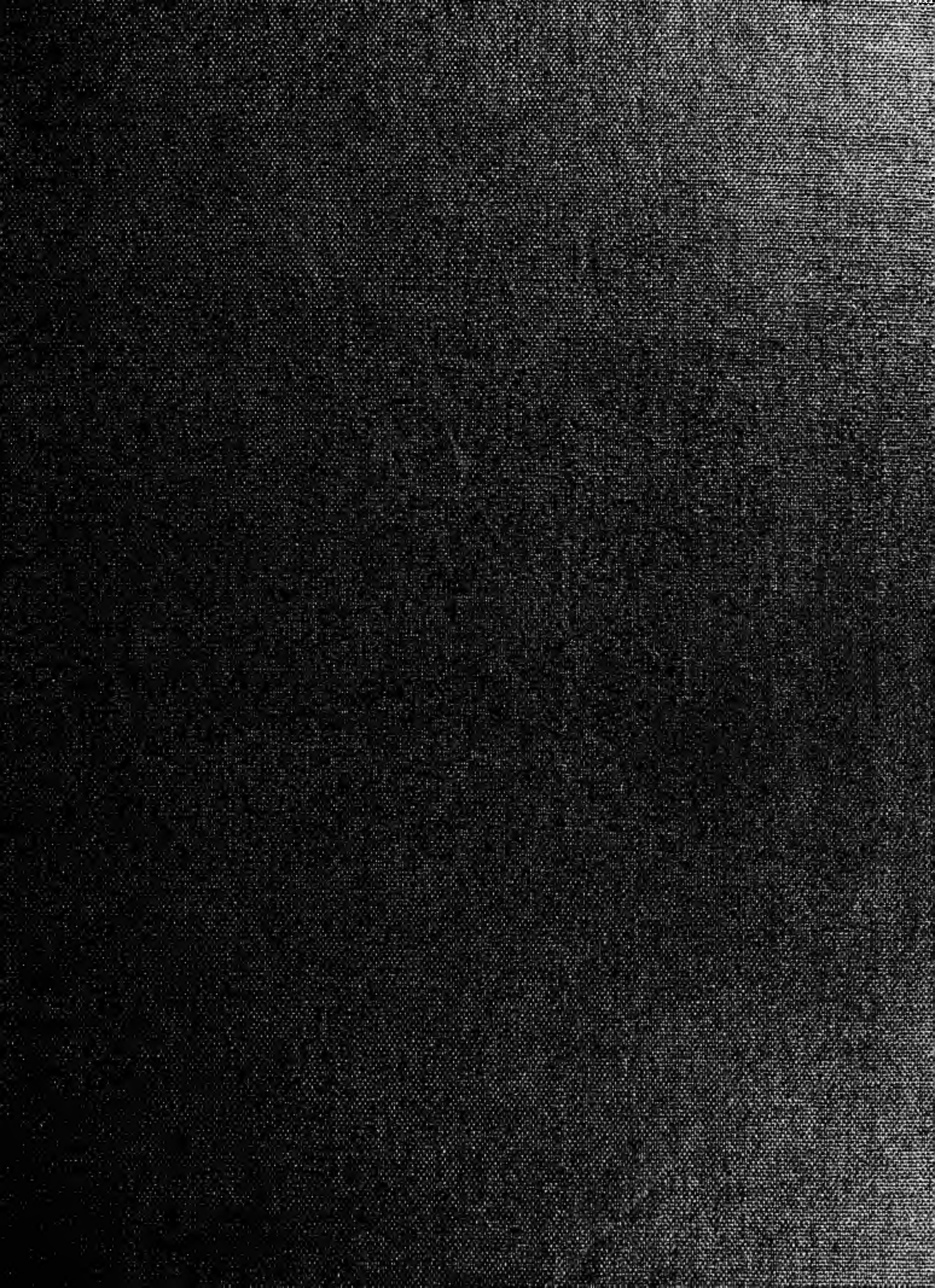

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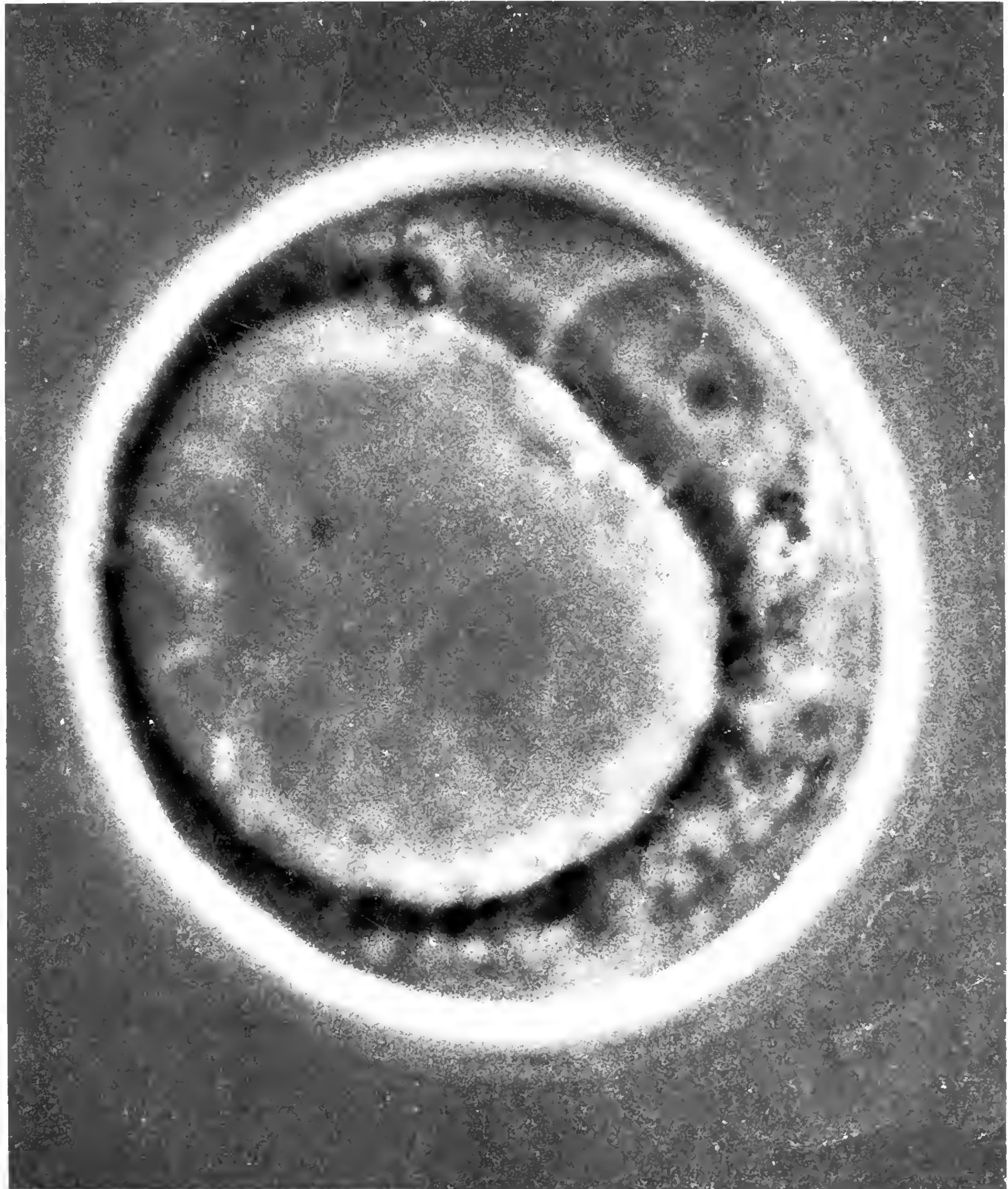

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PREFACE

The severity and continual spread of *Perkinsus marinus* disease and its increasing impact on the economic and ecological resources of the Gulf of Mexico and Atlantic coast of the United States have prompted urgent attention from the scientific community. This imperative has been recognized by the two federal agencies that supported the publication of this document—the Environmental Protection Agency through the Center for Marine & Estuarine Disease Research (CMED) and the National Oceanic and Atmospheric Administration through the Sea Grant Program.

