhabiting exposed rocks, those highest zoned pedally re-attached over the widest salinity ranges, and particularly under hyposaline conditions, with the highest-zoned *Cenchritis muricatus* reattaching at 01‰ salinity. Thus, salinity tolerance may influence maintenance of zonation, high-shore molluscs being capable of maintaining activity over a greater range of salinities than low-shore species. Observations on the shore supported this conclusion.

#### SMALL SCALE VARIATION IN RESPONSE OF INTERTIDAL MACROALGAE TO GRAZING BY WINKLES AND LIMPETS. C. D. McQuaid and K. Whittington-Jones, Dept. of Zoology & Entomology, Rhodes University, Grahamstown 6139, South Africa.

A detailed exclusion experiment was set up to examine the effects of different size classes of grazers on algal biomass on a rocky shore in South Africa. Grazers were classified as "macrograzers" (mainly limpets) and "mesograzers" (mainly winkles). There were seven treatments involving different designs of cages of  $50 \times 50\text{ cm}$ , with five replicates of each treatment. This allowed examination of the effects of each grazer type alone and in combination. The experiment was run in two seasons and at two shore heights. Repeated measurements were made at 2–4 week intervals of the cover of several functional groups (foliose algae, algal turfs, encrusting algae, barnacles).

The most important result was that variation among replicate treatments was enormous. Replicates which began with similar biomass of macroalgae responded completely differently to the same treatment. This produced large standard deviations which masked treatment effects. Analysis was by MANOVA, followed by ANOVA where this indicated a significant effect. Algal turfs and barnacles were affected by treatments in spring, but not winter, but meaningful biological interpretation of these results is difficult. Macrograzers seem generally to have had a more profound effect than mesograzers. More important is the conclusion that, while grazers very clearly had significant effects in some plots, different factors had overriding influences in others. Given the size of plots used, these factors must operate on scales of less than 1 m and appear to result in patchiness on very small scales on these shores. Small scale variations in recruitment success, topography and the outcomes of species interactions are all likely contributors to heterogeneity.

#### ASPECTS OF SHELL SHAPE IN LAGOONAL LITTORINIDS. Peter J. Mill, Andrea P. Clarke, and John Grahame, School of Biology, The University of Leeds, Leeds, LS2 9JT, England, and Delmont C. Smith, Department of Biological Sciences, State University of New York, College at Brockport, Brockport, New York 14420 U.S.A.

In the *Littorina saxatilis* complex, five morphs have been distinguished, including two which at various times have been af-

forded species status. One of these, *L. tenebrosa*, is sensu stricto, a lagoonal-dwelling morph which remains submerged and is found on, e.g., *Chaetomorpha*. It has a smooth, fragile high-spined shell which is black or dark brown and may be banded. There is another littorinid which occurs in coastal lagoons but which has a different shell shape, i.e., lagoonal *L. saxatilis*. In a survey of about 30 non-tidal, coastal lagoons over a stretch of some 150 km of coastline in eastern England, only one was found to contain *L. tenebrosa* with a further four containing lagoonal *L. saxatilis*. The one containing *L. tenebrosa* was largely surrounded by tall vegetation and was separated from the sea by a mature, tree-covered dune. Of the others, one was separated by a dune, the others by a shingle bank. In each case, inundation by the sea would be a very rare event. Shells from these populations were weighed and imaged, following which a number of shell variables were measured. The populations were compared with each other and with a sample of coastal *L. saxatilis* from the same region. Furthermore, comparisons were made with a population of *L. tenebrosa* from Ireland. In this case, the lagoon probably had periodic influxes of sea water. *L. saxatilis* were obtained from its seaward end; *L. tenebrosa* was confined to the landward end, adjacent to a freshwater input.

**IS LIFE ON A TROPICAL ROCKY SHORE REALLY SO HARD?** Dwayne Minton<sup>1</sup> and Deborah Gochfeld<sup>2</sup>, Hofstra University Marine Laboratory, Priory, Jamaica, W.I.

Interactions of biotic and abiotic factors are considered the principle mechanisms controlling the dynamics of rocky shore communities. Unfortunately, little research has examined how these factors affect community structure of tropical rocky shore assemblages. We examined the effect of wave action and desiccation on a rocky shore molluscan assemblage on the north shore of Jamaica. This assemblage exists entirely above mean high water (MHW) where physical factors were expected to be more important than biological factors. We compared the molluscan assemblage along ten vertical transects exposed to different levels of wave action and desiccation potential. In all, nineteen species of mollusc were observed, with thirteen occurring on >50% of our transects. Five species occurred on >80% of the transects and were dominant at three different vertical heights: *Cenchritis muricatus* occupied the highest position on the shore (1.0–1.3 m above MHW); *Nodolittorina dilutata* and *N. ziczac* occupied a middle region (approximately 0.4–0.8 m above MHW); and *Acanthopleura granulata* and *Notoacmaea antillarum* were found lowest on the shore (approximately 0.05–0.15 m above MHW). We found no differences in species number, individual densities, or the vertical distribution of the species between transects with differing wave action or desiccation potential, suggesting that these physical factors are not operating at the spatial scale studied. However, gastropod molluscs preferentially occupied pit and crevice microhabitats, which are believed to mediate physical stresses such as

desiccation and high temperature. The distribution of these rocky shore molluscs may be the result of the availability of, and competition for, rare microhabitats.

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**THE MANGROVE LITTORINIDS: EVOLUTION AND ADAPTATION IN THE GENUS LITTORARIA.** David G. Reid, Department of Zoology, The Natural History Museum, London SW7 5BD, U.K.

The littorinids found largely or exclusively upon mangrove trees are members of the genus *Littoraria*, a well-defined monophyletic group of 36 species occurring throughout the tropics. Some, but not all, of these species are notable for unusual morphological characteristics: they may be ovoviviparous (releasing veliger larvae or crawling juveniles), their shells may show striking color polymorphism (occurring in discrete yellow, brown, and pink morphs), and the central tooth of the radula exhibits a supposedly unique extra cutting edge (the so-called rachidian hood). In each case, workers have suggested that these attributes might be adaptations to the mangrove habitat, although this remains speculative in the absence of information about character evolution. In order to test these hypotheses in a phylogenetic context, a new phylogeny for the 36 species of *Littoraria* has been produced by cladistic analysis of morphological characters. By mapping habitat type and these three key morphological characters onto the phylogenetic tree, it is possible to test whether the characters are indeed derived features that have been selected within the mangrove habitat (i.e., adaptations), or whether they were ancestral features that permitted colonization of this habitat (i.e., exaptations).

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**ORIGIN OF NERITA ATRAMENTOSA REEVE 1855, A NERITE OF TEMPERATE ROCKY SHORES (NERITOP-SINA: NERITIDAE).** Bronwen Scott, Conservation Biology Unit, School of Life Sciences and Technology, Victoria University of Technology S008, P.O. Box 14228, Melbourne City MC, Victoria 8001, Australia, and Kerry Neil, Department of Marine Biology, James Cook University, Townsville, Queensland 4811, Australia.

The gastropod *Nerita* is characteristic of tropical and subtropical rocky shores and mangroves worldwide. At least twelve species occur in Australia, all but one confined to the warmer waters of the Indo-West Pacific. This exception is *Nerita atramentosa* Reeve 1855, which lives on rocky shores in temperate and warmer waters from southern Queensland to North West Cape, western Australia. It is also known from Norfolk Island, New Zealand and

the Kermadec Islands. The shell of *N. atramentosa* resembles both that of the African *N. senegalensis* and of the central Pacific *N. picca*, but the distribution of *N. atramentosa* in Australia is contiguous with that of the twelve Indo-West Pacific species. As the only temperate species, *N. atramentosa* poses a biogeographical problem: is its sister species from the tropical Indo-Pacific, the central Pacific or the western Indian Ocean? This study uses conchological and anatomical characters to investigate the phylogeny of the Australian nerites as a basis for biogeographical studies.

**RELATIONSHIPS AND POPULATION STRUCTURE OF *LITTORINA ARCANA* HANNAFORD ELLIS, *L. COMPRESSA* JEFFREYS, AND *L. SAXATILIS* (OLIVI) IN THE BRITISH ISLES USING SSCPS OF *CYTOCHROME-B* FRAGMENTS.** Maureen P. Small and Elizabeth M. Gosling, School of Science, Galway-Mayo Institute of Technology, and Microbiology Department, National University of Ireland, Galway, Ireland.

The *saxatilis* snail species complex are ubiquitous and important members of hard shore intertidal communities in the North Atlantic. The complex includes the recognized species *Littorina arcana* Hannaford Ellis, *L. compressa* Jeffreys and *L. saxatilis* (Olivi). We investigated the species and population structure of these snails from six locations in Ireland and Britain using a non-radioactive single strand conformational polymorphism (SSCP) analysis of a 375 base pair fragment of the *cytochrome-b* gene. Variability was high in this marker with 38 haplotypes found in 586 individuals. The most common haplotype in *L. arcana* and *L. compressa* was absent from *L. saxatilis* and the most common haplotype in *L. saxatilis* was found in low numbers in both *L. arcana* and *L. compressa*. Haplotypes restricted to *L. arcana* and *L. compressa* formed a cluster separate from haplotypes restricted to *L. saxatilis* in a maximum-likelihood tree, minimum spanning tree and multidimensional scaling analysis. In the same type of analyses examining population relationships, *L. arcana* and *L. compressa* formed a group separate from *L. saxatilis*. We concluded that *L. arcana* and *L. compressa* are more closely related to each other than either is to *L. saxatilis* and suggest that this is a resolution to a previous trichotomy among these species.

**EFFECT OF TEMPERATURE AND DESICCATION ON URIC ACID CONTENT OF *LITTORINA SAXATILIS*.** Delmont C. Smith, Department of Biological Sciences, State University of New York, College at Brockport, Brockport, New York 14420, U.S.A.

I have previously reported uric acid content of *Littorina saxatilis* that changes according to season and location on the shore. Such variation could be due to temperature and/or desiccation effects. To test this, winkles were maintained in controlled temperature rooms at 10 °C (designated as C) or 25 °C (H), and either

immersed in seawater (W) or dry (D). From an initial concentration of 100.8  $\mu\text{g g}^{-1}$ , animals maintained C/W showed a decline to 67.5  $\mu\text{g g}^{-1}$ . Animals that were C/D increased to 157.1  $\mu\text{g g}^{-1}$ , those that were H/W to 175.3  $\mu\text{g g}^{-1}$ , and those H/D to 219.8  $\mu\text{g g}^{-1}$ . If animals that had their uric acid raised by desiccation were then returned to seawater their uric acid concentration returned to initial levels within 48 h. During this time when uric acid concentration was declining, the animals also produced ammonia at rates nearly double those of snails kept in seawater. It therefore appears that nitrogenous wastes are stored as uric acid at times when water must be conserved, and that the uric acid is then eliminated when water again becomes available; most probably not as uric acid, but after conversion of uric acid to ammonia.

**TRANS-ZONAL MOVEMENTS IN WINKLES, *LITTORINA LITTOREA* (L.): REASONS AND CONSEQUENCES.** G. F. Warner, School of Animal & Microbial Sciences, The University of Reading, Whiteknights, Reading RG6 6AJ, U.K.

Two years of monthly samples of *L. littorea* at three levels on an estuarine shore at Southampton, U.K., have provided evidence of long-term movements up or down the shore. The zone occupied by *L. littorea* at Southampton is from about mid-tide level down into the sublittoral. Increases in population densities at the middle site of winkles in their second year of growth, indicate movements of young winkles towards the centre of their zonation range from both higher and lower levels. Increases in population densities of older winkles at the higher and lower levels indicate later dispersion away from the centre of the zonation range. Direct evidence of movements between the middle level and the lower level is provided by the presence or absence in the shell of bore-holes made by the polychaete *Polydora ciliata*, which recruits on winkle shells mainly on the lower shore. Thus, young winkles at the middle site with bore-holes on the spire have moved up from a lower level while older winkles at the lower level which lack bore-holes have moved down from a higher level. At the upper level, fluctuating population densities and observations of feeding fronts in the spring indicate seasonal migrations, down in winter and up in spring. Advantages and disadvantages of living at upper or lower levels are discussed and related to differences in growth rates and mortality at the different levels.

**CORRELATION OF MORPHOLOGICAL DIVERSITY WITH GENETIC DIVERSITY IN THE ROUGH PERIWINKLE *LITTORINA SAXATILIS*.** Craig S. Wilding, John Grahame and Peter J. Mill, The School of Biology, The University of Leeds, Leeds LS2 9JT, U.K.

Both morphological and genetical studies of rough periwinkles have been used in a variety of studies to examine the structuring of populations. However, the complementarity of these two different approaches has not been directly estimated, despite the fact that

such an approach could lead to a better understanding of the basis of shape variability in these morphologically diverse animals. In the present study, variation in both the mitochondrial DNA and in the nuclear genome (measured via RAPDs and PCR-RFLP of anonymous nuclear loci) of *Littorina saxatilis* is compared to, and correlated with, measures of phenotypic variability using multivariate approaches. Techniques involving Mantel matrix comparisons, and AMOVA (analysis of molecular variance) are considered. We show that in certain instances, a high degree of correlation between morphology and genetics can be uncovered, and this suggests that morphological approaches are of use for detecting population structure as well as environmental components of shell shape.

**VARIATION IN AMMONIA EFFLUX RATES WITH EMERSION OF THREE HONG KONG *NODILITTORINA* SPECIES.** R. F. Uglow, Department of Biological Sciences, University of Hull, Hull HU6 7RX, U.K., and Gray A. Williams, Department of Ecology & Biodiversity and The Swire Institute of Marine Science, The University of Hong Kong, Hong Kong.

On moderately-exposed Hong Kong shores, *Nodilittorina trochoides*, *N. radiata*, and *N. vidua* are distributed from the splash zone to the mid-eulittoral respectively where they experience long emersion times and, in summer, high rock and air temperatures. Individuals of these species were collected from the shore after varying periods of emersion for (0 min, awash and active controls, and 1, 4, and 22 h, inactive animals on natural rock). Ammonia efflux rates ( $n = 6$ ) were measured at fixed intervals (30 mins, 1 and 2 h) on reimmersion. The awash groups, after 30 mins, had mean efflux rates of 3.91, 6.01, and 3.53  $\mu\text{mol NH}_4 \cdot \text{g}^{-1} \cdot \text{h}^{-1}$  which were high compared with the 1.66, 2.02 and 0.32  $\mu\text{mol NH}_4 \cdot \text{g}^{-1} \cdot \text{h}^{-1}$  rates after 2 h for *N. trochoides*, *N. radiata*, and *N. vidua*, respectively. These data reveal interspecific variations in efflux rates and that handling may evoke enhanced ammonia excretion rates as a stress response.

Post-emersion efflux rates were always lower than those of the comparable control group—apart from the 30 min, post-1 h emersion rates of *N. vidua*, which were unchanged from control rates. *N. vidua* also showed the least diminution of efflux rates following 4 and 22 h of emersion—an interspecific difference

which correlates well with the generally greater activity and relatively lower tidal position of this species and the infrequency with which it may experience naturally 22 h of emersion, *cf* the other 2 species.

The energetic implications of these differences in post-emersion efflux rates are discussed in terms of emersion tolerance, hence limitations to vertical distributions on the shore. No evidence was found of a switch from ammonotelic to uricotelic during the lengths of emersion tested here, as no animal excreted detectable levels of urate during these experiments.

**NUCLEAR RIBOSOMAL DNA SEQUENCES AND THE PHYLOGENY OF THE LITTORINIDAE.** Birgitta Winpenninckx, Thierry Backeljau, Royal Belgian Institute of Natural Sciences, Vautierstraat 29, B-1000 Brussels, Belgium, and David Reid, Department of Zoology, The Natural History Museum, Cromwell Road, London SW7 5BD, U.K.

Phylogenetic relationships within the family Littorinidae were exhaustively investigated in several papers by D. Reid, who in particular dealt with the generic relationships in his 1989 paper (*Phil. Trans. R. Soc. Lond. B* 324: 1–110) and with the intrageneric arrangement of the genus *Littorina* in his book of 1996 (*Systematics and evolution of Littorina*, Ray Society). Most of these analyses were based on morphological characters, yet the phylogeny of *Littorina* was recently also investigated by means of mtDNA sequences. Despite all these efforts there still remain a number of unresolved or somewhat uncertain issues with respect to both the relationships within *Littorina* and the relationships among littorinid genera. We therefore attempted to investigate littorinid phylogeny at different taxonomic levels using complete 18S rDNA ( $\pm 1800$  bp) and partial 28S rDNA ( $\pm 770$  bp) sequences. We will present a first, restricted analysis of 18S rDNA data aiming at testing the suitability of this molecule to resolve relationships within the genus *Littorina* (with focus on the position of *L. striata*), while in a second analysis, we will present a very preliminary attempt to resolve generic relationships within the Littorinidae using both 18S and 28S rDNA sequences of representatives of 13 littorinid genera.

**ABSTRACTS OF TECHNICAL PAPERS**

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University College Cork  
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**MONITORING COASTAL SHELLFISH AS A SUSTAINABLE RESOURCE IN SOUTHERN ITALY.** E. Aloï Totàro, S. Costabile, S. Porco, and M. Totàro, Cattedra di Ecologia, Facoltà di Economia, Università degli Studi del Sannio (Benevento), Italy.

We consider the environmental characters and distribution of Gastropod and Lamellibranch culture along the south Italian coast.

The goal is to consider the possibility that this activity is a typical sustainable management. It is necessary to manage and to consider the sea as the most complex system of the Biosphere.