At the 1994 National Shellfisheries Association meeting in Charlestown, South Carolina, CMED sponsored a day-long symposium on *P. marinus* disease of oysters. The participants agreed to prepare review articles that would include recent progress and overall perspective in their areas of expertise. The valuable efforts of these scientists are included in this special issue of the Journal.

The issue also offers an opportunity to recognize three scientists for their creative and enduring investigations into *P. marinus* disease—Sammy M. Ray, Jay D. Andrews, and Frank O. Perkins. Dr. Ray was a member of one of the original teams that discovered the parasite in Gulf of Mexico oysters and, as a graduate student, developed the diagnostic culture medium and staining technique that has been used almost exclusively for over 40 years. Dr. Andrews was one of the earliest researchers to investigate the parasite in Chesapeake Bay, and in the ensuing 35 years, he has monitored resident and caged oysters in Virginia waters to establish much of our knowledge regarding epizootiology and natural transmission of the disease. Dr. Perkins has been intimately involved in the morphological description of the pathogen for over 20 years. His ultrastructural descriptions of *P. marinus* are unparalleled, and in recognition of this, the taxonomic description of this microorganism bears his name. The foreword and first two articles in this issue present historical perspectives from each of these scientists. Their work, as well as their enthusiasm and insight, has been at the core of our research in this field. Their additions to the special issue provide a continuity and context for the work that remains.

Completion of this issue would not be possible without the help and support of many people. From EPA, thanks are due to Robert Menzer and Courtney Riordan for their support during the initial stages of the project, and more recently to Sonny Mayer and Gil Veith; from Sea Grant, similar thanks are due to Bess Gillelan for her initial support, and more recently to Bill Rickards and James McVey. Several authors have reminded me of our mutual appreciation for the many peer-reviewers, most of whom provided excellent reviews and some on extremely short notice. I greatly appreciate the capable assistance and moral support of Jill Adams as well as the advice and publication assistance of Sandra Shumway, editor of *JSR*. The symposium and the special issue would not have been possible without the membership of the National Shellfisheries Association, whose continued interest and support of shellfish research have provided venues for these and many other productive projects and programs.

William S. Fisher
Editor

FOREWORD
Frank O. Perkins

Over 45 years have elapsed since John G. Mackin, H. Malcolm Owen, and Albert Collier of the Texas A & M Research Foundation and the Louisiana Department of Wildlife and Fisheries first noted that a protistan parasite was associated with mortalities of *Crassostrea virginica* found in the area of the Mississippi River delta. Due to the presence of cells of a parasite with a large eccentric vacuole containing a prominent inclusion, they concluded that the protist was a species of the genus *Dermocystidium* and named it *Dermocystidium marinum*. In the next two decades, it was well documented that the parasite was the causative agent of the oyster mortalities first observed in the Gulf of Mexico coastal waters and, in fact, could be found in oysters from Texas to New Jersey as well as other bivalve molluscs in that range. Although there were many researchers who contributed to our knowledge of the parasite during the 1950s and 1960s, Jay D. Andrews, John G. Mackin, and Sammy M. Ray provided the majority of information. Ray facilitated investigations of the parasite by providing the fluid thioglycollate medium (FTM) technique by which rapid and inexpensive detection of cells of the organism could be accomplished in large numbers of oyster tissue samples due to marked enlargement of the pathogen in the culture medium. Although not yet rigorously evaluated, there is good evidence that enlargement occurs without cellular multiplication. Almost 40 years later the Ray technique was redesigned to permit a quantitative estimation of the numbers of cells in selected tissues and in whole oysters by incubation in FTM followed by digestion in an NaOH solution and counting the number of *Perkinsus marinus* cell walls in the digest.

Detailed epizootiological and experimental ecological studies by Andrews, Mackin, and Ray yielded information of value to oyster growers and managers of oyster populations. It was soon determined that transmission of infections occur from oyster to oyster and the pathogen is most virulent at higher temperatures and salinities approximating 20 to 30°C and 20 to 30 ppt, respectively.

The structure and life cycle of the parasite was examined in greater detail in the 1960s and 1970s using electron microscopy. The demonstration of zoosporulation in sea water of cells (hypnospores) which had enlarged in FTM yielded the observation that infective, biflagellated zoospores were released. These cells were found to have an apical complex and other apicomplexan structures. Thus, evidence was presented that the organism is closely related to the Apicomplexa. Norman D. Levine in 1978 renamed the parasite *P. marinus* and placed it in a new class Perkinsea in the phylum Apicomplexa. A curiosity which remains to be explained is the fact that for about 20 years after its discovery, zoosporulation could be readily induced to occur in *P. marinus* under laboratory conditions by most cells of a hypnospore population. Since the late 1980s, this was found to occur in less than 1% of a hypnospore population and zoospore release failed to occur. On the other hand, isolates of *Perkinsus* spp. hypnospores derived from other bivalve hosts readily zoosporulate in sea water.

During the 1980s until present, research activity involving *P. marinus* and other species in the genus increased markedly on several fronts and excellent progress has been made. Epizootiological studies and investigations in experimental ecology both in the field and laboratory have centered around the effects of salinity and temperature in controlling expression of the disease, thus expanding on the

extensive studies reported earlier by Mackin and co-workers. The workers who followed them have confirmed that increased salinity and temperature enhance expression of the disease, and they have greatly expanded upon our knowledge of the details of that paradigm as well as the exceptions. Salinity has emerged as a more dominant factor than temperature in some studies and conditions. In other studies, temperature has been found dominant. The laboratory component of temperature and salinity studies has centered mainly around observations of hemocyte function and composition of hemocyte populations. Large-scale, field observations have revealed that epizootics of *P. marinus* in oysters are not induced simply by fluctuations in temperature and salinity but rather some other stimulatory factor or factors such as limited food supply or recruitment that occurs just before or at the same time as elevated temperatures and salinities. Thus, progress is being made toward constructing climatic models to predict the activity of the pathogen. Of significance may be the recent observation that *P. marinus* proliferation is enhanced by excess iron accumulation in the host. It is known that there is an increase in iron levels in oysters in the summer when *P. marinus* causes elevated mortalities. Thus, researchers will undoubtedly have to consider many more factors than just salinity and temperature in their modelling efforts.

Oyster hemocyte and *P. marinus* interactions have been evaluated *in vitro* by measuring reactive oxygen intermediates (ROI) primarily as expressed by the luminol-enhanced chemiluminescence (CL) response and by assaying for hemocyte lysosomal enzymes. Although there are conflicting results, it appears that *P. marinus* can either prevent ROI production or neutralize ROI in hemocytes with one hypothesis being that acid phosphatase produced by *P. marinus* inhibits superoxide radicals released by hemocytes. In the future, there will undoubtedly be increased research activity directed toward understanding how *P. marinus* is able to survive and multiply in hemocytes, recognizing that the hemocytes are the primary line of host defense against microbial agents. Related to this are ongoing investigations to identify and quantify substances that are produced by the pathogen and result in destruction of oyster cells.

The question as to whether anthropogenic chemicals in growing waters predispose oysters to mortalities caused by *P. marinus* continues to be one of major importance to managers and users of the estuarine environment. For over a century, oyster farmers and harvesters have cited pollution as the primary reason for the decline in oyster production with enhancement of microbially induced disease by pollution being a focus of their complaints. However, the evidence to support or refute their claims is not yet sufficient. In recent years, some insight has been obtained in working with compounds such as tributyltin and sediments contaminated by polynuclear aromatic hydrocarbons. It appears that some anthropogenic compounds can enhance the proliferation of *P. marinus* in oysters and can suppress the CL response. Furthermore, the matter of soluble iron (mentioned above) needs to be considered. It has been suggested that increased iron levels in industrially contaminated waters and/or sediments may enhance the expression of the disease. Therefore, the long-standing complaints of the oyster harvesters may prove to be correct. However, much is left to be determined before proof is forthcoming and informed management decisions can be made relevant to this issue.

Also of importance to managers and users of oyster populations is the question of whether *Perkinsus* sp. or spp., which are found in most (all?) other bivalve mollusc species co-existing with *C. virginica*, are *P. marinus* or another species of *Perkinsus*. There is probably at least one other species of *Perkinsus* in bivalve molluscs associated with *C. virginica*. It is found in *Macoma balthica* and *Macoma mitchelli* and is probably *Perkinsus atlanticus*. It can be induced to infect *C. virginica* most easily when its zoospores are fed to oysters. The observation of *Perkinsus* cells in other bivalve molluscs may in large part involve a carrier relationship with the pathogen, but multiplication of *P. marinus* is known to occur in many of those presumptive carriers. Whether they cause mortalities in those bivalves remains to be seen. This information is of interest to governmental regulators of bivalve mollusc transportations between estuaries and must be more completely investigated.

Recently, evidence has been obtained that there are probably strains or races of *P. marinus* with the Gulf of Mexico strains being less virulent than those along the mid-Atlantic Ocean coast of the U.S. Such preliminary information requires further clarification so that more informed decisions can be made concerning transportation of oysters.

Although some excellent biochemical and physiological studies were conducted using *P. marinus* cells isolated from infected oyster tissue, the lack of axenic cultures inhibited pursuit of such research. The problem was solved in late 1992 and early in 1993 followed by publication in 1993 of semi-defined culture media formulations by three different laboratories within months of each other. The contributions were significant and, as expected, have resulted in improved ability to investigate the biological characteristics of the pathogen. A further refinement has been made with the formulation of a defined culture medium which will permit even greater biochemical and physiological characterizations. The only word of caution has been that the few studies of transmission of infections using cultured cells have resulted in the observation that such cells do not appear to be as infective as *P. marinus* isolated from oysters and used directly in challenge experiments without being cultured. Identification of a culture medium that yields cells of the same infectivity as uncultured ones must be accomplished to lessen uncertainty as to whether naturally occurring characteristics are being observed when cultured cells are used. It is known that the cytological characteristics of many of the cells in culture differ in terms of size and cytokinesis from those observed in oyster tissues.

It has been established that infections of *P. marinus* occur from oyster to oyster, the developmental cycle in the oyster appears to have been well characterized, and it is known that zoosporulation can occur outside of the host to yield zoospores that are infective for other oysters. Nevertheless, the question has remained as to whether saprobic development can occur free of the host. The fact that the pathogen can be cultured in a variety of media leads one to suggest that *P. marinus*, as well as other species of *Perkinsus*, is a faculative pathogen. With the provision of fluorescein-labeled specific antibodies to *P. marinus*, cell DNA labeling with propidium iodide, and the use of flow cytometric analyses, it is now possible to detect cells of *Perkinsus* (not just *P. marinus*) in water and sediment samples. This will undoubtedly lead to greater insights into the life cycle with answers to the question of whether there is multiplication of the pathogen free of its host. Evidence that this may occur comes from the observation that enlarged cells (hypnospores) in sea water may not zoosporulate but rather may form hyphal-like outgrowths into which the cytoplasm flows and subdivides into daughter cells that are released into the sea water. These daughter cells are morphologically dissimilar to those found in the host. Whether these cells are saprobic forms in the life cycle or must enter a host to continue development remains to be determined.

Whereas most investigators accept that *Perkinsus* spp. are related to the Apicomplexa, the taxonomy and phylogeny of the pathogens remain a subject for scrutiny and reevaluation. In light of new phylogenetic alignments of the Protista and recent findings by molecular biologists studying nucleic acid base sequences of *P. marinus*, as well as the reinterpretation of the morphology of the pathogen by others, it is now realized that pathogens in the genus belong either with the Dinoflagellata, the Apicomplexa, or some intermediate taxon yet to be described. It has already been suggested that the Apicomplexa arose from the dinoflagellates with *Perkinsus* spp. being an early diverging group in the evolution of the Apicomplexa. This hypothesis may prove to be accurate when an adequate number of species in the two higher taxa are thoroughly evaluated.

The reader of this special issue of the *Journal of Shellfish Research* will find most of these research accomplishments described in greater detail in the papers that follow as well as other aspects not covered in this introduction. The accomplishments are considerable and much valuable information will undoubtedly continue to be provided in the years ahead. As measured by publications, the rate at which new information was being provided reached its highest level in the early 1990s and continues today. This is due in large part to funding from NOAA and in particular from NOAA's National Oyster Disease Research Program which has provided over $6 million in funding before being terminated this year. The U.S. Congress funded the Program with a special appropriation following an initiative by former Congressman Roy Dyson with particularly strong support from the Virginia and Maryland delegations; therefore, many of us who have worked on *P. marinus* are indebted to them. Once commercially viable answers to oyster mortalities caused by *P. marinus* have been found, oyster harvesters and farmers will also have these Congressional representatives to thank for much of the progress made in attaining that goal.

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HISTORICAL PERSPECTIVE ON *PERKINSUS MARINUS* DISEASE OF OYSTERS IN THE GULF OF MEXICO

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ABSTRACT A brief history of events and individuals involved in the discovery of the important oyster pathogen *Perkinsus marinus* (Dermo) is presented. Also a short review of the development of the fluid thioglycollate culture technique (FTM) for diagnosing Dermo is provided.