It is composed of two fundamental regions, the former is the coastal zone form, the latter is the ocean form. The coastal zone includes some coastal districts of variable width. Their development is linked, strongly, to the management capacity of coastal communities, so this development is also linked strongly to the correct type of government.

Italian coastal districts show increasing alterations of their biological resources such as the growing pollution caused by urban and industrial wastes, hydrocarbon inductions and the changes to the water temperature.

Recently an interest in new sustainable models for coastal regions and for new sustainable utilizations of marine biological resources, based on the increase of water bio-farms has developed. It is important to advertise these new methods of managing coastal ecosystems. These ecosystems will be considered as "controllable ecosystems". Consequently, it will be necessary to safeguard, to equip, to improve them in order to increase rational production of clams. But first one must locate specific coastal areas suitable for this production with an appropriate development strategy.

So to realize this program it is necessary:

- to revalue the human resource in this specific professional sector
- to restore the marginal areas and the non-used resources
- to create a real interest in the economic opportunities of clam restocking in the light of the sustainable development.

**ASSESSING THE EFFECTIVENESS OF BAY SCALLOP (*ARGOPECTEN IRRADIANS*) POPULATION RESTORATION ON THE WEST COAST OF FLORIDA, USA.** W. S. Arnold, D. C. Marelli, K. Hagner, M. Parker, P. Hoffman, and M. Harrison, Florida Marine Research Institute, 100 Eighth Avenue SE, St. Petersburg, FL, 33701-5095, USA.

Bay scallops (*Argopecten irradians*) were once abundant in the nearshore marine environment of western Florida. In recent years, however, many of the local populations that comprise the purported bay scallop metapopulation in Florida have collapsed, resulting in closure of the commercial fishery and implementation of severe restrictions on recreational harvest. Hypothesized causes of that collapse include overfishing, habitat degradation, and increased prevalence of red tide, but the exact cause is unknown. We are attempting to restore scallop populations in Florida by planting

cultured scallops in selected areas, but we are taking a stepwise approach to scallop restoration by first assessing the feasibility of this strategy before initiating a full-blown restoration effort. This experimental approach consists of first developing a thorough baseline of information concerning the local abundance and relative rates of recruitment in natural populations. We then spawn small groups of scallops for which we identify a unique mitochondrial marker. The resultant (genetically identifiable) broods are planted in cages within the location from which the parent stock was collected. We monitor the growth, survival, and reproductive development of each brood. Additionally, we employ artificial collectors to obtain juvenile scallops that are returned to the laboratory for genetic analysis. This allows us to estimate the proportional representation of cultured scallops in the recruiting population. Finally, we sample adult populations both at the planting site and at other sites throughout western Florida, to estimate the proportional representation of cultured scallops in the following adult year-class. If cultured scallops comprise a significant proportion of the subsequent year-class, or if we observe increased scallop abundance relative to background levels, then we will consider implementing a large scale restoration effort. If experimental enhancement fails, then we must assume that the causative factors of the collapse remain and must be mitigated before initiating full-scale restoration.

**TESTING ALTERNATIVE STRATEGIES FOR POPULATION ENHANCEMENT OF HARD CLAMS *MERCENARIA* SPP. IN THE INDIAN RIVER LAGOON, FLORIDA.** W. S. Arnold, D. C. Marelli, P. Hoffman, and M. Humphrey, Florida Marine Research Institute, 100 Eighth Avenue SE, St. Petersburg, FL, 33701-5095, USA.

Clams of the genus *Mercenaria* support a commercial fishery in the Indian River lagoon on the east coast of Florida, but that fishery is characterized by unpredictable availability of clams. We are testing three alternatives (spawner transplants, seeding, and direct larval injection) to enhance the availability of clams in the Indian River in an attempt to stabilize the fishery and reduce economic hardships visited upon the fishermen during periods of low clam abundance. Both spawner transplants and seeding are designed to increase the local abundance of mature clams, thereby enhancing fertilization success and resultant recruitment into the local habitat. Larval injection is designed to circumvent the natural spawning process by introducing fertilized eggs directly into the lagoon. For the spawner transplant study, we are harvesting clams from open shellfishing waters and transplanting them into two closed shellfishing areas during each of four seasons. Each seasonal group is evaluated every three months for survival and reproductive development. Seeding provides an alternative approach to enhancing spawner concentrations. We have planted three size classes of seed clams (2 mm SL, 8 mm SL, 16 mm SL) under each of four protective treatments (no cover, mesh cover, oyster shell cover, mesh+oyster shell cover), and we periodically assess the

survival, growth, and reproductive development of the planted clams. Ultimately, we hope to compare the relative biological and economic advantages of transplants versus seeding for enhancing hard clam spawner stocks in the lagoon. To evaluate the efficacy of direct larval injection, we have tracked introduced larval masses to monitor advection, survival, and settlement. The results of the larval tracking study will be used to evaluate the feasibility of this strategy for enhancement of the harvestable stock, and to identify modifications necessary to increase the proportion of introduced larvae that successfully set to the benthic environment.

**THE USE OF MATHEMATICAL MODELS TO ASSESS THE CARRYING CAPACITY OF EXPLOITED ECOSYSTEMS.** C. Bacher, CREMA, B.P. 5, 17137 L'Houmeau, France.

In carrying capacity assessments, the key notion is that there is an optimum standing stock, yielding a maximum production under some constraints due to the population dynamics, the culture practice and the food limitation.

The main objectives of this paper are therefore to present the background, the interest and the limitation of the mathematical models used in carrying capacity assessments. A simple model is presented first to illustrate how the dynamics of cultured population yields the stock/production relationship. This example is analysed more deeply to define the basic components underlying the carrying capacity modelling which are in turn illustrated with case studies. The first case study deals with the Thau lagoon (France) for which a mathematical model of the population dynamics was built to predict the evolution of the standing stock and the annual production, taking into account several sources of variability, the rearing strategy of the farmers, the fluctuation of the environmental conditions, and the growth variability between individuals. In a second case study (Aiguillon Bay, France), it is shown how the growth of cultivated filter feeders is affected by the trophic conditions. Modelling the ecophysiology, the cultured population dynamics and the ecosystem dynamics are then combined to assess the carrying capacity of the main French production area (Marennes-Oleron Bay).

As a conclusion, it is stressed that i) the carrying capacity assessment is of major importance in the scope of the sustainable development of aquaculture—with an example given of an EU/China project, ii) a full understanding of the underlying biological and physical processes is needed, iii) the socio-economic component has not yet been taken into account.

**THE IMPACT OF A NEPHROPS OTTER TRAWL FISHERY ON THE BENTHOS OF THE IRISH SEA.** B. Ball, B. Munday, and G. Fox, The Martin Ryan Marine Science Institute, National University of Ireland, Galway, Galway, Ireland.

The fishery for the Norway lobster (*Nephrops norvegicus*) is concentrated on a mud patch in the north western part of the Irish

Sea. The fishery commenced in the early '40's as a small-scale summer fishery, but the season has now extended to include most times of the year. Fishing intensity is estimated to be high (each square metre is trawled c. 5–10 times per year). No quantitative historical data on the benthos is available for the period prior to commencement of the fishery, although some limited qualitative data exists. This paper reports on studies of the benthos undertaken in the period 1994–1996. Short term effects of fishing on the benthos were investigated by means of samples taken both before and shortly after (c. 24 h) fishing activity. Studies of the medium to long term effects involved sampling the fauna of areas around wrecks (i.e. unfished pseudo-control sites) for comparison with fished grounds. From the available data, attempts were made to calculate the short, medium and long-term impact of the fishery on the benthos and surrounding environment. Direct (short-term) effects were not quantifiable at a heavily fished offshore site (75 m water depth), however, some changes were visible in a shallow (35 m water depth), low fishing intensity site. The medium to long-term effects are more easily detectable at the offshore site, while only minor changes are visible at the in-shore location. It would appear, therefore, that it is fishing intensity *per se*, rather than simply the direct impact from passage of the gear, that constitutes the major factor controlling long-term negative trends in the benthos of the Irish Sea *Nephrops* grounds.

**A MECHANISM TO REDUCE PLASTIC WASTE AND INCREASE MUSSEL PRODUCTION.** M. D. Barry, All in a shell Ltd., Dooniskey, Lissarda, Co. Cork, Ireland.

Ireland's suspended culture of mussels is almost exclusively based on the use of polyethylene tubular meshes. This is cheap material that is used once and discarded at harvesting. It has been a very successful system but has limitations.

The yield of mussels at harvest is in the range of 3–5 kg/m. Disposal of used polyethylene meshes is a growing environmental problem. Ireland plans to double its suspended mussel production over the next 5 years.

It will not be possible to double the existing area of mussel farms because of competition for coastal resources. Consequently, it is necessary to increase the yield/hectare on sites where this is possible.

ALL IN A SHELL is testing the new "Mussel Ladder" patent of XPLORE PRODUCTS, of Glasgow, Scotland using biodegradable cotton mesh and binding materials.

The object is the development of a growing and handling system that will yield more mussels/m, be more suitable to new more exposed sites and solve the growing environmental problems of disposal of used polyethylene meshes. Early results in Ireland, Scotland and Norway are very encouraging.

**THE EFFECT OF SCALLOP DREDGING ON IRISH SEA BENTHOS: EXPERIMENTS USING A CLOSED AREA.** C. Bradshaw, L. O. Veale, A. S. Hill, and A. R. Brand, Port Erin Marine Laboratory, Port Erin, Isle of Man, IM9 6JA, British Isles.

A 2 km<sup>2</sup> area off the southwest coast of the Isle of Man (Irish Sea) has been closed to commercial fishing with mobile gear since March 1989. This area was heavily fished for *Pecten maximus* prior to closure, and the seabed immediately surrounding the closed area is still one of the most heavily dredged in the Irish Sea. Two methods have been used to study the effect of scallop dredging on the benthos in this closed area and adjacent fished areas. Firstly, divers have carried out visual transect surveys of the epibenthos regularly since closure. Secondly, biannual fine-meshed dredge and grab sampling of experimental plots inside and outside the closed area since 1995 has enabled comparisons of the benthic infauna and epifauna of experimentally dredged plots, undredged control plots and plots exposed to commercial dredging.

Since 1989, there have been consistent significant increases in the mean numbers of many species in the closed area, including *Pecten maximus* and *Luidia ciliaris*, and upward trends in numbers of hermit crabs, spider crabs and brittlestars have also been recorded. Conversely, the common starfish, *Asterias rubens*, appears to be decreasing in abundance. Communities of experimentally disturbed plots have become less similar to adjacent undisturbed control areas and more similar to commercially dredged areas. At each sampling date, similarity between samples was greater outside the closed area than inside.

These results present strong evidence that scallop dredging alters benthic communities and suggest that the closure of areas to commercial dredging may allow the development of more heterogeneous communities and allow the populations of some species to increase. A common problem with studying fishing disturbance is the lack of good control sites and this work also demonstrates the value of closed areas to scientific studies of bottom fishing.

**HOMARUS GAMMARUS "COLOURMORPHS" THEIR INCIDENCE IN THE INDIGENOUS POPULATION AROUND THE IRISH COAST AND THEIR POTENTIAL USE AS BIOLOGICAL MARKERS.** R. Browne<sup>1,2</sup> and J. P. Mercer,<sup>1</sup> <sup>1</sup>Shellfish Research Laboratory, Carna, Co. Galway; <sup>2</sup>Taighde Mara Teo., Carna, Co. Galway, Ireland.

The marking of Homarid lobsters is an important means of obtaining data on their ecology, biology, population dynamics, survival and fisheries management. To facilitate the recovery of marked individuals in the absence of a concerted sampling programme, they need to be highly visible for up to 10 years.

Occasionally odd coloured (red, blue, white or yellow) *H. gammarus* and *H. americanus* have been landed. These colour variants have a genetic basis. It is important however to distinguish between genetically blue lobsters from those which can result from inadequate feeding and unnatural holding conditions in the labo-

ratory or that result in the wild for various environmental factors. One of the defining features used to describe the appearance of colourmorphs in this study was the colour of the antennae. Blue colourmorphs have blue antennae unlike the variants of normal coloured lobsters that have redish brown antennae.

Biologists in the 1960's deemed that the genetic colour variants were potentially valuable as a biological marker for research. This poster presents the variants recorded in Irish waters and looks at their occurrence in wild stocks. Over a five year period (1993 to 1997) 8 "electric" blue, 7 red, and 4 white *H. gammarus* (CL > 85 mm) have been reported or delivered to the Shellfish Research Laboratory (NUIG). This gives a very approximate estimate from the national catch records of natural occurrence of 1: 566,000, 1: 647,000 and 1/1,132,000 respectively.

Before they can be used as markers fundamental issues need to be addressed. Their behaviour, survival, and potential impact on lobster stocks need to be evaluated.

**HISTORICAL OVERVIEW OF THE IRISH LOBSTER (HOMARUS GAMMARUS) AND INSHORE DECAPOD CRUSTACEAN FISHERY WITH REFERENCE TO EUROPEAN (H. GAMMARUS) AND NORTH AMERICAN (H. AMERICANUS) LANDINGS.** R. Browne, Taighde Mara Teo., Shellfish Research Laboratory, Carna, Co. Galway, Ireland.

The historical development of Ireland's (*Homarus gammarus*) fishery is reviewed and compared to the landings in Europe and the North American *H. americanus* fishery. Lobster fishing (*H. gammarus*) has provided an essential form of income for over a century to many Irish coastal communities. There have been large fluctuations in landings over this time. In 1994 landings reached an all time high of 714 tonnes, valued at IR£6.3 million. In 1997 landings were 513 tonnes valued at IR£4.5 million.

The Irish lobster fishery regulations are: a) Minimum carapace length of 85 mm b) Ban on the landing of lobsters that have been V-notched or which have a mutilated tail fan c) Prohibition of capture of lobsters by SCUBA diving, and d) Licensing of all fishing vessels. Included are chronological details on legislation and innovations relating to the inshore crustacean fishery sector. *H. gammarus* forms an integral part of a complex mixed inshore commercial fishery for decapod crustacea.

Landings of *H. gammarus* exhibit signs of an overexploited stock with declines in catch per unit effort. There is a general decline of total landings for *H. gammarus* throughout Europe. Historically the management practices used have not proved to be successful as is evident in the catches. In contrast with North America, where there is a plethora of regulations governing the *H. americanus* fishery, landings appear to be in a healthier state. To effectively manage the interrelated inshore crustacean fisheries fundamental biological issues need to be addressed. Enlightened measures based on factual information are required to sustain and develop the lobster (*H. gammarus*) fishery in Irish waters.

**A DESCRIPTION OF THE FISHERY FOR *PALAEOMON SERRATUS* (PENNANT) IN THE CONNEMARA (WEST OF IRELAND) AREA AND POTENTIAL MANAGEMENT METHODS.** R. Browne<sup>1</sup> and M. Norman,<sup>2</sup> <sup>1</sup>Taighde Mara Teo., Carna, Co. Galway, Ireland; <sup>2</sup>Shellfish Research Laboratory (NUIG), Carna, Co. Galway, Ireland.

The caridean prawn *Palaemon serratus* are locally known as shrimp. For this reason they will be referred to as shrimp in this report. *P. serratus* is a valuable component of the Connemara inshore fishery. This survey was undertaken by Taighde Mara Teo. and the Shellfish Research Laboratory (NUIG) to investigate the effort expended into the fishery and yield of shrimp. The information obtained came from fishermen, shellfish merchants and experimental fishing. Aspects of *P. serratus* life cycle and biology are also discussed.

The shrimp *P. serratus* fishery in Ireland was established in the mid 1970s and landings have steadily increased in volume up to the 1990s where they appear to have reached a plateau of over 300 tonnes. It has become an important ancillary activity for many inshore fishermen and a sole fishery occupation for some. In 1996, 398 tonnes of *P. serratus* were landed nationally valued at IRE£2.03 million (Dept. of Marine). Commercial fishing predominantly occurs along the south and west coasts in suitable bays. The fishing season is weather and location dependent starting in July/August, ending in February/March. Approximately 22 tonnes of *P. serratus* were landed by up to 41 boats between Slyne head and Barna (Connemara) in 1997. *P. serratus* were purchased by four merchants for IRE£7 to £9 per kg, and exported to the continent. Management strategies are discussed in the poster. These include the increase of pot mesh size and a closed season.

**A REVIEW OF INNOVATIONS CONCERNING IRELANDS LOBSTER (*HOMARUS GAMMARUS*) FISHERY (1992 TO 1998).** R. Browne<sup>1,2</sup> and J. P. Mercer,<sup>1</sup> <sup>1</sup>Shellfish Research Laboratory, Carna, Co. Galway, Ireland; <sup>2</sup>Taighde Mara Teo., Carna, Co. Galway.

Lobster (*H. gammarus*) are a valuable component of Ireland's mixed inshore fishery. Consistent fishing pressure due to buoyant market demand has established keen interest in their biology and ecology. It is generally perceived both by fishers and governmental bodies that the pressure on lobster stocks is increasing and stocks are likely to suffer. National landings have varied from 715 tonnes in 1994 to 513 tonnes in 1997. In the past seven years, a number of innovations have occurred in the Irish lobster fishery which are discussed in this paper.

Due to the concerns expressed about declining catch per unit effort by lobster fishermen, two separate stock enhancement projects with hatchery produced lobsters were initiated (SRL, Carna and Wexford Lobster Co-op. Carne). The systems used to culture stage I larvae to postlarvae involved using two species of phy-

toplankton and *Artemia* in vigorously aerated hoppers providing routine survival of over 40%. The techniques of postlarval nursery cultivation, transportation, release and potential marking methods are discussed. A summary of lobster production is provided.