In 1946 the oystermen of Louisiana filed $30–$40 million lawsuits against several major oil companies and the Freeport Sulphur Company for alleged mortality of oysters due to in-shore petroleum operations. The primary allegation was that the discharge of "bleed" or "production" water into oyster-producing bays (primarily west of the Mississippi River delta) was responsible for abnormal losses of market-sized oysters. With the filing of these lawsuits, the defendants and plaintiffs began assembling teams of experts to investigate the allegations. Four major research groups were charged with determining the role, if any, of petroleum operations in oyster mortalities and determining the cause(s), if possible, of such high mortalities. They included:

1. Texas A&M Research Foundation (TAMRF) Project 9. This effort, which was by far the largest, was funded by several oil companies. The Project 9 investigators included Drs. Sewell H. Hopkins (head), John G. Mackin, and Winston Menzel as well as several chemists, supporting scientists, and technicians.
2. Gulf Oil Corporation (Gulf). Rather than join the TAMRF group, Gulf retained Albert W. Collier to lead its investigation. Gulf believed that more diversity would be introduced with two investigating groups. A. Wayne Magnitzky, Joe O. Bell, and Sammy M. Ray were hired by Collier to assist in the Gulf studies. Primary chemical support was from scientists at the Mellon Institute in Pittsburgh, PA.
3. Louisiana Wildlife and Fisheries Commission (LWFC). The LWFC investigation was led by Dr. H. Malcome Owen with the assistance of Robert M. Ingle, Fred (Red) Brigance, Lester W. Walters, and William Tolbert.
4. Freeport Sulphur Company (Freeport). Freeport's investigations were headed by Dr. A. E. Hopkins. To the best of my knowledge, these investigations were largely concerned with determining the effects of sulfur "bleed" or "production" waters on oysters. I recall only a few of the names of persons that assisted Dr. Hopkins, but they included Robert P. Hoffstetter, a cooperative student from Antioch College; John Boss, a cooperative student from Tulane; and Ted Ford. It is of interest to note that at least four of the major investigators of the Louisiana oyster mortality problem (S. H. Hopkins, J. G. Mackin, H. M. Owen, and Winston Menzel) had formerly worked at the Virginia Institute of Marine Science, Gloucester Point, VA.

By early to mid-1947 all groups launched extensive field and laboratory studies designed to determine the effects on oysters of Louisiana crude oil, "bleed" waters, water-soluble oil fractions, oil emulsions, and even associated oil production activities. Field studies by all oyster study groups generally showed two characteristic features of the Louisiana oyster mortalities: (1) major losses occurred in high-salinity areas during the warm months, and (2) market-sized oysters appeared to be much more susceptible than smaller oysters to mortality.

As the groups began to gather results from field and laboratory studies it became apparent to most investigators that oil and its associated operations were *not* the likely causes of continuing massive oyster mortalities in oysters transplanted from seed grounds east of the Mississippi River to oyster leases west of the river. A feature that supported this view was the lack of similar mortalities in oil fields located in low-salinity areas. This observation was also believed to be related to the fact that mortalities of the mid-1940s coincided with an extensive drought period in Louisiana.

With lessening concern about oil operations, several investiga-

tors began to consider other causes for the abnormal mortalities. The most prominent suspects being considered were *Thais* (southern oyster drill), *Nematopsis* (sporozoan), and *Polydora* (mud worm). Yet, extensive studies did not provide reasonable support for any of these primary suspects, particularly in relation to the high mortalities observed during the mid-1940s.

A year or two after the major investigations began, Albert Collier told me that he believed that an *unknown* microorganism was responsible for the oyster mortality. He showed me spherical organisms in fresh preparations of pericardial fluid from moribund oysters and pointed out that these bodies were not observed in healthy oysters. At this time he thought the organism was a "colorless" alga. A little later he showed me histological sections prepared of moribund oysters and pointed out "spherical" bodies that I now know were *Perkinsus* (*Dermocystidium*) cells. About the same time Drs. Mackin and Owen were apparently conducting similar studies of fresh preparations and histological sections of "healthy" and "moribund" oysters. Mackin, Owen, and Collier began to compare data and concluded that each was looking at the same "undescribed" organism that they suspected was the cause of abnormal warm-weather oyster mortalities in high-salinity areas.

This collaboration led to the publication in 1950 of the description of this unknown agent as *Dermocystidium marinum* (Dermo) by Mackin, Owen, and Collier (*Science*, III, 1950). Dr. Owen found Dermo in some preserved Louisiana oysters that had been exhibited at a World's Fair (Chicago?) circa 1920. This finding indicated that Dermo was present in Louisiana oysters for at least several decades prior to its discovery.

After the publication on *Dermocystidium* appeared in *Science*, the lawsuits began to unravel. Only weak evidence supported the allegations that oil operations were responsible for abnormal Louisiana oyster mortalities, yet there was strong epidemiological evidence linking them to *Dermocystidium* infections. By 1950, some of the lawsuits were dropped and the remaining ones were settled out of court on a nuisance basis for something on the order of $300,000–$400,000. It has been reported that the oil companies spent about $2 million on their investigations. I have no idea of the amount that the Louisiana Wildlife and Fisheries Commission spent, but Robert Ingle has indicated to me that it was rather small compared with oil company costs.

In addition to the discovery of a major disease-causing parasite of oysters, the Louisiana oyster investigations generated a strong impetus for establishment and expansion of marine science programs in Gulf Coast states. One notable example is the role the TAMRF Project 9 played in creating the Department of Oceanography at Texas A&M University at College Station. Many of the principals in the Louisiana oyster mortality investigations later became significant contributors to the field of marine science.

Prior to the discovery that Dermo was the likely cause of extensive warm-season mortality of market-sized oysters in high salinity areas west of the Mississippi River, Louisiana oystermen took steps to compensate for the great loss of oysters. In some areas, 75–100 percent of the market-sized oysters would die during the summer and early autumn. Since the oystermen wished to have a good crop of oysters for the harvest for the holiday trade (Thanksgiving and Christmas), they initially attempted to compensate for the summer mortality by doubling or tripling the seed plantings on leased grounds. This approach was possible because there was ample seed on the grounds east of the Mississippi River. This approach proved ineffective—the high mortality rate continued. Now that we know that Dermo may be transmitted directly, the excessive plantings probably exacerbated the spread of Dermo disease.

One thing was obvious to the oystermen. Sub-market-sized oysters that appeared to be growing well in spring would suffer extensive mortality during the following warm months of summer and early autumn. They learned that the extra 4–6 months required for the oysters to reach market size was also the greatest danger period. They correctly concluded that the second summer period in high-salinity areas was deadly for market-sized oysters and should be avoided if at all possible.

This realization prompted a drastic change in oyster culture strategy. In high-salinity areas west of the Mississippi River, seed planting was delayed until late summer and early autumn. These oysters were then harvested before the next summer. Using this timing of transplanting and harvesting, they avoided much of the summer mortality, which we now believe was caused by Dermo. Since most of these oysters were not large enough for the shucked or half-shell trade, they were canned. This method of marketing oysters proved to be very profitable. Although the period between transplanting and harvesting was rather short (6–8 months), the oystermen made money if they harvested a sack for canning for each sack planted. Oystermen were paid by the yield (cans per unit). In the event the harvest exceeded one for one, they did extremely well. The Louisiana canned oyster industry was eventually destroyed by cheaper imports of canned oysters.