Management of Irish lobster stocks in 1992 focused on a minimum size 85 mm (carapase length) and a ban on the capture of lobsters by SCUBA diving. The voluntary conservation practise of protecting selected reproductively mature lobsters by "V" notching their uropods began in 1993. In 1994 the Irish Lobster Association (ILA) was formed to represent regional organisations. The nationwide awareness campaign for management measures was strengthened by support from North American industry and scientific representatives. Also in that year legislation and governmental financial support served to give impetus to V notch programmes.

**SHELLFISH RANCHING IN THE UK.** C. A. Burton, J. T. MacMillan, and M. M. Learmouth, Sea Fish Industry Authority, Seafish Aquaculture, Marine Farming Unit, Ardtoe, Acharacle, Argyll PH36 4LD, UK.

There are six species of shellfish of commercial importance within the UK, which can be thought of as 'ranching' to varying degrees. Seabed ranching on natural habitats is where aquaculture and fisheries interact and some of the distinctions blur. Seafish, as part of its role in promoting the sustainable management of resources, is examining the current state of the industry.

Mussels (*Mytilus edulis*), Flat Oysters (*Ostrea edulis*) and, most recently, Cockles (*Cerastoderma edule*) are harvested as seed or part-grown animals and relayed on to controlled beds for on-growing to marketable sizes. This is the most extensive form of aquaculture. It is reliant upon natural spat fall and wild gathering for juveniles: the only intervention is to relocate the animals to more favourable growing areas. The move away from suspended culture in favour of on-bottom growing for King Scallops (*Pecten maximus*) has moved them from a semi-intensive to an extensively reared species. Hatcheries are in their infancy and most of the industry is reliant upon active collection of the wild spat as they settle from the plankton. These small juveniles are on-grown in rearing systems in the wild before being released on to suitable areas for growth to marketable sizes. Other than collection and protection of the juveniles, there is no other input. The species most often associated with the term 'ranching' is the European Lobster (*Homarus gammarus*). Egg-bearing females are gathered from the wild and transferred to controlled conditions in hatcheries where the larvae are hatched and the juveniles on-grown before they are released in to the wild. Until the recapture of the animals at market size as part of the wild fishery, there is no further human interference. Manila Clams (*Tapes philippinarum*) are a special case: as a non-native species, juveniles are produced in hatcheries and then seeded in to netted beds for on-growing under natural conditions. The netting protects the stock from predators and also provides containment.



### THE ROLE OF LOBSTER HATCHERIES IN RANCHING, RESTORATION AND REMEDIATION PROGRAMMES.

**C. A. Burton**, Sea Fish Industry Authority, Seafish Aquaculture, Marine Farming Unit, Ardtoe, Acharacle, Argyll PH36 4LD, UK.

Juvenile lobsters (*Homarus* spp) produced in a hatchery can be used for many purposes. Within the field of resource management the most likely uses are in ranching, restoration and remediation programmes. The decrease in the cost of animals, resulting from the commercialisation of the hatcheries, makes this increasingly viable.

**Ranching:** Successful trials in the UK proved that hatchery-reared juvenile lobsters could be released to the wild and that they would survive to market size. Their recapture in the commercial fishery and subsequent data demonstrated that these animals could have a positive effect on landings. The extension of UK legislation to confer protection on seeded stocks opened the way for ranching initiatives to progress. Sustainable resource management is also promoted by these projects. Other programmes within Europe and elsewhere, were able to draw upon these results to support their own work.

**Restoration:** Natural or anthropogenic environmental events can bring lobster stocks in an area to the brink of extinction. One location where this has occurred is the northern Adriatic Sea. Here, Italian researchers are using hatchery techniques to produce juveniles from available native broodstock. Initially, the animals are being on-grown and then released in to protected areas. They will be monitored and, if successful, the programme may be extended. Ultimately, it may restore not only the lobster population but also the fishery to health. Genetically distinct or other unique populations could be protected and restored in this way. This initiative has direct parallels with captive breeding and conservation efforts with terrestrial and avian species.

**Remediation:** Where human activity, for example, major civil engineering projects, directly affects the marine environment, it runs the risk of damaging fisheries. Sessile or habitat dependant species such as molluscs and crustacea are more frequently disadvantaged than mobile fish. Creation of suitable 'new' or 'alternative' habitat and stocking with hatchery derived juveniles may form part of the compensation package negotiated with fishery interests. Where appropriate, the design of the structure can be modified to produce the desired habitat. In other areas, where habitat has been destroyed (e.g., by dumping or dredging), a purpose-designed artificial structure may be used. Economic considerations have been cited for the non-progression of schemes in the past, but as costs fall, the balance may alter. Lobsters are a prime species for use in this way.

### THE ROLE OF THE MANILA CLAM, *TAPES SEMIDECUSSATUS* AS A TOOL IN ESTUARINE SEDIMENT TOXICITY ASSESSMENT.

**P. A. Byrne** and **J. O'Halloran**, Dept. of Zoology & Animal Ecology, National University of Ireland, Cork.

Increasing emphasis is being placed upon chemical analysis of sediments to determine the distribution and concentration of toxic

chemicals in aquatic environments. The resulting data are often used to characterise chemical accumulations, including delineation of 'hotspots.' These data alone, however, provide no information regarding the possible biological associations. Therefore, direct ecotoxicity testing is required.

Estuarine sediments frequently are repositories and therefore potential sources of anthropogenic contaminants. Many organic and metallic chemical compounds released into aquatic systems bind to particulates and so accumulate in the sediments, thus, sediments become repositories of contaminants in estuaries. These may also cause contamination through diffusion of porewater, re-suspension of particulates and dispersal of benthic fauna. There is a need to assess the biological affects of these anthropogenic contaminants because they may be toxic to infauna and bottomfish. Sediment toxicity bioassays are a means for carrying out such an assessment and primarily provide data on toxicity by measuring the effects on the test organism.

The Manila clam, *Tapes semidecussatus* was used to assess the ecotoxicity of estuarine sediments field-collected from five estuarine and coastal areas around the Irish Coast. The endpoints measured during the study were survival, behaviour, condition indices, biochemical and histochemical. Of these endpoints, the most sensitive were survival, behaviour and histochemistry. The potential of this cultured bivalve species as a sediment toxicity bioassay organism is discussed.

### DEVELOPMENT OF PCR AND *IN SITU* HYBRIDIZATION ASSAYS FOR DETECTION OF *BONAMIA OSTREAE* IN FLAT OYSTERS, *OSTREA EDULIS*.

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Rapid and sensitive methods for the detection of pathogens are needed for successful shellfish culture and restoration. Flat oysters (*Ostrea edulis*) infected with the microcell parasite *Bonamia ostreae* were used to develop a polymerase chain reaction (PCR) assay that will be more sensitive, faster, and less costly than standard histology. Genomic DNA was extracted from hemolymph of a Maine oyster and the gill of an Irish oyster. Using the PCR and primers tuned to protistan rDNA, a single, identical amplicon of 528 bp was obtained from both samples. This product spanned 341 bp of 18S rDNA and 187 bp of ITS1, and was determined by BLAST search to closely resemble rDNA genes belonging to members of the Phylum Haplosporidia. A PCR reaction specific for this sequence was designed and used to assay gill tissue of 71 Irish oysters independently scored for *B. ostreae* infections using hemolymph smears. A product presumed to be *B. ostreae* sequence was generated in 100% of heavily infected oysters; 73% of moderately infected oysters; 50% of lightly infected oysters; 0% of

scarcely infected oysters; and 3% of those apparently uninfected. Under these reaction conditions amplification was strongest when infection intensity was high, i.e., the relative amount of contaminating oyster DNA was low. Fluorescent *in situ* hybridization was used to confirm that the PCR product belonged to *B. ostreae*. Improvement of the sensitivity of both PCR and *in situ* diagnostic methods is ongoing.

**OYSTER REEFFUNCTION, ENHANCEMENT, AND RESTORATION: HABITAT DEVELOPMENT AND UTILIZATION BY COMMERCIALY- AND ECOLOGICALLY-IMPORTANT SPECIES.** L. D. Coen,<sup>1</sup> R. E. Giotta,<sup>1</sup> M. W. Luckenbach,<sup>2</sup> and D. L. Breitburg,<sup>3</sup> <sup>1</sup>Marine Resources Research Institute, SCDNR., Charleston, SC 29412, USA; <sup>2</sup>Virginia Institute of Marine Science, Eastern Shore Lab, P.O. Box 350, Wachapreague, VA 23480, USA; <sup>3</sup>The Academy of Natural Sciences, Estuarine Research Center, St. Leonard, MD 20685, USA.

Marine and estuarine habitats (e.g., seagrasses, mangroves, and salt marshes) have received a great deal of attention for their value as important habitats and hence of late have been protected. In contrast, oysters and the habitat they generate have been viewed historically to have value only as a resource. Consequently, virtually unregulated resource extraction has resulted in the significant destruction of both subtidal and intertidal oyster reefs. Traditional restoration efforts, therefore, have been directed at enhancing oyster landings depressed by either overharvesting or reduced environmental quality, via methods ranging from improving water quality to substrate addition, but all with the goal of resource enhancement. Of late, new emphasis has been placed on their direct and indirect ecosystem services, including filtering capacity, benthic-pelagic coupling, nutrient dynamics, sediment stabilization, provision of habitat, with a concomitant shift to restoring/enhancing ecological function. We discuss current restoration efforts in the U.S., their methods and goals and briefly review the findings and recommendations. We also discuss some of the issues associated with realizing the broader goal of ecological restoration, particularly in light of evaluating of oyster reefs as critical or Essential Fish Habitats (=EFHs), including current work comparing oysters with marsh and mudflats tidal creek habitats in SC. Finally, we review the impact harvesting and die-offs have on oyster reefs using experiments examining recruitment, growth, and survival of oyster spat as indicators of restoration potential by following recovery of manipulated sites after simulated harvesting, repletion, and/or major population die-offs.

**SOME GENETIC CONSIDERATIONS OF SHELLFISH RANCHING: A CASE-STUDY OF THE ABALONE, *HALIOTIS MIDAE* IN SOUTH AFRICA.** P. A. Cook and N. A. Swejld, Department of Zoology, University of Cape Town, South Africa.

Several recent studies, where molecular genetic techniques and markers have been applied to marine organisms, have revealed

various levels of population structure that were not predicted from previous studies. Some results have suggested that recruitment patterns may be relatively localised, even in broadcast spawners with pelagic larval stages. This has important implications for stock recognition and the delineation of biogeographic boundaries and may become more important as efforts at shellfish restoration or enhancement increase.

Abalone ranching and enhancement is being attempted in several countries, with varying degrees of success having been reported. These attempts have stimulated discussion on ecological and genetic implications and widely differing views have been put forward. A synthesis of this discussion will be presented and a specific case study will be reviewed.

The South African abalone, *Haliotis midae*, is distributed along at least 2000 Km of coastline, spanning three recognised biogeographical zones, but is currently managed as a single stock. A mtDNA analysis of wild and hatchery populations revealed at least two (possibly three), major genetic sub-divisions of the population. This is in contrast to results of a previous study that used more traditional methods (allozymes), which did not reveal any sub-divisions. The two stocks of abalone identified (east and west of Cape Agulhus), differ in terms of both haplotype frequencies and genetic diversity. Hatchery cohorts from two farms (one using east coast broodstock and one using west coast broodstock) both revealed significantly reduced genetic diversity and skewed haplotype frequencies relative to their source populations.

The implications of these results for mariculture and ranching of the South African abalone are discussed and their possible applicability to wider shellfish restoration issues is reviewed.

**SEASONALITY OF ECHINOID BEHAVIOUR IN A MARINE 'ISLAND,' LOUGH HYNE, IRELAND.** A. C. Crook and D. K. A. Barnes, Dept. Zoology & Animal Ecology, University College, Lee Maltings, Cork, Ireland.

Many echinoid species have been observed to display migratory and so-called 'covering' behaviours. The functional significance of these has yet to be quantified although some of the most popular theories have interpreted them as an adaptive response to avoid over exposure to light and/or as an anti-predator strategy. Predation pressure and light intensity may be seasonal as well as diurnal in nature but were not investigated in the context of echinoid behaviour until the present study. The aim of our research was therefore to examine, *in situ*, the potential seasonality of both diurnal migration and covering behaviour in a population of the purple sea urchin, *Paracentrotus lividus* at Lough Hyne, Co. Cork, Ireland. This is a species of commercial importance and one that has suffered severe declines in its natural population density through overfishing, particularly in the Mediterranean. At Lough Hyne, *P. lividus* has also undergone dramatic population fluctuations but these are not believed to be a response to overfishing.

Our results showed significant seasonal trends in the proportion of individuals displaying covering behaviour, the mean number and proportion of available items used to cover and the availability of covering items in *P. lividus* at Lough Hyne. In addition, a significant diurnal and seasonal pattern was found for *P. lividus* migratory behaviour. Predator density and light intensity were also shown to vary seasonally at Lough Hyne. A quantitative assessment of variables that may influence individual behaviours and thus subsequent growth patterns and survival may be of importance to the aquaculture industry.

**LIVING WITH BONAMIASIS: IRISH RESEARCH SINCE 1987.** S. C. Culloty and M. F. Mulcahy, Department of Zoology and Animal Ecology, National University of Ireland Cork, Lee Maltings, Prospect Row, Cork, Ireland.

The European flat oyster, *Osrea edulis*, was the major species of oyster grown in Ireland until 1987, when, following significant mortalities, the parasite *Bonamia ostreae* was diagnosed in oysters in Cork Harbour. Since then the parasite has been detected in a number of other major oyster-growing areas also. The impact of the disease, together with the field- and lab-based investigations and trials since that time are reviewed, with particular reference to the biology and specificity of the parasite and the development of resistance in oysters bred from survivors.

**AN ASSESSMENT OF THE POTENTIAL FOR THE SUSTAINABLE DEVELOPMENT OF THE EDIBLE PERIWINKLE, *LITTORINA LITTOREA*, INDUSTRY IN IRELAND.** V. Cummins,<sup>1</sup> N. Connolly,<sup>1</sup> and G. Burnell,<sup>2</sup> <sup>1</sup>Coastal Resources Centre, University College, Cork; <sup>2</sup>Zoology Department, University College, Cork.

The industry created from harvesting the common periwinkle *Littorina littorea* is valuable both in terms of employment and export earnings. Approximately £5 million worth of periwinkles is exported annually, primarily to France.

Little information is available on the common periwinkle *Littorina littorea* in Ireland with no scientific information available on the quantities harvested per year, or the impact of this harvest on the populations of periwinkles around the coast. One of the biggest problems facing the industry is its almost complete lack of regulation. In addition, many wholesalers have suggested a possible over-harvesting of the resource. They are finding it increasingly difficult to obtain medium to large sized winkles (greater than 16 mm in height), which the continental market requires.

There is clearly a need for a comprehensive study to review the current state of Irish periwinkle stocks. This project, currently in its second and final year, aims to assess the sustainable development of the edible periwinkle industry in Ireland. This is being

achieved by conducting an industry review in conjunction with a national stock assessment programme. Surveys of harvested shores show correlations between factors such as substrate type and periwinkle densities. For example, although occasionally found in sandy patches, periwinkles favour a stable substrate. Relationships also exist between shell height and distribution according to biological zones on the shore. Other results show that winkle densities are effected by exposure, with the highest density of winkles occurring in moderate environments.

The industry review indicates that ninety five percent of wholesalers would support a closed season to give the periwinkles a chance to grow, with the suggestion of a summer season receiving the most backing. The data accumulated throughout this project will be incorporated into a GIS. This will assist in creating an appropriate management strategy for the industry.

**RECOVERY OF INTERTIDAL MUSSEL BEDS IN THE WADDENSEA AFTER LARGE SCALE DESTRUCTION.** N. Dankers, A.G. Brinkman, A. Meijboom, and J. Zegers, Institute for Forestry and Nature Research, P.O. Box 167, 1790 AD Den Burg, Texel, The Netherlands.

Intertidal musselbeds almost disappeared from the Waddensea in the 1980s. The major cause was fisheries, combined with a period of low spatfall. In the three Waddensea countries different management options were adopted in order to reach the 'ECOTARGET', 'a larger area and more natural distribution of intertidal mature musselbeds.'

In Denmark large areas were closed for fisheries, and musselbed area is increasing. In Schleswig Holstein, the whole intertidal was closed and development of beds looks promising. In Lower Saxony, beds are still under fishery pressure which gave the opportunity to study the fishery impact. In the Dutch sector a co-management option has been developed. Some areas are closed; in the rest of the intertidal, areas with high potential for development of stable beds are left undisturbed by fishermen. A number of developing beds has been studied over at least 6 years. Surface area of individual beds did not change much, horizontal and vertical structure increased, several age classes are present, stability of the beds increased and in all beds spatfall was a common phenomenon, also when spatfall on the surrounding flats was limited. Based on the locations of mature beds in the past, and physical characteristics of these areas (exposure time, wave action, sediment characteristics etc) a model was developed in order to predict which areas have a high potential for the development of mature beds. The maps constructed on the basis of the model are used in sectoral management plans for closing areas for seed fisheries and restoration programs for mussel beds.

**ABALONE RANCHING IN SOUTH AFRICA: HOPE FOR SUSTAINABLE ABALONE PRODUCTION?** S. De Waal, N. Sweijd,<sup>1</sup> B. Godfrey,<sup>2</sup> P. Britz,<sup>2</sup> and P. Cook,<sup>1</sup> <sup>1</sup>Department of Zoology, University of Cape Town, Rondebosch 7700, South Africa; <sup>2</sup>Department of Ichthyology and Fisheries Science, Rhodes University, P.O. Box 94, Grahamstown 6140, South Africa.