Thus, as a matter of survival, the oystermen learned how to avoid the consequences of Dermo disease even before a large team of scientists was able to determine the cause. Some oyster biologists criticized the method devised by the oystermen as putting too much stress on the seed grounds. Had the canned oyster industry not collapsed, some predicted that the Louisiana oyster seed grounds east of the Mississippi River would have been ruined.

With settlement of the lawsuits in 1950, Gulf Oil Co. offered me a fellowship to attend either Rice University or Tulane University. My charge was to attempt to culture *Dermocystidium* in order to fulfill Koch's principles for this suspected pathogen of oysters. I chose Rice so that I could work under the late Dr. Asa Chandler, a world-renowned parasitologist, and began my studies in September 1950. The prevailing view at the time was that Dermo was a fungus, so my major efforts involved the use of various techniques generally employed to culture fungi.

With the failure to culture Dermo with the usual fungal techniques, Dr. Chandler and I considered that this organism might be an obligate parasite. We immediately changed the approach from attempting to culture Dermo to culturing oyster tissue to provide a medium for culturing Dermo. We realized that this was a formidable undertaking because tissue culture science was in its infancy at this time. Also a research of the literature indicated that there had been little success in developing molluscan tissue cell lines in the early 1950s.

The immediate problem was to develop procedures for obtaining sterile oyster tissue. Initially, excised pieces of gill tissue were stored for 24 hours in sterile sea water fortified with penicillin and streptomycin to inhibit bacterial growth. Gill tissue was selected since ciliary activity could be used readily as a gauge of tissue survival. Excised gill tissues appeared to survive antibiotic treatment. Thus the next step was to determine if the treated gill tissues were sterile. Since fluid thioglycollate medium (FTM) is commonly used to test various items for sterility, the treated tissues were placed in tubes of sterile FTM and incubated for 48 hours to

test for sterility. The tubes of FTM showed no evidence of bacterial growth after 48 hours' incubation.

The next step was to examine the treated gill tissues microscopically. My initial reaction was that the cultured gill tissues were filled with large "oil droplets." Upon closer examination the spherical structures appeared to have a definite wall and I discounted their being oil droplets. An examination of controlgill tissues (continuously stored in sea water with antibiotics)did not show the large spherical bodies noted in the cultured tissues.

Up to this point I had relied solely on microscopic examination of pericardial fluid of oysters to determine if they were infected with Dermo. This system worked fairly well in cases of moderately to heavily infected oysters. As a safeguard, however, every oyster used in my studies was fixed for possible histological examination if verification was required. Fortunately, I recalled having seen very large spherical bodies in the pericardial fluid of one oyster several months earlier. At the time sketches were made of the bodies and in my data notebook I recorded "these cells look like Dermo but they are too large." Thus I dismissed the thought that these bodies were Dermo. After seeing the large bodies in gill tissues cultured in FTM, stained histological sections (the first for my Rice research) were prepared of the oyster with the large cells and gill tissues (cultured in FTM) with large bodies. The bodies from both sources appeared to be the same—leading both Chandler and me to believe we were probably looking at enlarged Dermo cells.

Verification of this belief was accomplished by studying a time-sequenced series of stained sections of cultured gill and mantle tissues of infected oysters. The tissue series included control tissues (uncultured) and a series of tissues incubated in FTM at various intervals ranging from 2 to 48 hours. The controls showed the usual forms of Dermo found in histological sections of infected oysters. In FTM-cultured tissues, a slight enlargement could be detected after 2 hours' incubation and the cells appeared to reach maximum enlargement at 48 hours. As the cells enlarged with increased incubation, the typical Dermo cells began to disappear until none could be detected after 24–48 hours. With these data we were satisfied that Dermo did, in fact, enlarge when incubated in FTM as well as in other nutrient media. Moreover, we were convinced that little, if any, multiplication occurred during FTM culture. Thus, we believed that a simple, reliable technique for diagnosing Dermo disease in oysters had been discovered.

In retrospect, this discovery resulted from a combination of good luck, good recordkeeping, and logic. It was a matter of "luck" that the first tissues cultured in FTM came from a heavily infected oyster. Another important factor was the chance observation of enlarged Dermo-like cells in a live oyster, which (fortunately) was preserved for possible further examination. The logic came from "connecting" the large spherical bodies from two sources as possibly being Dermo cells.

The above comments prompt me to quote the late Dr. Sewell H. Hopkins with regard to luck. Since the late Drs. Hopkins and J. G. Mackin were actively working on Dermo, we wished to share the discovery with them and have their comments concerning the validity of our data. These scientists agreed that we had made a significant discovery, which should be published and made available to the scientific community as soon as possible. Near the close of our meeting Dr. Chandler said, "Sammy will be the first to admit that he was lucky." And I replied, "Yes." Dr. Hopkins' next comment is one that I shall never forget. He said, "You know luck is a strange thing—a person that works 16 hours a day has twice as much luck as one who works 8 hours a day." This comment on luck by Dr. Hopkins gave my morale as a graduate student a great boost.

With this diagnostic tool, which circumvented the use of time-consuming histological verification, and my limitations as a married graduate student with a family, I made the calculated decision to achieve my immediate goal—complete my graduate studies and obtain immediate answers to the most important questions. Since my studies were partially supported by the G.I. bill, I have estimated that my 4-year study at Rice cost the Gulf Corporation about $20,000. My studies occurred just before the era of big-time sponsored research at universities. Gulf gave no money, no overhead, and no other remuneration to Rice University.

During the workshop held on *Perkinsus marinus* at the National Shellfisheries Association meeting in Charleston, SC, in April 1994, a couple of current researchers told me that I was very close to successfully culturing *Perkinsus* during my studies at Rice and they wondered why I stopped working on this aspect. The above comments are given to explain my reason for shifting from the culture to other aspects of the problem. I am greatly impressed by the progress that has been made in the continuous culture of *Perkinsus* in an artificial medium as well as improvements in the sensitivity of the fluid thioglycollate diagnostic technique.

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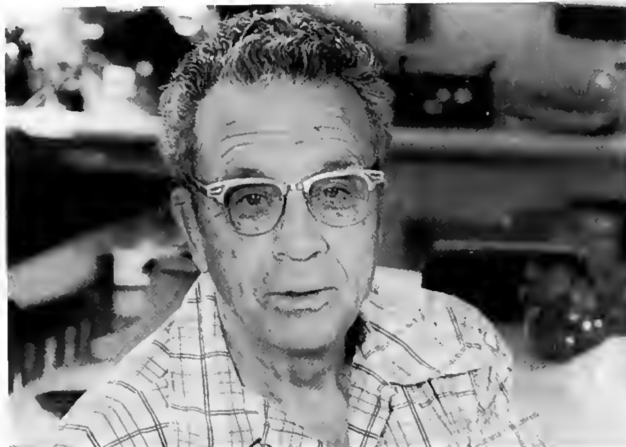
HISTORY OF *PERKINSUS MARINUS*, A PATHOGEN OF OYSTERS IN CHESAPEAKE BAY 1950–1984



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ABSTRACT The pathogen *Perkinsus marinus* (Dermo) was discovered in Chesapeake Bay in 1950. It was already widely distributed in the Bay and caused annual mortality below the mouth of the Rappahannock River. Annual mortality in trayed oysters at the Virginia Institute of Marine Science (VIMS) varied annually from 24% to 57% at this most favorable site for the disease. Over 2 million bushels of seed oysters from the James River public beds were transplanted annually to private beds in 4 major growing areas. These were Hampton Roads, lower Bay proper, Mobjack Bay at mouth of York River, and the Rappahannock River. The introduction of *Haplosporidium nelsoni* (MSX) in 1959 resulted in killing most oysters throughout the Bay, and private planting was abandoned. Extreme dry weather during the decade of the 1980s allowed both diseases to spread widely throughout the Bay, and the oysters became scarce everywhere. MSX retreated to its endemic area below the mouth of the Rappahannock River when salinities returned to average levels. Dermo destroyed oysters in the seed area of the James River, and it has persisted there tenaciously with low mortality. Market-oyster production dropped from 2 to 3 million bushels annually during the 1950s to 6,000 in 1993. No seed oysters are available, and planting of private beds has ceased. Recovery is slow, and the oyster industry in Virginia was destroyed.

KEY WORDS: History, diseases, Chesapeake Bay, pathogen, mortality, distribution, oyster culture

ORIGIN AND LIFE CYCLE OF *PERKINSUS MARINUS*

The origin of *Perkinsus marinus* is obscure. The pathogen is widely spread throughout SE Asia; possibly it was introduced by ship transport during World War II, but mortality of oysters was reported before 1940 in Virginia. Numerous small introductions of Pacific oysters (*Crassostrea gigas*) have been made along the east coast of North America from the west coast (Andrews 1979). The disease has not been a problem along the west coast of North America, or in Europe where oceanic climates and upwelling keep waters much cooler than on east coasts. The Pacific oyster was introduced along the west coast of North America before 1900; seed oysters in commercial quantities were imported regularly from Japan to Washington and California after World War II (Andrews 1980). Dermo has not occurred along the western shores of Europe despite many tons of introductions of Japanese oysters in late 1960s and early 1970s.

Dermo causes a warm-season disease of eastern oysters (*Crassostrea virginica*) in Chesapeake Bay (Andrews 1988). At temperatures above 20°C, the pathogen multiplies and kills oysters about a month after infection. For rapid proliferation, the disease requires temperatures of 25°C which prevail for about 5 months in Chesapeake Bay waters (Andrews and Hewatt 1957). Mortality ceases by 1 November when water temperatures decline below 20°C; during the 1950s, oysters gradually expelled infections, and from February through April most samples showed no infections by Ray's FTM test (Ray 1952). However, oysters placed in 25°C water during late winter and spring revealed about 20% infection within a month (Andrews and Hewatt 1957). These hidden infections became patent in June when temperatures reached 25°C. These over-wintering infections caused deaths by 1 August, and two more generations of infections occurred before mid-October with prevalences of Dermo often at 90% to 100%.

A comparison of the life cycle of Dermo in the Gulf of Mexico and in Chesapeake Bay is revealing (Andrews and Ray 1988). Higher winter temperatures in the Gulf allow the pathogen to persist in oysters with patent infections throughout the winter, although intensity and prevalence decline. In Louisiana where most Gulf oysters are grown, salinities fluctuate widely depending upon Mississippi River flow, resulting in wide fluctuations of the disease by years and areas. Planters there must search for disease-

[1]VIMS Contribution No. 1884.

free oysters in low-salinity areas to transplant into high-salinity areas for growth, fattening, and early marketing.

Dermo had a wide distribution in Chesapeake Bay at the time of its discovery in 1950. It spread more widely into marginal salinity areas during the mid-1960s' invasion of upper Virginia and Maryland oyster beds. It spread into the James River only during the dry period of the 1980s. It persisted tenaciously at low levels of infection most winters. Only during dry summers did the pathogen kill oysters in areas with late-summer salinities <20 ppt. Scarcity of oysters in the lower bay limited the distribution of Dermo to manmade structures and creeks which had regular recruitment of new year-classes. Beds leased by private growers became barren because those bottoms are soft and oysters sink in time. This fact is important to any efforts to grow oysters in isolation once disease-free seed is available.

TRANSMISSION OF *P. MARINUS* DISEASE

Transmission of Dermo is direct from infected dying oysters to other hosts of the species (Mackin 1962). Proximity to gapers (dying oysters) is necessary because large dosage is required to achieve rapid infection (1×10^{5}) zoospores (Roberts, Virginia Institute of Marine Science [VIMS], pers. comm. 1984). All stages appear to be infective or become so when the host dies and prezoosporangia are released into marine waters. From 1,000 to 2,000 zoospores are estimated to be produced by one large sporangium (Perkins 1966). Zoospores are produced from prezoosporangia after culture in thioglycollate medium for 24 to 48 hours. Feeding or injecting small amounts of macerated gaper tissues produces infection in nearly all oysters. Infection occurs apparently through the digestive tract as indicted by the location of foci of infection in sectioned live oysters. The role of zoospores in open water infections is unknown. They must be an infective stage, but difficulty in production of this stage in the laboratory has prevented completion of the life cycle for the pathogen after 45 years of research. Distances for isolation of oysters from the disease are speculative, which hampers planning for repopulation of oysters in Chesapeake Bay.

A host of scavengers live on oyster beds to feed on oysters killed by predators and diseases (Andrews 1988). Blue crabs and mud crabs (Xanthids) kill small oysters whereas nereid worms, spider crabs, and several small fishes such as blennies, gobies, and clingfish are scavengers quick to snatch bits of loose flesh from gaping oysters (SCUBA observations).

OVER-WINTERING OF *P. MARINUS* IN CHESAPEAKE BAY

The level of over-wintering infection is critical to the infectivity and mortality caused by *P. marinus* disease the following summer. There is a 5-month period of temperatures above 20°C that favors multiplication by the pathogen. If there were no over-wintering infections, the disease would die out; no alternate host has been identified. During the early 1950s, Delaware Bay planters imported oysters from the eastern shore peninsula of Virginia and introduced the disease there (Ford 1992, 1996).

During the 1950s, when Dermo was monitored without interference by *Haplosporidum nelsoni* (MSX) disease, Ray's FTM tests showed low levels of over-wintering infection at VIMS pier in samples from February through April. Yet development of a few infections was found in June and July after temperatures reached 20°C to 25°C. Oysters placed in warm water for a month in April had about 20% infection. Disease-free oysters imported in April from the upper James River did not develop infections until August, and mortalities were far less than those of acclimated oysters from a previous year's transplanting. This pattern of infection and mortality was derived from 30 years of FTM tests on Virginia oysters. It was apparent that hidden infections were over-wintering, but the stage and site in oysters were not known (Andrews 1988).

SPREAD OF *P. MARINUS* DURING DROUGHT OF 1980S

The droughty decade of the 1980s allowed Dermo to spread widely into Maryland and up the James River seed area which is vital to oyster culture in Virginia (Andrews 1988, Burreson and Andrews 1988) (Fig. 1). Record high salinities and well-populated contiguous oyster beds allowed the disease to spread rapidly and to kill most oysters in the James River except on a couple of upriver beds. Continued harvesting by oystermen helped to deplete the 15-mile-long area of seed oysters and broodstock. Yet the pathogen persisted by wintering in quite low-salinity waters with temperate winters. Over-wintering prevalences of 100% were found at Point of Shoals, a rather upriver site (Ragone Calvo and Burreson 1994). Probably planters, who transported infected oysters from lower river beds to their upper river private beds, helped spread the disease. For a low price, these planters bought market-size oysters in late spring and held them on upriver beds through the summer for high fall prices.