South Africa's commercial abalone fishery for the perlemoen (*Haliotis midae*) is in crisis due to over-exploitation caused by large scale poaching, increasing recreational fishing effort and ineffective law enforcement. This threatens the livelihood of people currently employed in the industry, as well as the effective implementation of South Africa's Marine Living Resources Act which aims to achieve equity in access rights to fishery resources. Land-based abalone mariculture has been successfully implemented at several locations along the South African coastline, and subsequently, abalone seeding with hatchery produced spat is being considered both for stock enhancement and commercial abalone ranching. Abalone ranching offers an incentive-based mechanism for co-management of defined areas, and hope for sustainable abalone production. The assumption is that 'ranchers' will have an incentive to protect the seeded areas against poaching. Pilot abalone ranching trials are being conducted in heavily poached areas on the South African East coast (Indian Ocean), as well as on the West coast (Atlantic Ocean) where abalone do not occur naturally. Effective protocols for seeding hatchery reared abalone have been developed, and results to date indicate that commercially viable growth and survival is achievable. Genetic studies (using mtDNA and microsatellite DNA markers) are being conducted on both wild and hatchery populations in order to assess the genetic implications of ranching. These studies aim to contribute to the development of appropriate hatchery genetic management programmes and also to evaluate the potential of genetic tags as a tool to assess survival.

**SHORT-TERM IMPACT OF BLUE MUSSEL DREDGING (*MYTILUS EDULIS* L.) ON A BENTHIC COMMUNITY.** P. Dolmer, T. Kristensen, M. L. Christiansen, M. F. Petersen, P. S. Kristensen, and E. Hoffmann, Danish Institute for Fisheries Research, Charlottenlund Castle, DK-2920 Charlottenlund, Denmark.

The short-term effect of mussel dredging in a brackish Danish sound was studied. A diver identified a commercial dredging track and an analysis of the species composition inside the track and at an adjacent control area showed that dredging changed the community structure by reducing the density of small polychaetes. In order to investigate the extent and the duration of the dredging impact experimental dredging was conducted. The experimental dredging removed 50% of the mussels in the two dredged areas. Immediately after dredging, a significantly lower number of spe-

cies was measured inside the mussel beds in dredged areas compared to control and boundary areas. This effect lasted for at least 40 days. The analysis of the species composition showed that the dredged area had a significantly lower density, particularly of small polychaetes compared to the boundary area. An increased number of species was recorded outside the mussel beds just after dredging, but this effect lasted for less than 7 days. After dredging, brown shrimps, *C. crangon* invaded the dredged areas. This species is an important predator of smaller invertebrates, and it is suspected that it was feeding on small vulnerable polychaetes exposed at the sediment surface after dredging. The dredging process was observed to form 2–5 cm deep furrows in the seabed, but the sediment texture and the organic content of the sediment were not affected. The biomass accumulation of individual blue mussels was significantly lower in the dredged area compared to the boundary area. This indicates that the disturbance of the mussel bed structure reduced growth and that the lowering of intraspecific food competition caused by a reduced density of mussels did not increase the accumulation of biomass in the mussels that remained in the dredged area.

**INTEGRATING FIELD AND REMOTE SENSING DATA—AN EXAMPLE FROM A HARMFUL ALGAL BLOOM EVENT.** D. L. Eslinger,<sup>1</sup> M. E. Culver,<sup>1</sup> P. Tester,<sup>2</sup> M. Soracco,<sup>1,3,4</sup> and K. Waters,<sup>1</sup> <sup>1</sup>NOAA NOS Coastal Service Center, 2234 South Hobson Ave., Charleston, SC, 29405, USA; <sup>2</sup>NOAA NOS Center for Coastal Fisheries Habitat Research, Beaufort Laboratory, 101 Pivers Island Rd., Beaufort, NC, 28516, USA; <sup>3</sup>Systems Engineering and Security, 7474 Greenway Center Drive, Greenbelt, MD, 20770 USA; <sup>4</sup>NOAA NESDIS Office of Satellite Data Processing and Distribution, FB4, Suitland, MD, 20746, USA.

Several National Oceanographic and Atmospheric Administration (NOAA) offices are developing applications which integrate a variety of field observations with remotely sensed data, with a goal of increasing our understanding of biological and physical processes in the upper ocean. Although coastal managers have a need for timely, accurate information about events such as harmful algal blooms, they may not have the sophisticated computer technology needed to easily utilize new remote sensing data sources. We are developing methods of delivering integrated data sets over the world wide web and in geographical information system environments.

We will demonstrate some of these integrated data products, including the integration of surface wind, temperature, currents, and bathymetry data with remotely sensed sea surface temperature and chlorophyll fields. Examples from a harmful algal bloom will be shown.

**TOOLS FOR TURNING THE TIDE OF DETERIORATING WATER QUALITY IN SHELLFISH GROWING AREAS: A DECADE OF EXPERIENCE IN PUGET SOUND, WASHINGTON STATE, USA.** D. Fagergren, Puget Sound Water Quality Action Team, P.O. Box 40900, Olympia, WA 98504-0900, USA.

Shellfish production and consumption in the Pacific Northwest has historic and economic significance. In the mid 1980s major acreage was downgraded due to non-point source pollution from a rapidly-growing, human population along the shores of Puget Sound.

Local governments, tribes, state and federal agencies, shellfish growers and citizens combined forces to halt the decline of water quality. These watershed efforts focused primarily on correcting failing on-site (septic) systems, decreasing polluted stormwater runoff and improving animal keeping practices.

Turning the tide didn't come easily; many of the planning efforts were funded through new state grants, but heavy competition for these funds resulted in stalled efforts in implementing actions at the local watershed level.

In 1992, shellfish protection legislation was passed to make mandatory the formation of programs and districts, funded by local fees, whenever a downgrade in growing waters occurred. A shellfish closure response strategy was also employed. Progress was slow at first; in some instances well-organized citizen opposition fought the mandatory districts designed to identify and correct fecal coliform problems.

Over time, many downgraded areas began to show signs of improvement, and in 1998 a record four major commercial growing areas, approximately 4500 acres, were officially upgraded. This represents 21% of the total acreage downgraded in the past decade (1989–1998), and 42% of the total acreage upgraded during that time. Another tool, the 'early warning system', was developed to alert local governments, shellfish growers and citizens in advance of a downgrade and was designed to jump-start corrective actions.

In early 1999, three areas are threatened by downgrades, illustrating the difficult and continuing challenge of protecting water quality. Changing priorities for natural resource protection and competition for scarce monetary resources have also compromised our ability to deal with long-term corrective actions designed to preserve and restore important shellfish growing waters.

**A NEW FISHERY FOR RAZOR CLAMS (*ENSIS SILIQUA*) ON THE EAST COAST OF IRELAND.** E. Fahy, Marine Institute, Fisheries Research Centre, Abbotstown, Castleknock, Dublin 15.

An investigation of the biology and the fishery for razor clams (*Ensis siliqua*) on the most important bed to have been discovered to date in Ireland, provides basic information on which a manage-

ment plan for sustainable harvesting of this resource might be devised. The clams occur with a number of common interstitial invertebrate species. The clams range in age between 0 and 19 years-old. Males grow at a faster rate than females and the largest animals on the bed are males. These findings are in general agreement with what has been discovered of the biology of the species elsewhere but there are some significant differences. The Gormanstown clams appear to be slower growing than the species in Britain or in Portugal where it has been investigated in greatest detail. The characteristics of the gonadal cycle are fairly similar but *Ensis siliqua* spawns later in the year at Gormanstown than off the Portuguese coast. It is reckoned to reach first maturation at three to four years of age at Gormanstown, one in Portugal.

Samples for the biological investigations were provided by commercial vessels whose skippers and the processors also cooperated in making documentation available. The average age of clams captured in 1998 was 9.34 years; the following year the average age had fallen to 8.34 years and a cohort (?) of very small individuals were encountered for the first time.

The bed at Gormanstown has been estimated from GPS data to be 2100 hectares in extent. It is situated between the 7 m depth isopleth and it runs into the intertidal area. The original clam biomass of the bed has been calculated using data on landings which incorporates figures on rejection rates provided by processors and from breakage rates observed in the samples obtained from the commercial boats—at 1500 tonnes. To the beginning of July 1999 it is estimated that more than 1,000 tonnes of clams have been removed. This bed is believed to have supplied virtually the whole Spanish market for razor clams during the past two years.

**ATTEMPTS TO ALLEVIATE FISHING PRESSURE ON STOCKS OF BROWN CRAB (*CANCER PAGURUS*) CAUSED BY THE WHELK FISHERY IN THE SOUTH WESTERN IRISH SEA.** E. Fahy, Marine Institute, Fisheries Research Centre, Abbotstown, Castleknock, Dublin 15.

The fishery for brown crab has, in keeping with the trend for inshore fisheries generally, greatly intensified in recent years. The animals are harvested for their claws in the spring and whole meat in the autumn. An important by-product for this fishery is bait for the whelk fishery and this use of brown crab is general wherever whelk are fished in Britain and continental Europe.

Crabs are sold to the processors who also buy whelk and the crab is exchanged in the ports for whelk supplied to the factory. Crab bait is estimated to weigh 7% of the whelk harvested, although where the stocks of whelk have been run down, the crab bait ration remains the same and it can amount to 20% of the whelk catch by weight.

At the peak of the fishery it was estimated that some 470 tonnes of brown crab were going into the whelk fishery each year causing concern among crab fishermen and conservation authorities alike.

Much of the crab is supposed to originate in the claw fishery which involves discarding the bodies of the animals. However, examination of the bait used revealed that a large proportion of the crab used as bait still had claws attached. There have been reports of undersized crab being used as whelk bait and of fishing effort directed on brown crab for use as bait only.

The thrust of the work described here is to examine alternatives to the present combinations of crab and dogfish. Two lines of enquiry were pursued: one sought alternatives among fish offals of various kinds. Whitefish, particularly cod, proved almost as good. Pelagic species (herring, scad) did not yield acceptable results. The other was to test an artificial bait incorporating some crab meat among a list of other ingredients but reckoned to reduce demand for brown crab by some 60%. Results from these trials did not differ statistically from trails using natural crab bait rations.

The brown crab fishery will always produce a discard and it is desirable to utilize it in some way. There is a thin line between this and the abuse of the fishery leading to inevitable over-exploitation.

**AZASPIRACID: DETECTION OF A NEWLY DISCOVERED PHYCOTOXIN *IN VITRO*.** A. F. Flanagan,<sup>1,2</sup> M. Kane,<sup>2</sup> J. Donlon,<sup>1</sup> and R. Palmer.<sup>2</sup> <sup>1</sup>Department of Biochemistry, National University of Ireland, Galway, Ireland; <sup>2</sup>National Diagnostic Centre, BioResearch Ireland, National University of Ireland, Galway, Ireland.

Azaspiracid is the name given to a previously unknown phycotoxin responsible for an outbreak of shellfish based diarrhetic food poisoning in Holland in 1996. Although azaspiracid was previously classified as a diarrhetic shellfish poison (DSP), azaspiracid toxicity has since been classified into a class of its own, called azaspiracid poisoning (AZP). Animals exposed to azaspiracid by intraperitoneal injection react differently than those exposed to other DSPs. The marine azaspiracid response is characterised by hopping, scratching and progressive paralysis leading to death of the animal within 60–90 min.

The development of alternative diagnostic strategies for the detection of phycotoxin contamination in shellfish is driven by scientific, ethical and financial concerns. To address this, an assay has been developed based upon the cytopathological responses of cultured mammalian cells to okadaic acid type toxins. The primary response of these cells to any of the okadaic acid family of toxins is to 'round-up' and lose their distinctive morphology. This effect, which is evident less than three hours after sample application, is due to the action of toxins as phosphoprotein phosphatase inhibitors. Cells treated with okadaic acid type toxins don't exhibit any significant decrease in viability until more than 48 h post toxin application. Azaspiracid does not cause the 'rounding up' effect on cultured cells. Cells exposed to azaspiracid exhibit a slight morphological

change (the cells appear to 'crenate' slightly) but their cellular viability, as measured by an MTT assay, drops to less than 90% of the viability of control cells after 18–24 h. Combining cell morphology observation at three hours with 24-h viability measurement enable the detection of both okadaic acid type toxins and azaspiracid in shellfish.

**A MONITORING TOOL FOR ASSESSING OYSTER PERFORMANCES IN DIFFERENT FARMING AREAS: THE IFREMER REMORA NETWORK: METHOD AND FIRST RESULTS (1993–1998) IN BRITTANY (FRANCE).** P. G. Fleury, E. Goyard, J. Mazurié, S. Claude, J. F. Bouget, A. Langlade, and M. J. Le Coguic, IFREMER, Regional Shellfish Laboratory of Brittany, 12 rue des résistants, F-56470 La Trinité-sur-mer, France.

The network IFREMER/REMORA has watched every year, since 1993, from February to December, mortality, growth and quality criteria of two oyster class-size ("juveniles" of the first-year class and "adults" of the second year class), distributed among various stations of the French oyster areas. This has provided a standard and simple annual assessment of the rearing results, generally tallied with professional observations. From year to year, data series have been obtained, from which mean values (references), chronological trends or spatial differences can be analysed. Results in Brittany, from 1993 to 1998, show that, in a "normal" year, 30 g oysters grow to 60–80 g at the end of the year, with a mortality rate of 10–15%. Mortality occurs mainly in spring for adults and in summer for juveniles. Beyond annual variations, mainly connected to climatic and hydrological influences, differences between monitoring stations remain high, which gives data for characterization of sites. On the other hand, unusual mortalities (in 1995), lack of growth (1998) or problem of quality (infestation by the worm *Polydora*) could be pointed out and quantified. Moreover, unusual results could be observed, which might not be explained only by annual trends or geographical specificities. This gives REMORA network a role of alert and advice in the collective oyster management. Lastly, REMORA data, especially when linked to climatological or hydrological data series, may allow studies in many areas: description of oyster quality, biological indicators in coastal water monitoring, explanatory models of the oyster-farming ecosystems.

**THE HARD CLAM TASK FORCE.** G. E. Flimlin, Jr., Rutgers Cooperative Extension, NJ Sea Grant Marine Extension Program, 1623 Whitesville Rd., Toms River, NJ 08755 USA.

Since 1996, Gef Flimlin has been the Organizer for a group of Industry, Research Scientists, Resource Managers, and Extension Personnel who have an interest in the wild harvest and aquaculture of clams. The Hard Clam Task Force (HCTF) was formed because there was a prevailing perception among many in the "Northern

Quahog" *Mercenaria mercenaria* industry that clam harvests coast wide have been in decline for several decades, yet researchers and industry felt frustrated that little money has been directed toward this organism. Initially the group comprised of representatives from Atlantic Canada to Florida, however it was decided to include West Coast representatives working on Manila clam (*Tapes japonica*) since many of their issues are common with those surrounding. The group has identified several focus areas where a positive impact could be felt: the need to attract research money, serve as an effective conduit to standardize sampling protocols and data collection efforts so that results can be comparable, identify a number of research priorities that are designed to fill the information voids, and to use the Internet to leverage the expertise of our members and use each other as resources for experimental design, information resources, and sources of ideas for rehabilitation, education and outreach in the field of hard clam research.

Using the expertise of other members, the agent has begun a stock restoration program for hard clams in New Jersey with Industry assistance. There have also been several other groups formed from the membership which address topics such as a Stock Assessment Group, Hydroacoustic Study Group, an Interstate Transport Issues Group, and a decision to bring more industry to the table to participate in the process, probably through a large regional meeting. The Task Force aims to marry science and management with contributions from academia and industry, just as the ICSR does.

**THE INFLUENCE OF DREDGE DESIGN ON THE CATCH OF *CALLISTA CHIONE*.** M. Gaspar,<sup>1</sup> L. Chicharo,<sup>2</sup> M. D. Dias,<sup>3</sup> P. Fonseca,<sup>4</sup> A. Campos,<sup>4</sup> M. N. Santos,<sup>1</sup> and A. Chicharo,<sup>2</sup> <sup>1</sup>Instituto de Investigação das Pescas e do Mar, Centro Regional de Investigação Pesqueira do Sul, Av. 5 de Outubro, 8700 ñ 305 Olhão, Portugal; <sup>2</sup>Universidade do Algarve, UCTRA, Campus de Gambelas, 8000 Faro, Portugal; <sup>3</sup>Instituto de Investigação das Pescas e do Mar, Del. de Setúbal, Av. Jaime Rebelo 29 A, 2900 ñ 409 Setúbal, Portugal; <sup>4</sup>Instituto de Investigação das Pescas e do Mar, Av de Brasília, 1400 Lisboa, Portugal.