The spread of Dermo into the James River seed area during the 1980s, after 30 years of freedom from disease, was unprecedented (Andrews 1988). The prolonged dry weather during the decade, as well as the complete absence of hurricanes to depress salinities during summers, was critical. Each of three earlier decades had one or more hurricane flooding periods. Salinity regimes in Maryland were very high with a one-time record of 25 ppt at the Bay Bridge above the oyster-growing area. Dry summers increased salinity levels.

Many years of observation of coastal plains estuaries, such as the Great Wicomico and Piankatank Rivers, show that Dermo can persist indefinitely in rather low-salinity rivers once established; only when bay waters are salty from low runoff do the two pathogens, Dermo and MSX, cause appreciable mortality. These estuaries are dependent on the bay for their salinity regimes, and little freshwater runoff is available to reduce salinity. Typically, these estuaries get to 15 ppt only in late summer and fall when time for disease development is limited. Importantly, these estuaries are effective in producing seed oysters with quite regular spatfalls. The diseases have not affected setting rates in the coastal plain estuaries because low populations of broodstocks are adequate. The James River requires very high oyster populations for production of seed oysters.

SUSCEPTIBILITY OF OYSTERS TO *P. MARINUS* BY SIZE AND ORIGIN

Few seed oysters have been imported commercially from the Carolinas into Chesapeake Bay; however, in the early 1950s when New Jersey planters were transplanting oysters from eastern shore of Virginia, a scarcity for local planters induced a trial of South Carolina oysters. Seaside (Virginia) oysters were found to be highly susceptible to MSX, but Dermo was not found on these beds perhaps because fast growth allowed early harvesting. South Carolina oysters matured rapidly in Virginia waters and were somewhat resistant to Dermo. Native yearling oysters from James River, where no selection had occurred, showed strong resistance to Dermo infection in open waters. Heavy dosage in aquaria produced infections.

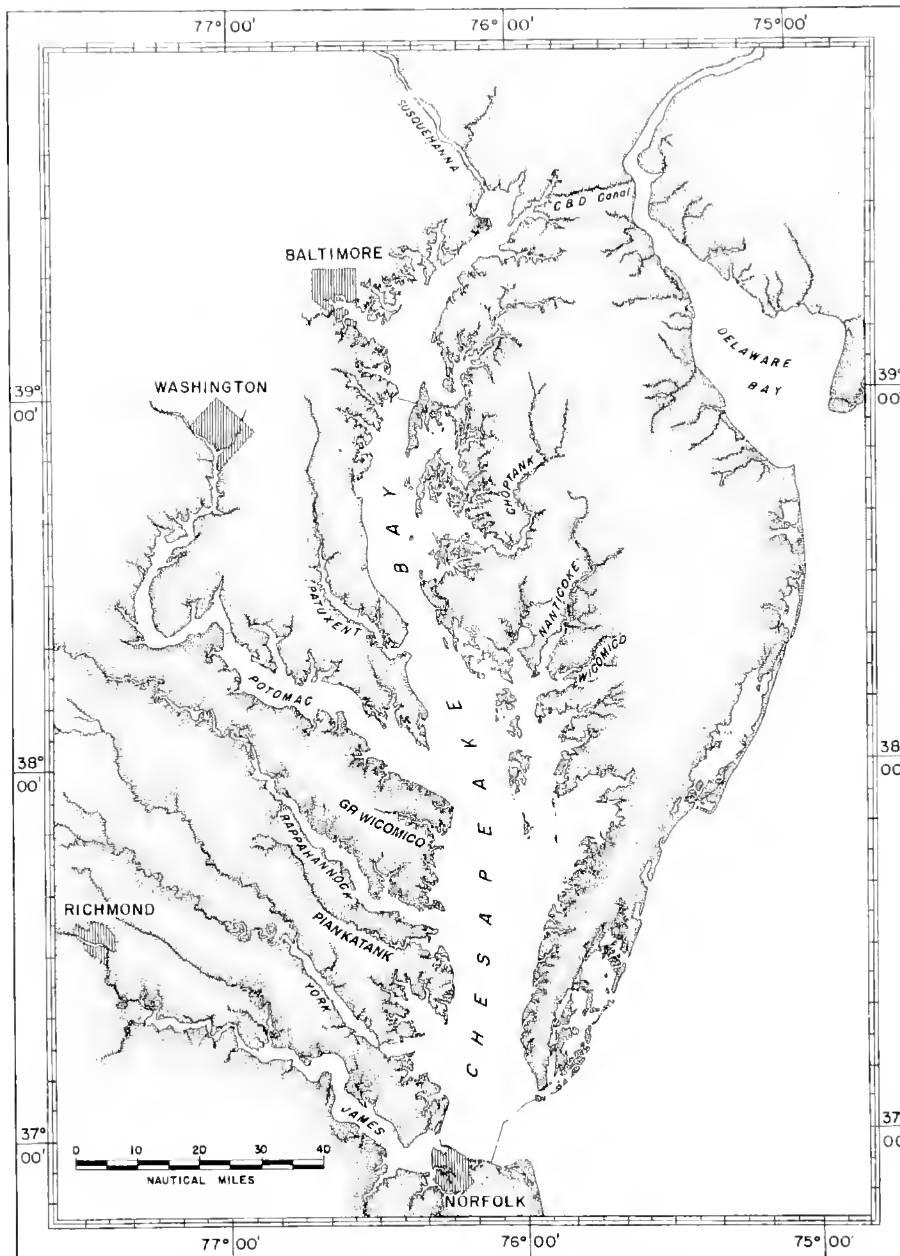

Figure 1. Map of Chesapeake Bay showing major rivers where oysters were grown as discussed in text.

This resistance of yearlings to Dermo may allow production of oysters on isolated beds if moved before their second summer of exposure. If a coastal plain estuary were declared strictly for production of young seed oysters, by prohibiting private plantings along the shores, a disease-free supply of oysters could be produced annually for transplanting to barren areas in higher salinity waters. Early harvesting would be necessary. This method should be tried because recovery of setting and decline of Dermo in the James River seed area are unpredictable. The price of a pint of west coast oysters in local stores is $8 now, which deprives the author of a seafood that was cheap throughout most of his 40 years of study of oyster diseases. I miss them.

INTRODUCTION OF *H. NELSONI* DISEASE IN 1959

The introduction of *H. nelsoni* (MSX) (Ford and Haskin 1982) complicated disease problems in Chesapeake Bay. This disease moves rapidly up and down the bay with changes in salinities. It requires salinities of 12 to 15 ppt to infect oysters, but it is easily discharged at 10 ppt. This disease invaded the Maryland part of the bay in the mid-1960s, causing heavy mortality (Andrews 1967), and again in the mid-1980s. The source of infection by MSX is unknown. Fresh-water flows from the large drainage areas of the James, Potomac, and Susquehannah Rivers reduce salinities in winter and spring, and that sets the patterns of disease for different areas. MSX invades the upper bay rapidly in one year and is usually discharged the following winter. Hurricanes play an important role in controlling MSX by lowering bay salinities during summers, and it is expelled in winters. There have been no significant hurricanes in the Chesapeake area since 1973.