An important bivalve fishery takes place along the Southwest coast of Portugal. At present, in this part of the Portuguese coast, the most important commercial species is the clam *Callista chione*. This species constitutes the target of a specific fishing activity carried out by an artisanal fleet. Dredges are the gear used in this fishery. The shape and structure of the Portuguese dredge has been maintained constant throughout time and consists of a small, heavy semicircular iron structure, with a net bag and a toothed lower bar at the mouth. Welded to this iron structure are three metal shafts forming a kind of hen's foot where the towing cable is attached. This year, some fishermen from the region of Setúbal, have been trying to introduce a new type of dredge in the fishery, debating, therefore, being a gear that maintaining the fishing efficiency relatively to the target species, carries less damages to the accompa-

nying species. The basic difference between both dredges resides in the retention structure of the bivalves. In fact, in the new dredge, the net bag is replaced by a rectangular metallic grid. To evaluate a possible introduction of this new dredge in the fishery, IPIMAR has conducted a study with the objective to compare the efficiency of the gears and to evaluate the impact produced by both dredges in the benthic community. The experiments were carried out during March 1999, on the Southwest coast of Portugal, from a site off Troia. Three tow duration 5, 10, and 20 minutes were investigated. A total of 24 hauls was completed, 4 for each tow duration and dredge. The experiments were conducted by attaching to the gear a cover bag with a 20 mm mesh. After each haul the catches in the bag and in the cover were sorted separately. All animals retained are allocated scores on a scale of 1–4 in which 1 equate to good and 4 equate to dead. The results obtained show that the catches coming from the traditional dredge (TD) are composed of a great fraction of juveniles of *C. chione*, where the new dredge (ND) are composed of a quite great number by individuals with a superior size to the minimum legal length (50 mm). This result is an indicator that the mesh of the bag of the TD used in the exploitation of this resource is not adequate. For the 3 tow duration, the mean fishing yield obtained for the ND was always superior to the TD, due to its great efficiency in capture. For all 3 tow duration, the impact caused by both dredges on the target species and upon the macrobenthic community in general (percentage of damaged individuals) was similar and low. The greatest advantage in the usage of the ND relatively to the TD, is to allow the smallest individuals (independently of the species) to escape rapidly through the metallic bars on the grid, increasing their probability of survival.

**THE AFFECTS THAT AMNESIC SHELLFISH POISONING HAS ON SCALLOP PROCESSORS AND COMMERCIAL FISHERMEN.** R. Gidney and J. Hermse, Scallop Dredging Association, 5 Boat Croft, Kemnay, Aberdeenshire, AB51 5GZ.

Algal toxin monitoring in Scotland is carried out by the Fishery Research Services, Marine Laboratory in Aberdeen. The monitoring is required under EU directive (91/492). There have been sea areas closed to commercial scallop fishermen due to Amnesic Shellfish Poisoning (ASP) for some 10 weeks (July–September 1999) now on the Scottish West Coast. Smaller closed areas have also been in force around the Orkney Islands and East of Aberdeen. In some cases the levels of ASP toxins have been extremely high (231 ug/g) when the statutory maximum level is 20 ug/g.

The ASP toxin is affecting the commercial fishing of king scallops (*Pecten maximus*) and queen scallops (*Aequipecten opercularis*). Fishermen and processors have been extremely diligent in observing and protecting the closed areas in order to preserve consumer confidence. However, the industry is becoming restless as the financial burden of such a lengthy ban begins to bite. The

size of closed areas, the integrity of sampling procedures, the testing methodology and the causes of the high levels of toxins are amongst the questions now being asked. A meeting of the Scottish Parliament (at which the SDA will make a presentation) will shortly debate the problem and make recommendations.

The Scallop Dredging Association (SDA) is suggesting a pooling of end product testing results and other resources with the scientific community in order to better co-ordinate the testing and sampling procedures. The SDA is requesting that research is required into the forecasting, causes and effects of Harmful Algal Blooms.

**THE INFLUENCE OF OYSTER CULTURE STRUCTURES ON BIRDS IN A SHELTERED IRISH ESTUARY. G. Hilgerloh, J. O'Halloran, T. Kelly, and G. Burnell, Dept. of Zoology & Animal Ecology, University College Cork, Ireland.**

In an estuarine bird feeding area on the south coast of Ireland (Cork Harbour) a commercial company had installed oyster growing tables (trestles), to which bags of oysters were attached. The tables were arranged in long rows separated by lanes left for the tractors. The study investigated birds in the shellfish culture area. The occurrence of the different species and their behaviour were compared to a nearby area free of aquaculture within the same estuary. First results showed that all species which occurred in the aquaculture free area could also be observed in the trestle-area. The most frequently observed species were oystercatcher *Haematopus ostralegus*, redshank *Tringa totanus*, dunlin *Calidris alpina*, curlew *Nuneniuss arquata*, black-headed gull *Larus ridibundus* and common gull *Larus canus*. The percentage of feeding birds per species did not differ in the two areas of comparison. However, there were significant differences in density in four of the six studied species.

**BREEDING PONDS AS A BASIS FOR FLAT OYSTER (*OSTREA EDULIS*) CULTURE AND THEIR USE TO DEVELOP RESISTANCE TO THE DISEASE *BONAMIA OSTREAE*. D. Hugh-Jones, Loch Ryan Shellfish Ltd., c/o the Thatched Cottage, Penberth St., Buryan, Penzance, England TR 6HJ.**

Flat oyster production in Cork over the last quarter of a century has relied entirely on its own production of spat from shore-based breeding ponds. Over this period, selection for fast growth has led to the routine production of market-size oysters of 70–120 g in 3 years.

Since the stock was almost totally destroyed by the disease, *Bonamia*, in 1987, the breeding objective has since concentrated on mass selection for resistance to the disease. This work, which in currently being evaluated in Ireland, France and Holland, appears to be showing promising results, with production in Cork Harbour, where oysters are exposed to the disease for their entire growing period, rising to as much as 80% of former levels.

**ARTIFICIAL REEFS FOR SHELLFISH HABITAT: RESULTS AND IDEAS TO DATE. A. Jensen, School of Ocean and Earth Science, University of Southampton Oceanography Centre, Southampton.**

Artificial reefs are used worldwide in a variety of roles: promotion of fishery catch, habitat protection and recreation being the most common. In Europe the majority of artificial reefs have been placed for habitat protection (*Posidonia* and other seagrass meadows) and developing finfish fisheries yield.

The use of artificial reefs for shellfish culture/ranching is in its infancy in Europe, existing as a reality in the Adriatic sea where mussel and oyster cultivation has become possible because of artificial reef deployment and as a ranching proposal in Northern Europe where several strands of the knowledge needed to promote lobster ranching have been established but still require some research before development can occur.

In the Adriatic Sea the combination of eutrophic, shallow water and significant natural mussel larval production have provided the conditions which have facilitated development of mussel and oyster cultivation in association with Italian 'pyramid reefs'.

In northern Europe the deployment of artificial reefs, development of lobster hatcheries and research showing the effective survival of hatchery reared lobsters and inclusion in a fishery are the basis for further work to make lobster ranching a reality.

This paper summarises the work undertaken in Europe (and elsewhere) to date and speculates about the future possibilities of promoting shellfish cultivation by using artificial reef technology.

Japanese workers have pioneered the use of artificial reefs for abalone habitat and developed coastal structures to influence larval settlement. In order to make artificial reefs an economic reality in Europe, we must learn from their example, particularly in the large scale of their operations.

**THE COMMERCIAL POTENTIAL OF THE COMMON SEA URCHIN *ECHINUS ESCULENTUS*. M. S. Kelly, P. Pantazis, and P. Owen, Scottish Association for Marine Science, PO Box 3, Oban, Argyll, PA34 4AD, Scotland, UK.**

As over fishing continues to delibitate world sea urchin populations there is increased interest in echinoculture as a means of meeting market demand for urchin roe. Inherent in this is the investigation of the commercial potential of species previously considered unviable. The regular echinoid *Echinus esculentus* is widely distributed around the British Isles. While islanders and maritime communities once considered this species as a food resource, the gonad is now generally considered to be of a poor quality and unsuited to the European palette. The skins of roe are however of a similar size to those of the Japanese species harvested for the domestic market. Therefore an investigation of its commercial potential was initiated.

A survey was conducted of gonad content and colour in *E. esculentus* from the Scottish west coast from June to August 1998.



The survey found the gonad content was variable in terms of weight and colour. A relatively small proportion of urchins (35%) had a gonad which was considered marketable in terms of both yield and colour. Urchins with a marketable yield of roe were found where algal growth or encrusting organisms were most prolific. Good roe colours and high roe yields were not necessarily coincident. Tank based diet trials were then conducted to assess the ability of this species to utilise pelleted feeds. *E. esculentus* were fed either a commercially available salmon feed, an artificial diet designed for sea urchins or a more natural macroalgal diet. The artificial diets enhanced gonad growth. The urchin diet also beneficially influenced gonad colour. The data suggest a fishery is likely to be impractical economically and could potentially be very destructive for the wild urchin population. A fully integrated culture approach is therefore the most feasible option for commercial exploitation of this species. An artificial diet could be used to enhance gonad growth, colour and flavour. The aim would be to produce urchins of test diameter 45–50 mm, within 2 years, with a premium quality roe export to the far eastern markets.

**ECOLOGICAL EFFECTS OF FISHERY DISTURBANCE TO OYSTER REEF HABITAT IN EASTERN NORTH AMERICA.** H. S. Lenihan<sup>1</sup> and G. W. Thayer,<sup>2</sup> <sup>1</sup>University of North Carolina at Chapel Hill, Institute of Marine Sciences, 3431 Arendell St., Morehead City, North Carolina, 28557; <sup>2</sup>National Oceanic Survey, Beaufort Laboratory, 101 Piveris Island Rd, Beaufort, North Carolina, USA, 28516

Eastern oysters, *Crassostrea virginica*, are ecosystem engineers that create biogenic reef habitat important to estuarine biodiversity, benthic-pelagic coupling, and fishery production in North America. Oysters and oyster reefs have declined dramatically over the last century due to overfishing, habitat destruction, reduced water quality, and/or the introduction of diseases. We provide evidence from experiments conducted in the Neuse River, North Carolina, that oyster loss results from interactions among multiple environmental disturbances. Our results indicate that destructive harvest practices reduce the height of oyster reefs, causing flow speeds across reefs to decrease. Reduced flow speeds on reefs caused increased sedimentation and decreased the quality of suspended food material for oysters. In turn, these factors helped explain why oysters on harvest-disturbed reefs grew slowly, were in relatively poor condition, had relatively high incidences of parasitism, and died at relatively high rates. Oysters on harvest-disturbed reefs located in deep water were exposed to bottom-water hypoxia/anoxia during estuarine stratification, killing oysters and other reef-associated invertebrates and demersal fishes. We also found that oyster reefs were utilized by 23 species of commercially valuable fishery organisms (fishes and the crab, *Callinectes sapidus*), many of which recruited to reefs and/or used them as foraging substrate. Fishes occupying harvest-damaged reefs in deep water were forced into shallow water during a hypoxic/

anoxic period. Escaping fishes accumulated in high densities on reefs in shallow water where they decimated prey populations. Therefore, indirect trophic interactions in undisturbed habitats result from multiple human disturbances in remote, disturbed habitats. In general, interactions among environmental disturbances imply a need for the integrative approaches of ecosystem management to restore and sustain estuarine habitat.

**THE INTEGRATION OF REMOTE SENSING DATA WITH LOCAL AND STATE MONITORING DATA.** D. L. Leonard, National Oceanic and Atmospheric Administration, National Marine Fisheries Service, Habitat Conservation Office, 1315 East West Highway, Silver Spring, MD 21401, USA.

The coastal states of the United States are all required under the National Shellfish Sanitation Program to prepare Marine Biotoxin Contingency Plans in anticipation of harmful algal events. In the last 15 years these HAE events have affected 20 of the 23 coastal states. The costs to the states and the industry are estimated at 50 million dollars annually. Emerging technologies such as remote sensing, spectral analysis, and internet access can provide an early warning system for states and industry while new laboratory methods can expedite identification of toxin type and levels. On the local level the mussel watch programs maintained by states and plankton sampling supported by volunteers can be used to verify the data received from the federal government. In response to the growing need for support the US HAB National Contingency Plan will use a National Clearinghouse for direct transfer of information and access to the technical expertise of federal and state agencies, academia, and private industry on a day-to-day basis and an early alert system for most toxic effects. A National Event Response Coordinator will alert all federal agencies of an event and arrange for the specific expertise and technical assistance to be provided on a national and local level by appropriate agencies.

**UNDERSTANDING THE FACTORS THAT INFLUENCE EUROPEAN LOBSTER RECRUITMENT: A TRANS-EUROPEAN STUDY OF COBBLE FAUNA.** A. Linnane,<sup>1</sup> B. Ball,<sup>1</sup> J. P. Mercer,<sup>2</sup> G. van der Meeren,<sup>3</sup> C. Bannister,<sup>4</sup> D. Mazzoni,<sup>5</sup> B. Munday,<sup>1</sup> and H. Ringvold,<sup>3</sup> <sup>1</sup>Martin Ryan Science Institute, National University of Ireland, Galway, Ireland; <sup>2</sup>National University of Ireland, Galway, Shellfish Research Laboratory, Carna, Co Galway, Ireland; <sup>3</sup>Institute of Marine Research, Austevoll Aquaculture Research Station, N 5392 Storebo, Norway; <sup>4</sup>CEFAS, Lowestoft laboratory, Pakefield Road, Lowestoft, Suffolk NR33 OHT, UK; <sup>5</sup>DIPROVAL, via del Guasto 5/B, Università Degli Studi di Bologna, 40126, Bologna, Italy.

The re-stocking or enhancement of Homarid populations has been the focus of several research projects in recent years. However, despite being able to successfully rear lobsters in captivity, there are few reports of newly settled European lobsters (*Hom-*

*marus gammarus*) in the wild. This is of serious concern for fishery managers when trying to ascertain if restocking or enhancement programmes are successful at the fishery level.

Based on published work from the eastern Atlantic seaboard cobble and boulder substrata have been identified as an important nursery habitat for early benthic phase (EBP) American lobsters (*Homarus americanus*). In Europe, cobble and boulders are utilised by adult lobsters yet little is known of the associated fauna of these substrata, primarily due to the difficulties involved in sampling such sites. This paper, based on an E.U. funded research project (LEAR), describes the results of quantitative airlift suction sampling from cobble habitat in Norway, the UK Ireland and Italy. Overall, crustaceans and molluscs were the most abundant species in all countries. While the collective densities of animals per m<sup>2</sup> of cobble were similar to that of the United States, the species diversity in Europe was significantly higher. Among the crustacea, the reptant decapods dominated at all sites. These findings tend to support the modern day "exclusion hypothesis" that competition and predation from other species limits the successful recruitment of EBP European lobster to the benthos. The results are being used to devise both models and experiments designed with the aim of providing a greater insight into the factors that influence the recruitment of *Homarus gammarus*.

**APPLICATIONS OF UNDERWATER IMAGING TECHNIQUES IN THE MONITORING AND RESTORATION OF COASTAL HABITATS.** U. Lobsiger and J. L. Manuel, Tris-Mar Research Inc., One Research Drive, Dartmouth, NS, B2Y 4M9, Canada.

Underwater imaging techniques are being applied in many applications of coastal zone management. A review of such techniques is presented with specific case studies and two novel developments that are particularly relevant to coastal shellfish resources will be discussed in some detail.

The relative merits of tethered video with surface display vs. autonomous image acquisition will be demonstrated using a number of habitat monitoring examples. They include: A winter survey of benthic energy dynamics in a high-Arctic inlet; the "Mermaid" remotely operated imaging vehicle; the "Video Grab", the "CAMPOD", as well as time lapse stereo and sediment-profiling photography of polychaete assemblages. Other examples include the direct observation of fishing gear in action with MANTA and in situ cameras on scallop rakes and fishing trawls. It will be further shown that quantitative sea-floor imaging can serve as ground-truthing for synoptic techniques, including side-scan sonar, RoxAnn, QT Seabed, and hyperspectral airborne imaging. Thanks to new technology developments in image acquisition, storage, communication and interpretation, novel applications with much better cost/benefit ratios of information value over logistical costs are now possible. Smaller size, lower cost, real-time options of

satellite and internet communication and powerful PC-platform image analysis software render imaging as a powerful adjunct and as a primary tool of investigation for many coastal habitat surveys and monitoring programs.

The MarineCanary™ (patent pending), a concept that uses the feeding behaviour of bivalve mollusks as keystone species of coastal ecosystem health will be discussed in detail. Behaviour patterns of a statistically relevant number of animals are monitored in situ with time lapse imaging over days and weeks, and behaviour is correlated with growth and reaction to contaminants and toxins.

A new ultra rugged and cost-effective digital time lapse camera system will be introduced.

**OYSTER REEF RESTORATION IN VIRGINIA, USA: REHABILITATING HABITATS AND RESTORING ECOLOGICAL FUNCTIONS.** M. W. Luckenbach, J. Harding, R. Mann, J. Nestlerode, F. X. Ó Beirn,<sup>1</sup> and J. A. Wesson,<sup>2</sup> <sup>1</sup>Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062, USA; <sup>2</sup>VMRC, 2400 Washington Street, Newport News, VA, USA.

Repletion efforts in response to declines in abundance of the eastern oyster, *Crassostrea virginica*, have historically relied upon transplanting of oyster seed and planting of a suitable settlement substrate. These efforts have generally failed to revitalize the fishery because they (1) failed to rehabilitate degraded reef habitat and (2) placed little emphasis upon reestablishing a population age structure capable of sustaining a self-supporting reef. More recently restoration efforts in Virginia have focussed on reconstructing 3-dimensional reef habitats and establishing brood stock sanctuaries with an emphasis on restoring lost ecological functions of reefs. Manipulative studies of reef placement, construction material and interstitial space have lead to the development of design criteria for maximizing oyster recruitment, growth and survival on constructed reefs. Further, we have characterized the successional development of resident macrofaunal communities on restored reefs and have begun to relate that development to specific habitat characteristics. Utilization of these restored reef habitats by transient species has been characterized through extensive field collections and underwater video observations; gut analyses of finfish are beginning to elucidate trophic linkages between the reefs and adjacent habitats. In addition, these structures appear important to the early developmental stages of juvenile fishes, some of which have considerable recreational and commercial importance. These studies are helping us to (1) clarify the ecological functions supported by oyster reef habitat, (2) define design criteria for reconstructing reefs and (3) establish success criteria for such restoration projects. While destructive fishing of oyster reefs appears inconsistent with meeting these goals, an emerging paradigm is that reef sanctuaries can be used to support desired ecological functions as well as supply recruits to adjacent areas which can be managed from a fisheries perspective.

**HABSOS: A PILOT PROJECT OF THE US GLOBAL OCEAN OBSERVING SYSTEM AND THE NATIONAL ASSOCIATION OF MARINE LABORATORIES.** T. Malone and C. Horn, Point Laboratory, UMCES, PO. Box 775, Cambridge MD 21613 USA.

Estuarine and coastal marine ecosystems are subject to convergent inputs of materials and energy from terrestrial, atmospheric, oceanic and anthropogenic sources that vary over a broad range of time-space scales. As a consequence, coastal ecosystems are experiencing unprecedented changes related to climate change, rapid increases in the number of people living near the coast, the exploitation of coastal resources, atmospheric deposition, and land-use practices in coastal watersheds. In this regard, there are some indications that the occurrence of "harmful algal blooms" is increasing in both time and space. However, the evidence for such a trend is not compelling and the causes of HAB events are poorly understood.

It has become painfully clear that the scarcity of observations on coastal ecosystems of sufficient duration, spatial extent, and resolution and the lack of real-time data telemetry, assimilation and visualization are major impediments to the documentation of pattern and to the development of a predictive understanding of environmental variability and change in coastal waters. This problem is particularly evident in the case of HABs. This paper describes an effort to network coastal marine laboratories and government agencies to design and implement an observing system for HABs (HABSOS) that will provide timely access to data and information on HAB events, the environmental conditions under which they occur, and their impacts. The goal is to systematically document the time-space dimensions of HAB events and their effects, to forecast their occurrence and issue alerts, and to mitigate deleterious effects.

**THE MANAGEMENT OF FRESHWATER CATCHMENTS.** P. McGinnity and K. F. Whelan, Marine Institute, Salmon Management Services, Furnace, Newport, Co. Mayo, Ireland.

The basis of holistic catchment management can be traced back over twenty years, but it is only during the past five years that its true significance has been recognised by resource managers. There is increasing recognition that the exploitation of natural resources such as the processes associated with agricultural production, forestry and other forms of resource usage impact negatively on lands and waters. This is well recognised in fisheries management. However, current responses to such phenomena are often poorly planned and lack a strategic dimension. The development of an alternative approach to resource management, a catchment or watershed management approach, which better integrates the maintenance of ecological values with resource exploitation and development, has been identified as an essential response. This paper traces the history of catchment management in Ireland and its evolving role as a fundamental strategy in the long-term management of fisheries and freshwater resources. Although integrated

catchment management has become a buzzword amongst the resource management sector, to date it lacks a clear-cut definition. This paper describes in some detail the practical field programmes which has recently been undertaken to test the feasibility of developing data acquisition technologies to support a clearly defined catchment management strategy. Finally the paper highlights the pragmatic and philosophical hurdles which must be quickly overcome if catchment management is to fulfil its initial promise as a novel, fundamentally sound pillar in modern integrated resource management.

**A COMPARISON OF THE ARKHELL CLAMS, ANADARA OVALIS AND NOETIA PONDEROSA, AS POTENTIAL MARICULTURE SPECIES ALONG THE ATLANTIC COAST OF THE UNITED STATES.** K. A. McGraw<sup>1</sup> and M. Castagna,<sup>2</sup> <sup>1</sup>State University of New York, P.O. Box 2000, Cortland, NY, 13045-0900, USA; <sup>2</sup>Virginia Institute of Marine Science, Eastern Shore Laboratory, Wachapreague, VA, 23480, USA.

A new fishery for arkshell clams (*Anadara ovalis* and *Noetia ponderosa*) began along the coast of Virginia in 1991. Before that time, arkshell clams were considered a useless by-catch with the harvest of the hard clam, *Mercenaria mercenaria*, and simply discarded. Since no regulations exist for the harvest of arkshell clams in Virginia, fishermen quickly depleted stocks in some areas to meet a constantly increasing market for the clams. Our studies over the last several years on *A. ovalis* and *N. ponderosa* indicate that the former is a good candidate species for mariculture. Although both species have been spawned and reared previously using standard hatchery techniques, Von Bertalanffy growth curves show that the growth rate of *A. ovalis* is about twice as fast as that of *N. ponderosa* in Virginia waters (2.5–3 years for *A. ovalis*, compared to at least 6–8 years for *N. ponderosa* to reach average market size of 56 mm in shell height). In addition, *A. ovalis* averages about 10% more meat for a given size category because it has a much thinner shell than *N. ponderosa*. We plan to pursue a pilot study using hatchery-reared *A. ovalis* seed clams and a conventional off-bottom culture technique to test the feasibility of large-scale mariculture endeavors. A good market for arkshell clams exists in the United States, and, if successful, mariculture of *A. ovalis* could provide a reliable source of seed clams and market-sized clams, help restock depleted arkshell stocks, and alleviate some of the overfishing of arkshell clams occurring in the oceanside lagoon systems of Virginia.

**THE CANADIAN SHELLFISH SANITATION PROGRAM—HOW SHELLFISH CLOSURES ARE LEADING TO IMPROVED WATER QUALITY.** E. McKnight<sup>1</sup> and H. Nelson,<sup>2</sup> <sup>1</sup>Marine Environment Division, Environment Canada, 351 St. Joseph Blvd., Hull, Quebec, K1A 0H3, Canada; <sup>2</sup>Pollution Prevention and Assessment Division, Environment Canada, 224 West Esplanade, North Vancouver, British Columbia, V7M 3H7, Canada.

The Canadian Shellfish Sanitation Program (CSSP) is a tripartite program administered by three federal agencies to ensure the

safe consumption of molluscan shellfish. Based on the U.S. National Shellfish Sanitation Program (NSSP), the first critical control point of the CSSP is the proper classification of shellfish growing areas. Classifications, based on sanitary shoreline evaluations and water quality surveys, are Environment Canada's responsibility under the CSSP. Shellfish closures made under the CSSP have focused the need for improved water quality in Canada's coastal areas.

Environment Canada's major ecosystem initiatives in its coastal regions empower local communities to address their environmental challenges, including those related to water quality. On Canada's east coast, Environment Canada provides seed funding to thirteen east coast communities to develop and implement comprehensive environmental management plans.

On the west coast of Canada, the major environmental stewardship program is the 5 year Georgia Basin Ecosystem Initiative (GBEI) which began in 1998. This initiative resulted from a growing concern in the early 1990 is that the health of the marine environment was deteriorating rapidly in relationship to population growth and development along the coast. The degradation was clearly demonstrated by the closure to harvesting of an increasing number of commercial and recreational shellfish beds.

Concerted efforts at the community level to identify and remediate non-point pollution are gaining momentum and some of the early initiatives from the mid 1990s are showing positive results. New programs and funding agreements through the GBEI and new regulations under the Canada Shipping Act to restrict sewage discharges from vessels will be discussed. Classification upgrades to shellfish growing areas on the west coast of Canada reflect a growing improvement in general marine environmental quality and shoreline amenities.

**IRISH COASTAL DINOFLAGELLATE BLOOMS AND SHELLFISH TOXICITY.** T. McMahon,<sup>1</sup> J. Silke,<sup>1</sup> and B. Cahill,<sup>2</sup> <sup>1</sup>Marine Institute, Fisheries Research Centre, Abbotstown, Dublin 15, Ireland; <sup>2</sup>Marine Institute, Irish Marine Data Centre, 80 Harcourt Street, Dublin 2, Ireland.

Since the mid 1980s the Marine Institute's Fisheries Research Centre has carried out a monitoring programme in Irish Coastal waters involving both the identification and quantification of phytoplankton species present in near surface waters and the detection in shellfish of toxins of algal origin.

Recurrent blooms of the dinoflagellate *Gyrodinium aureolum* have been recorded, particularly along the southwest coast. In several cases mortalities of marine fauna, including farmed finfish and shellfish, have been associated with these blooms. Recent studies have shown that the blooms originate offshore and are subsequently advected into the bays under a particular set of meteorological conditions. Details of the deployment of two moored instrument arrays, with near real-time data telemetry, designed to provide an early warning of these bloom events are described.

Results from the algal toxin monitoring programme are presented and discussed. The results show that Irish shellfish can have a very complex toxin profile. Toxins (Gonyautoxin-2 and Gonyautoxin-3) causing Paralytic Shellfish Poisoning (PSP) associated with the presence of the dinoflagellate *Alexandrium tamarense* have been detected in one production area. Toxins (Okadaic acid and DTX-2) causing Diarrhetic Shellfish Poisoning (DSP) associated with the presence of the dinoflagellates *Dinophysis acuta* and *Dinophysis acuminata* have been detected in many of the main shellfish production areas in the country. More recently a novel toxin, azaspiracid as well as several of its analogs, of unknown origin have been also been detected in shellfish in Ireland. Shellfish toxicity has occurred during both summer and winter months and therefore monitoring is now a year round activity.

*A. tamarense* is known to produce a cyst stage as part of its life cycle. The cysts can remain viable in the sediments for several years. A survey of the distribution of cysts of *A. tamarense* in the surface sediments in Cork Harbour was carried out in order to determine if a "seed bed" of toxic cysts was present in the area. The results of the survey are presented and discussed.

**EXOTIC SPECIES: IMPLICATIONS FOR COASTAL SHELLFISH RESOURCES.** D. Minchin, Marine Institute, Fisheries Research Centre, Abbotstown, Dublin, Ireland.

Introduced species form the basis for the development of many important economies in Europe and worldwide. Many of these species are globally in production and the successful development of a species in one part of the globe is followed by trials elsewhere. Unfortunately there is a dependence on a small number of species and it is likely that there are many other species that could be profitably used. The ICES Code of Practice forms the basis for a responsible procedure for the introduction of 'new' species for culture.

Exotic species are in constant transit whether in with live-trade or inadvertently with the transport ships. Although the great majority of these species are not known to have great impacts there are some that will have consequences that man affect human health or may compromise coastal shellfish resources. The trade of live species, and in particular the trade of half-grown oysters, continues to be implicated in the range expansions of molluscan pests. Measures taken to reduce the spread and impacts of harmful species in the case of those species moved by shipping is difficult to achieve. Treatment measures are being actively researched. It appears that the continued expansion of phytoplankton toxins may in part be due to inoculations from ballast water discharges by ships. The close proximity of shellfish resources to ballast water discharge sites and berthing regions could compromise shellfish production. There are indications that TBT may have influenced the populations of scallops in Cork Harbour and the improving water quality there may lead to a recovery of these populations. It may also provide more suitable conditions for invasive species. It is therefore predicted that once the

planned discontinuation of the usage of TBT takes place that there will be more frequent exotic species invasions.

This paper examines some species that are likely to become introduced to Ireland in the coming early century and the consequences of these introductions for the shellfish economy.

**ON THE DYNAMICS OF INTER-TIDAL AND SUB-TIDAL STOCKS OF BLUE MUSSELS IN THE DANISH WADDEN SEA. S. Munch-Petersen and P. Sand Kristensen,** The Danish Institute for Fisheries Research (DIFRES), Charlottenlund Castle, DK-2920 Charlottenlund, Denmark.

As part of a monitoring programme for the commercially exploited stock(s) of mussels (*Mytilus edulis* L.) in the Danish Wadden Sea, samples have been collected regularly from 1990 to 1998, both from sub-tidal and inter-tidal settlements. These samples are the basis for the estimated size composition of the mussels as well as estimates of total biomass by locality.

The observed size distributions have been used for identification of cohorts. Cohorts are easy to identify and follow during the first year after settlement. Later, however, the size of the individuals in the cohorts will vary to an extent such that the size distributions seemingly merge into one single group. By slicing the size distributions into components representing cohorts using the available growth parameters for *Mytilus* in the Wadden Sea and assuming certain values of standard deviation of the mean length-at-age this development of the composed size distributions is demonstrated.

Estimates of annual production are compared to production figures for similar localities in the German Wadden Sea.

**DECREASE IN HETEROZYGOSITY IN SUSPENSION-CULTURED BLUE MUSSELS FOLLOWING THEIR TRANSFER TO GROW-OUT SITES AND ITS POTENTIAL CONSEQUENCE ON MUSSEL FARM PRODUCTIVITY. B. Myrand,<sup>1</sup> R. Tremblay,<sup>2</sup> and J.-M. Sève,<sup>3</sup>** <sup>1</sup>DIT-MAPAQ, C.P. 658, Cap-aux-Meules, Canada, G0B 1B0; <sup>2</sup>GIROQ, Université Laval, Québec, Canada, G1K 7P4; <sup>3</sup>Institut Maurice-Lamontagne, MPO-Canada, 850 Route de la Mer, Mont-Joli, Canada, G5H 3Z4.

In the Magdalen Islands (southern Gulf of St. Lawrence, Canada), spat is collected in a small lagoon, placed in mesh sleeves, and transferred to other sites for grow-out in suspension-culture. We observed a significant decrease in multilocus heterozygosity (MLH) estimated at 7 allozyme loci for mussels sleeved at two densities (usual density:  $2.44 \pm 0.14$  and high density:  $2.14 \pm 0.21$ ) compared to that of the spat ( $2.95 \pm 0.08$ ) used for sleeving. This decrease in MLH occurred within the first year after the transfer to the grow-out site. It was not the result of adverse con-

ditions at the grow-out site. We hypothesize that more heterozygous individuals are more active and thus get out of the mesh sleeves more rapidly. Doing so, they are more prone to fall-offs, mainly those resulting from turbulence created by heavy winds over the shallow lagoons while they are still weakly attached. This decrease in MLH may have important impacts on the productivity of the local mussel industry. First, we observed an inverse relationship between MLH and basal metabolism, so that more heterozygous individuals have lower metabolic needs. Further, we observed that more heterozygous individuals were significantly more resistant to various stressful conditions. Indeed, the MLH of survivors to stressful conditions was significantly higher than that of the controls during two successive experiments (August and September 1997). The LT50 of 50–60 mm suspension-cultured mussels (MLH $\ddagger$ :  $2.70 \pm 0.16$  in August and  $3.08 \pm 0.14$  in September) was also systematically lower than that of 50–60 mm wild mussels taken from the spat collection site (MLH $\ddagger$ :  $4.00 \pm 0.18$  in August and  $3.82 \pm 0.21$  in September) under identical stressful conditions. It seems that the mussel growers are not taking full advantage of the spat potential as they are losing the more heterozygous individuals while in suspension-culture.

**EVALUATING DESIGN CRITERIA AND RECRUITMENT LEVELS IN THE RESTORATION OF OYSTER REEF-SEMBLAGES. F. X. O'Beirn, J. A. Nestlerode, and M. W. Luckenbach,** Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062, USA.

The construction of reef structures to promote shellfish habitat restoration represents a significant investment of public and/or private resources. Thus, it is essential that effective design and construction protocols be established and evaluated on sufficiently large spatial and temporal scales to ensure success. These scales often exceed those of standard manipulative field experiments. We describe findings from large-scale field experiments, begun in 1995–1996, addressing the interactive roles of substrate type and tidal elevation on the early development of oyster reef assemblages. In addition to the development of oyster populations, we examine successional trends in macrobenthos associated with the reefs and relate these to reef design characteristics. We observed a particularly strong interaction between substrate type and regional recruitment level (i.e., high vs. low recruitment years) on the development of reefs, adding emphasis to the importance of scale in restoration. The relationship between the development of oyster populations and a variety of community parameters we hope will resolve the temporal and spatial scales that must be applied to oyster reef restoration efforts. Concomitantly, we hope to determine more accurately the appropriate success criteria for similar restoration endeavors.

**CO-ORDINATED LOCAL AQUACULTURE MANAGEMENT SYSTEMS (C.L.A.M.S.).** T. O'Carroll, BIM, PO Box 12, Crofton Road, Dun Laoghaire, Co. Dublin.

As distinct from national aquaculture policy and development programmes the concept of Co-ordinated Local Aquaculture management Systems (C.L.A.M.S.) focuses at the local bay level (while still taking on board relevant national policies). The concept is to amass relevant baseline data and in conjunction with the formation of a local C.L.A.M.S. group, to formulate an aquaculture development plan for the bay while incorporating and extending the successful concepts of Single Bay Management to all farmed species (including extensive culture).

C.L.A.M.S. will provide the following tangible outputs:—

- A concise description of the bay/area in terms of physical characteristics, history, aquaculture operations, future potential, problems, etc
- Integration of a series of codes of practice for current aquaculture operations and translation of those national codes to the specific circumstances of each bay or coastal region
- Expansion of the concept of SBM to species other than salmon
- A development plan for aquaculture in the bay
- Information on other activities in the bay
- A local and national communication network, with "bottom up" and "top down" dialog capacity

In addition C.L.A.M.S. intends to incorporate the development plans of the local individuals as well as integrating the management practices of the various species sectors that may be operating in the same bay.

Over the next few years the concept of Coastal Zone Management will become an applied legislative reality. It is envisaged that C.L.A.M.S. could form the basis for the aquaculture section of any new local Coastal Zone management initiatives.

**ABSOLUTE DETERMINATION OF CHLOROPHYLL CONCENTRATION AND OPTICAL PROPERTIES OF WATER BY AIRBORNE HYPERSPECTRAL REMOTE SENSING.** E. O'Mongain and A. Collins, Spectral Signatures Limited, Roebuck, Belfield, Dublin 4, Ireland.

The absorption characteristics of the pigments of chlorophyll modulate the observable optical properties of marine and inland waters. In clear open ocean waters the blue pigment of chlorophyll is used for chlorophyll estimation by remote sensing. In coastal and inland waters, the blue pigment is masked by other absorption processes. In these waters it is the red pigment which is observed by spectral remote sensing. Airborne measurements over Wexford and Cork estuaries show the distribution of near surface chlorophyll concentration. The technique compares modelled and measured hyperspectral reflectance in a way which utilises the known absorption characteristics of pure water to provide measurement calibration. An absolute measure of the total absorption in the 580 nm region of the spectrum, which is strongly related to visibility or sechi depth, is also obtained. Thus sediment and chlorophyll dis-

tribution are independently monitored for validation of hydrodynamic and eutrophication modelling projects.

For surface based validation measurements the ChloroFlow instrument system similarly utilises the red pigment of chlorophyll to provide in-vivo photometric chlorophyll estimates in real time on a flow-through basis. This instrument also provides measurements of the full spectral absorption curve for investigation of the role of other pigments and sediments and for reflectance modelling exercises. Absolute measurements of spectral absorption (+ back-scattering) in a long pathlength cuvette is the basis of this system which has gone into service in U.K. marine waters this season to assist in the monitoring of sensitive waters.

**CHEMICAL EFFECTS OF HYPOXIC AND ANOXIC WATERS ON SHELLFISH.** M. J. Orren, Oceanography Department, The National University of Ireland, Galway, Ireland.

Normal, ("healthy") coastal sea waters are 90%, or more, saturated with dissolved oxygen (DO). The inflow of new, freshly-oxygenated seawater balances, or exceeds, the demand of DO required by aerobic (oxic) microbial decay of organic matter. However, if inflow is restricted, such as in a stagnant pool, or if the input of labile organic matter, say, from man-made inputs, exceeds the assimilative capacity of the DO to meet this demand, then DO declines rapidly. When DO levels fall to about 60 to 20 micromol DO/kg (1.5 to 0.5 ml DO/l), a hypoxic system, the chemistry changes dramatically. Nitrate converts to nitrite and some denitrification occurs, releasing nitrogen and nitrous oxide gas, while previously insoluble metal oxides begin to become solubilised. When DO becomes undetectable, an anoxic (anaerobic) system, there is no DO for animals to breathe and sulphate ion, relatively abundant in seawater, is microbially (anaerobically) converted to highly toxic sulphide chemical species. But what other chemical reactions occur? Precipitated metal oxides re-dissolve, rapidly releasing their adsorbed load of highly toxic trace elements, including mercury, lead and cadmium, while adsorbed organics are also released. Particulate sulphur compounds are produced and may be ingested, while other co-existing reduced organic and inorganic compounds may be extremely toxic to all shellfish and other living matter.

Anoxic waters destroy shellfish beds since, unlike finfish, or mobile crustaceans such as lobsters, the sessile animals cannot take avoidance action. In this paper the onset of anoxia will be traced and the chemical mechanisms which occur will be discussed, with some comment on precautions to minimise shellfish mortalities from anoxia.

**ENIGMAS IN THE REPRODUCTIVE BIOLOGY OF PACIFIC OYSTERS IN IRELAND.** G. O'Sullivan and M. F. Mulcahy, Department of Zoology & Animal Ecology, National University of Ireland, Lee Maltings, Prospect Row, Cork, Ireland.

The study aims to assess the number of segments of an oyster which should be examined histologically to obtain an accurate

conclusion on the total gonadal variation between male, female and hermaphrodite. Comparison of results from previous studies raised the possibility of variation in gonad from one part to another, (Steele 1998, Sato 1998). The reason for the failure of oysters to spawn in Cork harbour when comparable oysters at comparable temperatures and phytoplankton levels in Dungarvan did spawn, (Steele 1998), is being examined.

Oysters in Cork harbour became ripe but reabsorbed the gonad, without spawning, (Steele 1998). The spawning capability and larval survival of ripe oysters brought in from Dungarvan and from Cork are being assessed.

The reproductive cycle and gonad development are being studied over a 12 month period and examined histologically before being staged, using a modified version of the method devised by Mann (1979). Temperatures at both sites are being monitored daily, chlorophyll *a* levels are recorded on a regular basis. Mean condition index and shell index are calculated for each sample, as TBT is a suspected factor in Cork harbour, (Minchin et al. 1996).

**A NATIONAL HARMFUL ALGAL BLOOM DATA MANAGEMENT SYSTEM.** M. C. Paraso and M. D. Ford, Coastal Ocean Laboratory/NODC/NOAA, 1315 East-West Highway, Silver Spring, MD, 20910, USA.

The National Oceanographic Data Center (NODC) is developing a system which will provide access to physical, chemical, and biological information acquired from various sources to assist in harmful algal bloom (HAB) management and research. Initially, a prototype system will be developed for the Gulf of Maine and the Gulf of Mexico. Later, the system will expand to all US coastal areas affected by HABs. Sources of data include routine monitoring efforts, event driven monitoring, topical research initiatives (ECO HAB), and the NODC archive.

Routine monitoring data and biological data will be held in a new database. A common interface will link this database to other databases that hold HAB-related data such as NODC's Ocean Profile Database and Ocean Current Time Series Database. A large data management system like this will allow researchers and resource managers to access pertinent data for a particular region for decision making, or to compile a historical perspective on events in a particular region. The combination of physical, chemical, and biological data from many sources in this system will provide researchers with a tool to assist in developing forecast models for harmful algal blooms.

**USE OF VIDEOGRAPHY TO ASSESS DIFFERENCES BETWEEN RESTORED AND NON-RESTORED AREAS IN CHESAPEAKE BAY.** K. T. Paynter and T. E. Koles, Chesapeake Biological Laboratory and Department of Biology, University of Maryland, College Park, MD 20742 USA.

Restoration of shellfish beds has at least two obvious applications: commercial and ecological. While the commercial value of

restored areas to the shellfish industry may be obvious, the ecological value of restored shellfish beds has been difficult to quantify. Oyster bar restoration in Chesapeake Bay has only recently begun in earnest. In 1995 and 1996 10 acres of oyster "bottom" was restored with 1 million hatchery produced oyster spat per acre. In 1997 and 1998, additional areas were restored in the Choptank, Patuxent and Chester Rivers. As the laboratory charged with monitoring these efforts, we have experimented with various techniques of assessing oyster bar restoration. Initial sampling was conducted by scuba divers using quadrats. Surface shell was collected from within the quadrats and returned to the laboratory. Live oysters were counted, measured and tested for condition, gonadal maturity and parasite presence and abundance. Organisms associated with the shell collected including anemones, curved mussels and barnacles were also quantified. Although these samples provided an accurate measure of oyster abundance and health, they were inadequate for ecosystem-level observations.

Recently we have employed underwater videography to assess differences between restored and non-restored areas. This technique appears to hold great promise as a method for learning more about what restored oyster bars may produce at the ecosystem-level. Quadrat sampling rarely represented motile species such as blennies, gobies and crabs while videography not only showed those species were present but also showed how those organisms were related to the physical structure of the reef. We believe the use of quantitative videography will be an important and useful tool in demonstrating the ecological value of oyster and other shellfish reefs.

**THE APPLICATION OF RANDOMLY AMPLIFIED POLYMORPHIC DNA (RAPD) MARKERS TO STOCK DISCRIMINATION OF THE WHITE SEA URCHIN, *TRIPNEUSTES VENTRICOSUS*, IN THE EASTERN CARIBBEAN.** M. Pena,<sup>1</sup> C. Gomes,<sup>2</sup> and W. Hunte,<sup>2</sup> <sup>1</sup>Marine Resource and Environmental Management Programme (MAREMP), University of the West Indies, PO Box 64, Cave Hill, Barbados; <sup>2</sup>Natural Resource Management Programme (NRM), University of the West Indies, PO Box 64, Cave Hill, Barbados.

The white sea urchin, *Tripneustes ventricosus*, supports artisanal fisheries in several countries in the eastern Caribbean, but the implementation of sound management practices for the fisheries requires an understanding of population stock structure in the region. Genetic differences among populations of urchins from Anguilla, St. Lucia, Barbados, Carriacou and Grenada were investigated in this study using randomly amplified polymorphic DNA (RAPD) analysis.

Five arbitrarily designed primers detected 52 polymorphic loci in 182 individual sea urchins. Analyses of variance detected a high and significant level of population differentiation among all populations, both when populations were simultaneously analysed ( $-ST = 0.872$ ;  $P < 0.001$ ), and when they were analysed in population pairs ( $P < 0.001$  in all cases). Estimates of gene flow ( $Nm$ ) ranged

from 0.009 to 0.078, indicating limited gene flow between populations. Cluster analyses of similarity and percent match indices revealed subdivision of the populations into five distinct genetic groups, which aligned perfectly with geographical location. Gene flow was significantly correlated with geographical distance ( $P < 0.05$ ), but distance explained only 38.25% of the variation in gene flow. The implication of these results is that there are five unique genetic stocks of *T. ventricosus* in the eastern Caribbean. This is surprising, given the small geographical distances separating the stocks and the presence of a planktonic larval phase in *T. ventricosus*. We are currently attempting to verify these results using EPIC primers of the nuclear allelic system.

**A SATELLITE AND FIELD PORTRAIT OF A *GYRODINIUM AUREOLUM* BLOOM OFF SOUTH-WESTERN IRELAND; AUGUST 1998.** R. Raine,<sup>1</sup> S. O'Boyle,<sup>1</sup> T. O'Higgins,<sup>1</sup> M. White,<sup>1</sup> J. Patching,<sup>1</sup> T. McMahon,<sup>2</sup> and B. Cahill,<sup>3</sup> <sup>1</sup>Martin Ryan Institute, National University of Ireland, Galway; <sup>2</sup>Marine Institute, Fisheries Research Centre, Dublin; <sup>3</sup>Marine Institute, Marine Data Centre, Dublin.

An extensive surface bloom of the dinoflagellate *Gyrodinium aureolum* (Hulbert) (= *Gymnodinium mikimotoi*) occurred off southwestern Ireland during August 1998. The bloom was evident both from remotely sensed satellite ocean colour data, and as visibly discoloured water. The bloom extended from the mouth of Bantry Bay around towards Cork, extending some 40 nautical miles offshore. The timing of the bloom co-incident with a field survey in the area. This paper compares the surface distributions of chlorophyll and *G. aureolum* concentrations with satellite ocean colour and thermal infra-red SST pictures, from which may be derived the origins of the bloom. It would appear that weak coastal upwelling transported a thermocline population of *G. aureolum* up to the surface in the region of the Fastnet Rock, from where it was wind-dispersed eastwards across the northern Celtic Sea.

**TRIALS ON CLAM (*CHAMELEA GALLINA*) BEDS OF AN INNOVATIVE HYDRAULIC DREDGE WITH VIBRATING AND SORTING BOTTOM.** E. Rambaldi, G. Priore, G. Prioli, N. Mietti, T. Pagliani,<sup>1</sup> and M. L. Bianchini,<sup>2</sup> <sup>1</sup>Consorzio Mediterraneo s.c.r.l., Via Nazionale 243, 00184 Roma, Italy; <sup>2</sup>P. F. Raisa/CNR, Consiglio Nazionale delle Ricerche, Via Tiburtina 770, 00159 Roma, Italy

To solve some of the productive and environmental problems related to the use of the hydraulic dredge in bivalve mollusc fishing, an experimental gear with vibrating bottom grid and other technical changes has been tested on clam (*Chamelea gallina*) beds. Comparative fishing surveys have pointed out a significantly different selectivity of the vibrating dredge, with respect to a standard gear: in fact, undersize clams are sieved out during the fishing process, and almost no juveniles were caught. Speaking of the

product quality, laboratory analyses show that the internal sediment is significantly lower in the catch from the modified dredge, thanks to a sort of "alarming" device. Nevertheless, the number of damaged clams suggests a greater mechanical stress of the vibrating grid. As for the environmental effects, the vibrating bottom is selective for the associated fauna too, as it is shown by the mean weight of all the by catch species, which is higher in the experimental gear. Moreover, the riddling goes on continuously, allowing the immediate release of the sorted out organisms, which are repositioned in the area origin, thus avoiding a "contagious" distribution. In conclusion, these preliminary indications suggest a positive evaluation of the modified dredge, especially when considering its innovative design, still with wide margins for improvement.

**EFFECT OF INCREASED PRIMARY PRODUCTION IN A FJORD ON GROWTH OF BLUEMUSSELS AND SCALLOPS.** K. I. Reitan, G. Oie, Y. Olsen, and H. Reinersten, SINTEF Fisheries and aquaculture, Group of Bio-resources, Trondheim, Norway.

The growth of scallops (*Pecten maximus*) and blue mussels (*Mytilus edulis*) is dependent on both the availability and the quality of the food, and physical factors as temperature and water current in the fjord. The primary production in a fjord normally fluctuates during the season. A controlled addition of nitrogen, phosphorous and silicon (N:Si:P = 16:8:1, where P = 0.4 mg m<sup>-3</sup> day<sup>-1</sup>) to a closed small fjord (Hopvåggen in Norway) increased the primary production with 50% compared to control locations. However, this addition of nutrients gave no increase in biomass of phytoplankton in the fjord.

The increased primary production in Hopvåggen resulted in higher growth of scallops compared to the control. This increased growth of scallops was probably due to a higher growth rate of phytoplankton in the fjord when extra nutrients were added.

The growth pattern of blue mussels differed from that of scallops. No significant difference in shell height was obtained for blue mussels with and without extra addition of nutrients to the water.

**NITROGEN EXCRETION BY THE PACIFIC OYSTER, *CRASSOSTREA GIGAS*, AS A CONTRIBUTOR TO ESTUARINE NUTRIENT CYCLING IN TOMALES BAY, CALIFORNIA.** L. Righetti, San Francisco State University, Ca; Romberg Tiburon Center, CA.

Because of its importance as an aquaculture commodity, the filtering capacity and dietary requirements of the Pacific Oyster, *Crassostrea gigas*, have been studied in some detail. Most inquiries have focused on the question of nutrient up-take by the animal, and the portion of their intake that is converted to meat production. Excreted organic matter may be returned cycling systems, in such forms as NH<sub>4</sub>, or removed from these systems. As



in estuarine systems primary production is influenced by biotic and abiotic factors. The activity of filter-feeders can alter the composition of available nutrients in the water column, thus influencing phytoplankton food webs. This study examines the organic matter excreted by *C. gigas*, and considers the resultant impact of oyster aquaculture on the estuarine food web of Tomales Bay, California.

**MORTALITY AND DISPERSAL IN A BENTHIC SUB-TIDAL DECAPOD COMMUNITY AND OF HATCHERY REARED LOBSTER *HOMARUS GAMMARUS*. M. Robinson and O. Tully, Zoology Department, Trinity College Dublin, Dublin 2, IRELAND.**

Depletion of decapod stocks and increasing market prices has led to a number of stock enhancement programs in Northern Europe. One such method utilised for the enhancement of European lobster *Homarus gammarus* involves the release of hatchery-reared juvenile lobster onto the seabed. Due to the scarcity of data pertaining to the habitat requirements of wild juvenile *H. gammarus*, stage V reared lobsters are commonly released into habitats similar in characteristics to those occupied by adults. The fate of hatchery-reared lobster, and their affect on the decapod communities resident in the release areas is unclear. Certain release methodologies result in localised, short-term high densities of juvenile lobster.

Juvenile lobsters were released at high density into enclosed and unconfined experimental plots containing existing wild fauna within a commonly utilised release ground. Twelve percent of the initial seeded lobsters were recovered from enclosures after one month, compared to a one percent recovery from unconfined plots. Overall density, 4.8 individuals-1 m<sup>2</sup>, may represent a rough approximation to the saturation density of juvenile *H. gammarus*, although wild densities are unlikely to reach this level. Enclosure and/or the presence of juveniles reduced the abundance of young of the year porcellanid crab *Pisidia longicornis*, but did not affect any other species, or community structure as a whole.

Dispersal can play an important role in regulating density-dependant processes acting on mobile benthic decapod crustacea. The influence of dispersal may become less important for species below saturation density resident in physically complex habitats with a plentiful food supply. Movement in densely populated areas, subject to losses to demersal and benthic predators, may result in increased mortality. The numbers of released lobsters that survive to recruit to the fishery, and the resultant financial viability of this enhancement method, remains unclear.

**THE IMPACT OF SCALLOP DRAGS ON SEA URCHIN POPULATIONS AND BENTHOS IN THE BAY OF FUNDY, NEW BRUNSWICK, CANADA. S. M. C. Robinson, S. Bernier, and A. MacIntyre, Biological Station, Dept. Fisheries and Oceans, St. Andrews, New Brunswick, EOG-2XO, Canada.**

The fishery for the green sea urchin (*Strongylocentrotus droe-*  
*bachiensis*) began in New Brunswick in the late 1980's in response

to increasing demand from Asian markets. Fishing was initiated by members of the scallop fishery and the harvesting practices were based on familiar gear and similar operations in Maine. Because of the potential harvesting impact on the shallow water benthic populations a study was initiated in 1993 to document 1) the proportion of sea urchins damaged during the harvesting operation, 2) the impact on and subsequent recovery time of the associated benthic flora and fauna and 3) the impacts on the bottom substrate. Two representative sites were chosen for the study. At each site, a towing lane was created parallel to shore and divided into a treatment section and control section. Divers used a fixed line transect method to survey the control and treatment plots prior to and after the harvesting operation (2 m Digby drag). Further surveys were scheduled three and six months later. The results from the study showed a significant increase in the number of broken sea urchin tests after the harvesting operation from 0.05 m<sup>-2</sup> to 1.4 m<sup>-2</sup> at the Passamaquoddy Bay site. Similar trends were found in Grand Manan. On both sites in the experimental plot, there was an increase in the density of mobile predators such as hermit crabs, starfish, whelks and sculpins. While the lobster density declined to zero in the experimental plot, the lack of body parts suggested they moved out of the area. The dragging operation also adversely affected a number of macrophytes.

**THE U.S. HAB PROGRAM: ONE-HALF OF A U.S.-IRELAND HAB EXCHANGE. K. G. Sellner, Silver Spring, MD, USA, 20910.**

ECOHAB (Ecology and Oceanography of Harmful Algal Blooms) is the U.S. national program that provides support for research on blooms that impact living resources, coastal economies, and public health along the U.S. coast. Five agencies, NOAA, NSF, EPA, ONR, and NASA, provide funding for research to 1) determine the linkages between physics, water quality, and HAB biology, ecology, physiology, behavior, and toxicity in order to develop regional forecasting capabilities for expression of HABs in U.S. waters, 2) expand general knowledge on HAB species, and 3) investigate and determine species- and region-specific prevention, control, and mitigation strategies for reducing coastal impacts. Results from the ECOHAB regional studies should be considered as resources for Irish goals of developing an Irish-specific program, where bathymetry, circulation, and HAB taxa characteristics of Irish coastal areas and species might fit with similar sets of conditions in the U.S., permitting transfer of U.S. results (like models) to Ireland as a foundation to build upon. Irish results from mariculture and its own species would, in turn, expand U.S. knowledge on N. Atlantic taxa and impacts that conceivably might at some time develop in U.S. waters. Additionally, the U.S. ECOHAB Program is part of a larger U.S. effort, that includes other areas of likely interest to Irish HAB management communities, including Federally-supported HAB monitoring and assessment programs, event response activities, education/outreach

programs, and public health-related epidemiological and cohort studies, yielding a fairly comprehensive national HAB program. Cross-Atlantic exchange of HAB information, results, and management approaches is an extremely important area for future U.S.-Irish HAB communities.

**HARMFUL ALGAL BLOOMS AND SHELLFISH RESTORATION: PERMANENT OBSTRUCTIONS OR TEMPORARY NUISANCES?** S. E. Shumway, Natural Science Division, Southampton College of Long Island University, Southampton, NY 11968 USA.

It is now generally accepted that the number and frequency of harmful algal bloom (HABS) is increasing and they are increasingly blamed (sometimes wrongly) for the destruction or demise of shellfish beds and aquaculture operations, or pointed to as a source of major concern with respect to siting of aquaculture and restoration ventures. These HABS occur worldwide and, in some areas, they are a common and seasonal occurrence. In other areas they are sporadic or unique. These blooms have far-reaching impacts on ecosystem integrity, species interactions, aquatic animal health, population growth, human health, economy, industry, and ecology. For obvious reasons, algal species known to be associated with human health risks have received the most attention and commercially important fish and filter-feeding shellfish have been the primary species of concern. Algal species which cause human illnesses are not the only species of importance with regard to animal or environmental health or economics, and commercially important fish and shellfish are not the only organisms impacted. Current discussions frequently focus on mitigation and control of adverse effects of these harmful algae.

This presentation will review our knowledge of harmful algal-shellfish (molluscan and crustacean) interactions worldwide and discuss ways in which shellfish restoration efforts may be undertaken successfully in the face of these imposing threats. The discussion will stress science-management interaction and ways in which restoration efforts may be undertaken without imposing undue environmental stress and expense.

**DINOFLAGELLATE RESTING CYSTS IN CORK HARBOUR: IMPLICATIONS FOR SHELLFISH AQUACULTURE.** J. Silke and T. McMahon, Marine Institute, Fisheries Research Centre, Abbotstown, Dublin 15, Ireland.

In Ireland a monitoring programme for the detection of algal toxins in shellfish is carried out by the Marine Institute's Fisheries Research Centre. The monitoring programme is carried out under EU Directive 91/492. The North Channel area of Cork Harbour has, to date, been the only location in Ireland where toxins causing Paralytic Shellfish Poisoning (PSP) have been detected in shellfish above the regulatory limit.

During the summer of 1996, 1997, and 1998 mussels (*Mytilus edulis*) from the North Channel area were found to contain PSP toxins above the regulatory limit for a short period and a ban on harvesting was imposed. Oysters (*Crassostrea gigas*) remained below the regulatory threshold. The dinoflagellate *Alexandrium tamarense*, a known vector of PSP toxins, was observed in the area during each of the toxic events. The exact origin of the populations of *A. tamarense* was unknown.

**SHELLFISH CARRYING CAPACITY AND ECOSYSTEM PROCESSES.** A. Smaál and M. van Stralen, Netherlands Institute for Fisheries Research RIVO-DLO, Centre for Shellfish Research, PO Box 77, 4400 AB Yerseke, The Netherlands.

Shellfish culture in the Netherlands consists of bottom culture of mussels (*Mytilus edulis*) and oysters (*Crassostrea gigas*, *Ostrea edulis*) and fisheries and experimental relaying of wild cockles (*Cerastoderma edule*).

Annual surveys show high densities of shellfish and consequently they are the most abundant functional group in the cultivation areas Wadden Sea and Oosterschelde estuary. Annual yields of shellfish culture and fisheries are registered by the fishery board; as all mussels are delivered to the auction, detailed long-term data are available of condition (% flesh) and annual yield from the various production areas.

The "exploitation" carrying capacity of the Oosterschelde ecosystem—defined as the standing stock of the exploited species at which the yield of the marketable cohort is maximised—was evaluated before and after completion in 1987 of a large scale coastal engineering project consisting of the construction of a storm-surge barrier. This project resulted in decreased current velocities, increased water residence time, decreased nutrient loads and increased water transparency. The phytoplankton population showed a resilient response by maintaining primary production while species composition adapted to changed light and nutrient conditions. Phytoplankton turnover increased significantly.

It was demonstrated that the average annual condition of mussels delivered to the market showed a significant negative correlation with the annual shellfish standing stock in the pre-barrier phase (1980–1984) and in the first period of the post-barrier phase (1987–1990). There was also a significant correlation between mussel growth and the annual primary production in both periods. It was therefore concluded that the carrying capacity of the ecosystem was fully exploited and that the primary production determined the carrying capacity. In third period (1991–1997), however, the correlation appeared to have vanished.

Meanwhile, shellfish culture has adapted to the new conditions in the Oosterschelde estuary. Mussel lease sites were relocated in response to changed hydrodynamic conditions, cultivation techniques have evolved and lease sites are now used in a more extensive way. Although the standing stock has maintained, the an-

nual yield has increased, residence time of mussels on the lease sites has decreased, hence the turnover has increased. Yet, the condition of market delivered mussels has not changed. Apparently, the mussel farmers made the choice to increase yield rather than quality. Total standing stock of shellfish has furthermore shown a decrease of cockle densities due to a lack of spatfall, and a dramatic increase of Pacific oysters. The latter has spread from lease sites to virtually everywhere in the estuary, and is now considered a threat to other shellfish.

The interaction between shellfish and the ecosystem can be described in terms of primary production, grazing pressure and water renewal. For the Oosterschelde it will be shown that phytoplankton turnover and shellfish filtration have increased, while water renewal has decreased. The interactions have intensified, and the consequences for carrying capacity estimation will be discussed.

**OPENING REMARKS FOR GEAR IMPACT AND REMEDIATION SESSION.** G. W. Thayer, Beaufort Laboratory, Beaufort, North Carolina, USA.

The alteration of benthic habitat by fishing activities is not well understood, yet it is generally acknowledged that some fishing gears may influence species composition and diversity and alter habitat complexity. It is also considered that the potential effect of fishing activities on benthic habitats is a key issue facing the long-term sustainability of our coastal and marine living resources. In the United States the eight Fishery Management Councils (FMCs), which are responsible for managing fisheries, have been required to develop a comprehensive habitat plan for their respective management areas. These plans are to identify Essential Fish Habitat (defined as "those waters and substrate necessary to fish for spawning, breeding, feeding, or growth to maturity" where "waters" includes aquatic areas and their associated physical, chemical, biological properties that are utilized by fish). As part of the Essential Fish Habitat amendments to the Magnuson-Stevens Fishery Conservation and Management Act (December 1996) all FMCs were to identify adverse impacts of fishing activities on Essential Fish Habitat, and protect Essential Fish Habitat from fishing and gear impacts. As one might expect, this has been highly controversial.

It is evident that the effects of fishing in estuaries and coastal waters can be diverse and far-reaching, but effects are obscured by uncertainty for a number of reasons because systems are dynamic, many systems already have a long history of harvesting, and impacts may not be seen due to inappropriate scales of observation (Blaber et al. Draft MS. "Effects of fishing on the structure and functioning of estuarine and near shore ecosystems." Special issue of ICES Journal of Marine Science). It also has proven difficult to separate the effects of fishing from other sources of variability. Currently, several environmentally-related agencies in the United States are developing joint funding initiatives directed at the effects of fishing activities on benthic habitats. These initiatives are

addressing the relations between the biological and physical effects of gear to the geological characteristics of benthic habitats. Workshops have and are being convened not only to address gaps in our knowledge but also to generate research priorities and plans. This is a very hot topic.

The workshop convened here at the International Conference on Shellfish Restoration has brought together a series of presentations and videos dealing with (1) impact of fishing gear in shallow estuarine and nearshore waters, approaches to management and restoration of these areas and (2) with management considerations and impacts of gear to deeper water benthos and benthic habitats. Our (co-chairs Gordon Thayer, US Department of Commerce, NOAA and Michael Kaiser, School of Ocean Sciences, Menai Bridge, UK) goal has been to bring together experts in the arena of gear impact to benthic shellfish habitats to provide an improved basis for environmental management decisions regarding not only gear types and potential impacts to benthic habitats but also restoration of habitats impacted by fishing gear.

**RESTORATION OF LOBSTER (*HOMARUS GAMMARUS*) POPULATION EGG PRODUCTION IN DEPLETED STOCKS.** O. Tully, Zoology Department, Trinity College Dublin, Dublin 2, Ireland.

Stocks of lobster (*H. gammarus*) in Ireland have declined significantly over the past 30 years as indicated by trends in CPUE. The current stock biomass may therefore be below the environmental carrying capacity as larger stocks previously existed. The decline has presumably occurred because of recruitment overfishing or because of environmental factors that reduced larval production and supply to the seabed or survival of juvenile lobsters.

Restoration of population egg production is being achieved in Irish lobster fisheries by conservation of marked females (using a tail v-notch) which have lifetime legal protection from fishing. In one fishery this program has been successfully implemented over a 5 year period. Population egg production has been increased by 23 million eggs per annum and possibly by 25%. Based on estimates of growth, mortality and spawning frequency this contribution will increase in the future without the addition of new marked females. Predicting the effects on recruitment is hampered by the lack of information on temperature and hydrodynamic effects on larval supply to the seabed and post settlement mortality rates (essentially the stock recruit relationship of a closed population). Fishery log book data is being used to monitor subsequent effects on recruitment into the fishery. This requires a long time series of data. CPUE from 1995 to 1997 was stable. In 1998 a 75% increase in the catch rate of undersized lobsters was recorded compared with the previous year. This may have been due to favourable conditions for larval survival 3 years previously in addition to the added egg production potential in the population. The efficacy of this method relative to other technical measures is currently being assessed for all Irish lobster fisheries.

**CLAM FARMING AND TOURISM—A DIFFICULT COMBINATION? THE SOCIO-ECONOMIC ROLE OF *RUDITAPES DECUSSATUS* CULTIVATION IN THE ALGARVE.**

**J. C. Wallace**, Centre of Marine Sciences (CCMAR), University of Algarve, Campus de Gambelas, 8000 Faro, Portugal.

The oldest and most traditional, marine aquaculture in Portugal is clam farming. About 90% of the national cultivated bivalve production is produced in the Algarve, on the coastal wetlands known as the Ria Formosa. More than 100 hectares of these tidal wetlands are used for the production of 'amêijoa boa', *Ruditapes decussatus* (= *Tapes decussatus* = *Venerupis decussata*), [eng. carpet-shell clam; fr. palourde; ger. teppichmuschel; esp. almeja fina]. The production involves, essentially, the tending of family plots, of various sizes, which have been sown with clam 'seed' collected from the natural population of the Ria. The Ria Formosa, extending for several kilometers along the Algarvian coast, is also the recipient of sewage from several towns, which, during the tourist season, receive tourists in numbers far exceeding those of the resident populations. The peak tourist season also coincides with the period of maximum sea temperatures and, due to evaporation, highest salinities. These factors combine to produce a deterioration in water quality in the Ria. It is, however unclear whether or not the clam production is affected directly, or simply stressed sufficiently to become weakened and more susceptible to parasitic diseases. The paper considers the recent history of this aquaculture, as well as the present situation and possible future developments.

**DEVELOPMENT OF HYDROLOGIC MODIFICATION INDICATORS TO SUPPORT WATERSHED-BASED RESTORATION OF SHELLFISH RESOURCES IMPACTED BY FECAL COLIFORM CONTAMINATION.** **N. W. White, L. E. Danielson, and M. V. Holmes**, Brooks Hall, School of Design, Box 7701, NC State University, Raleigh, NC 27695-7701, USA.

The Jump Run Creek Project in North Carolina is a multi-disciplinary, multi-agency, watershed-based restoration project that combines in-column water quality data, stormwater quality data, storm and stream flow measures, with spatial analysis, and community involvement to investigate causes and solutions of bacterial loading contributing to shellfish closure management. Preliminary project efforts were reported at ICSR '98, Hilton Head showing data indicating that increased volume and velocity of stormwater flows may be the primary transport vector. However, the impervious surface area in this watershed is less than 5%—well below the threshold of 12%–25% cited in the literature as the level at which nutrient loading causes water quality degradation. Very little investigation has been conducted to review the effects of land use modification on bacterial loading. This effort reports on one approach to measure impacts to watershed hydrology indicat-

ing conditions contributing to excessive bacterial loading and bed closure. Using scanned images of aerial photos of the watershed from the 1960s through the 1990s, overlain with current parcel data, and linked to GIS classification ranks are assigned to each parcel indicating the degree of hydrologic modification. Parameters being used are vegetative cover, ditching, and impervious surface area. Using regression techniques, spatial and temporal models of hydro-modification will be regressed against thirty years of bacterial data collected by North Carolina Division of Environmental Health—Shellfish Sanitation Branch to investigate relationships. The aerial and GIS have been completed. The hydro-modification database is currently under construction. Regression analysis will be conducted during the summer months; so that a report on the results can be made to the conference in September. Over the coming year, this information will be used in two ways: 1) to help watershed citizens understand land use activities that contribute to bacterial loading; and 2) in conjunction with watershed citizens, to design and locate BMPs for mitigation and restoration.

**THE ACQUISITION AND INTERPRETATION OF DIGITAL ACOUSTICS FOR CHARACTERIZING LOUISIANA'S SHALLOW WATER OYSTER HABITAT.** **C. A. Wilson, H. H. Roberts, J. Supan, and W. Winans**, Oyster geophysics Program, LSU, Baton Rouge, Louisiana 70803, USA.

Louisiana's oyster industry generally ranks first nationally in production, averaging 10–12 million pounds of shucked meat, with an average total economic value of approximately \$56 million annually. However, coastal Louisiana, like many deltaic landmasses, faces continued landscape alteration from natural processes and anthropogenic impacts that are and will affect oyster production. Many steps are being taken at both State and Federal levels to slow/mitigate these changes. Most promising of these strategies is river diversions, which introduce freshwater and sediment to river-flanking environments (lakes, bays and associated marshlands). Two such diversion projects planned by Louisiana Department of Wildlife and fisheries and US Army Corps of Engineers, Caernarvon and Davis Pond, are designed to nourish marshes with water and sediment as well as to help establish ideal isohalines over historic oyster grounds. Critical to the success of these programs is a rapid and accurate means to qualify and quantify changes in oyster habitat.

Digital high resolution acoustic instrumentation linked to state-of-the-art acquisition and processing software is available for building a baseline of information that can be used for evaluating future changes in shallow water bottoms with special application to oyster habitat. Application of digital side-scan sonar (100 and 500 kHz) and a broad-spectrum sub-bottom profiler (4–24 kHz) for rapidly acquiring both surficial and shallow subsurface data (average water depths 0.7 to 3.0 m) has now been accomplished.

These data sets "calibrated" with coring, surface sampling, and other "ground truthing" techniques have enormous potential for understanding (a) distributions of bottom sediment types, (b) locations of oyster reefs and distributions of scattered oyster clumps and shells, (c) fisheries habitats, (d) areas of active sedimentation and erosion, and (e) shallow subsurface configurations that influence surface conditions. This approach of linking high-resolution acoustic data with various direct sampling techniques has been verified in pilot studies.

Geo-referenced side scan sonar mosaics of oyster lease areas surveyed were incorporated into a GIS database. Using image-processing techniques to analyze mosaic reflectance patterns, we estimated the percent and total acreage of several bottom types. Results were calibrated with field collected ground truth measurements.

#### **CO-ORDINATING SHELLFISH AND FINFISH AQUACULTURE SYSTEMS. D. Jackson and T. O'Carroll.**

As CLAMS (Co-ordinated Local Aquaculture Management Systems) is introduced to the various bays around the country a process will be undertaken to open dialogue with and between local aquaculture operators and to facilitate the development of an appropriate plan. The steps in this process are outlined and the key elements necessary to facilitate the preparation of an effective CLAMS document are identified.

In a workshop setting the practicalities of establishing an effective local CLAMS group are explored under the following headings:

- Introduction to concept of CLAMS
- The Paperwork
- Information and Integration
- Risk Assessment
- Feedback to Regulatory agencies
- Development Plan

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**COVER PHOTO:** New Zealand greenshell mussels, *Perna canaliculus*, courtesy of Rebecca Holland, National Institute of Water and Atmospheric Research Ltd., Auckland, New Zealand

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