H. nelsoni became the dominant pathogen during the 1960s and 1970s in lower Chesapeake Bay. It kills quicker than Dermo, and it has no need for proximity to infected oysters to produce infec-

tions. Dermo was suppressed by scarcity of oysters in the lower bay, but given 2 or 3 years of exposure, it eventually got into trays and oyster beds. Populated beds in the lower bay were gone after 1961 from MSX ravages. Only when the high-salinity years of the 1980s occurred throughout the bay did Dermo become the dominant disease in upper bay estuaries. Sparse populations of oysters were being killed in the lower bay by both diseases. Oyster planting in the lower bay had ceased in 1961 after MSX was imported in 1959. The endemic zone for MSX was from the mouth of the Rappahannock River down-bay, including the lower James River and all of the York River. In up-bay low-salinity areas some oysters were still being planted. Dermo was explosively dominant in the upper James River where it had never appeared before. The quick invasion and strong persistence in James River have not been explained adequately, although the cause was definitely high salinities through many very dry years in the 1980s. During the 1990s, 5 years had winter-spring runoff in the bay less than half-average flow. Dry summers sustained the high salinities. Expulsion of Dermo from the James River seed area is slow and the possibility of its removal is questionable. Wet, cold winters may be helpful. It may take many years before the river develops adequate broodstock to begin repopulating the famous river that produced 2 to 3 million bushels of seed oysters annually without fail until the 1980s.

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EPIZOOTIOLOGY OF *PERKINSUS MARINUS* DISEASE OF OYSTERS IN CHESAPEAKE BAY, WITH EMPHASIS ON DATA SINCE 1985

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ABSTRACT Since 1987 *Perkinsus marinus* has been the most important pathogen of the eastern oyster, *Crassostrea virginica*, in Chesapeake Bay because of its widespread distribution and persistence in low salinity areas. The pathogen became established on all oyster beds in the Chesapeake Bay as a result of natural spread during the consecutive drought years from 1985 to 1988 or by movement of infected oysters during the same period. Elevated salinities resulting from drought conditions and concomitant warm winters allowed *P. marinus* to proliferate in what were historically low salinity areas. Oyster mortality was high on most beds and landings of market oysters declined to record low levels in both Maryland and Virginia during the late 1980s and early 1990s. The seasonal periodicity of *P. marinus* is primarily controlled by temperature. Both prevalence and intensity of infections begin to increase in June as temperature increases above 20°C and overwintering infections begin to proliferate. Maximum values of prevalence and intensity occur in September immediately following maximal summer temperatures. Infection regression occurs during winter and spring as temperature declines resulting in minimum prevalence and intensity values in April and May. Prevalence and intensity of *P. marinus* infections in oysters from the James River, VA, over a five year period were significantly correlated with temperature when temperature data were lagged three months. Temperature explained 39% of the variability in prevalence and 46% of the variability in intensity. The relationship between temperature and annual variability in *P. marinus* abundance is somewhat obscure, in part because of the difficulty separating salinity and temperature effects. Nonetheless, data from 1988 to 1994 from the James River, VA, suggest that abnormally warm winters have a more significant impact on summer *P. marinus* abundance than abnormally cold winters. Salinity is the primary environmental factor that controls local distribution and intensity of *P. marinus* infections. Long-term oyster disease monitoring along a salinity gradient in the James River, VA, revealed a statistically significant relationship between salinity and *P. marinus* prevalence and intensity. *P. marinus* infections remain light in intensity and no oyster mortality results if salinity is consistently less than 9 ppt. However, infections may persist for years in low salinity areas. If summer/fall salinities range from 9 to 15 ppt some infections may progress to moderate and heavy intensity, but oyster mortality is relatively low. If summer/fall salinities are consistently greater than 15 ppt, moderate and heavy infections may be numerous and oyster mortality may be high. Field studies in the York River, VA, suggest that new *P. marinus* infections are acquired from July through early October, but peak infection acquisition occurs during late August and is correlated with oyster mortality. The early infection process in oysters and the role of zoospores in transmission dynamics in nature are poorly understood. No direct link between oyster defense mechanisms and control of *P. marinus* infections has been established. If oyster defense mechanisms do modulate *P. marinus* infections, the components have not been identified. There is little evidence to support the common perception that pollution is responsible for the dramatic increase in *P. marinus* abundance since 1985. Pathogen abundance is clearly correlated with salinity increases resulting from drought conditions in the late 1980s, although there may be subtle effects of toxicants or poor water quality on the host/parasite interaction.

KEY WORDS: *Perkinsus*, oyster disease, annual cycle, transmission, epizootiology, salinity effects, temperature effects

Since 1987, *Perkinsus marinus* (Mackin et al. 1950), the causative agent of Dermo disease, has been the most important pathogen of the eastern oyster *Crassostrea virginica* (Gmelin) along the east coast of the United States south of Delaware Bay. The origin of *P. marinus* is obscure, but it probably always has been an associate of oysters. It was first reported in Chesapeake Bay oysters in 1949 (Andrews and Hewatt 1957). The pathogen was first described as *Dermocystidium marinum* because of apparent affinities with fungal parasites of freshwater fishes (Mackin et al. 1950). It was later reclassified as *Labyrinthomyxa marina* because of observations of gliding cells similar to those present in slime molds (Mackin and Ray 1966). Ultrastructural observations (Perkins 1976) of an apical complex in the motile zoospore stage led Levine (1978) to establish the new genus *Perkinsus* for the pathogen within the phylum Apicomplexa. Taxonomic placement of *P. marinus* in the Apicomplexa has been controversial because of the presence of a number of morphological and life cycle characteristics more typical of the Mastigophora (flagellates) than of the Apicomplexa (Vivier 1982). Molecular sequence data (Fong et al. 1993, Goggin and Barker 1993) and a recent phylogenetic analysis based on sequence data (Siddall et al. 1995) do not support inclusion of *P. marinus* in the phylum Apicomplexa, but suggest a recent common ancestry with the dinoflagellates.

Along the east coast of the United States prior to the late 1980s, *P. marinus* was restricted to high salinity portions of coastal bays and estuaries south of Delaware Bay, although it apparently was absent from the seaside bays of the eastern shore of Virginia and Maryland (Andrews 1988). In the Chesapeake Bay, *P. marinus* was prevalent in the lower Bay, but was restricted to the mouths of the major tributaries in Virginia and southern Maryland (Fig. 1). There were a few localized concentrations of *P. marinus* in Maryland, primarily in Fishing Bay and Eastern Bay. The pathogen was observed locally in Delaware Bay in the mid-1950s, as a result of importing infected oysters from Chesapeake Bay, but it never caused significant mortality in oysters and appeared to die out as importations stopped in the late 1950s (Ford 1992). North of Delaware Bay the parasite was absent or at least undetectable.

In endemic areas *P. marinus* has always been responsible for some oyster mortality, but it did not significantly affect harvest most years because of the large natural sets on public beds and good seed-oyster availability for private planters in Virginia. An excellent review of the history of research on this pathogen and of

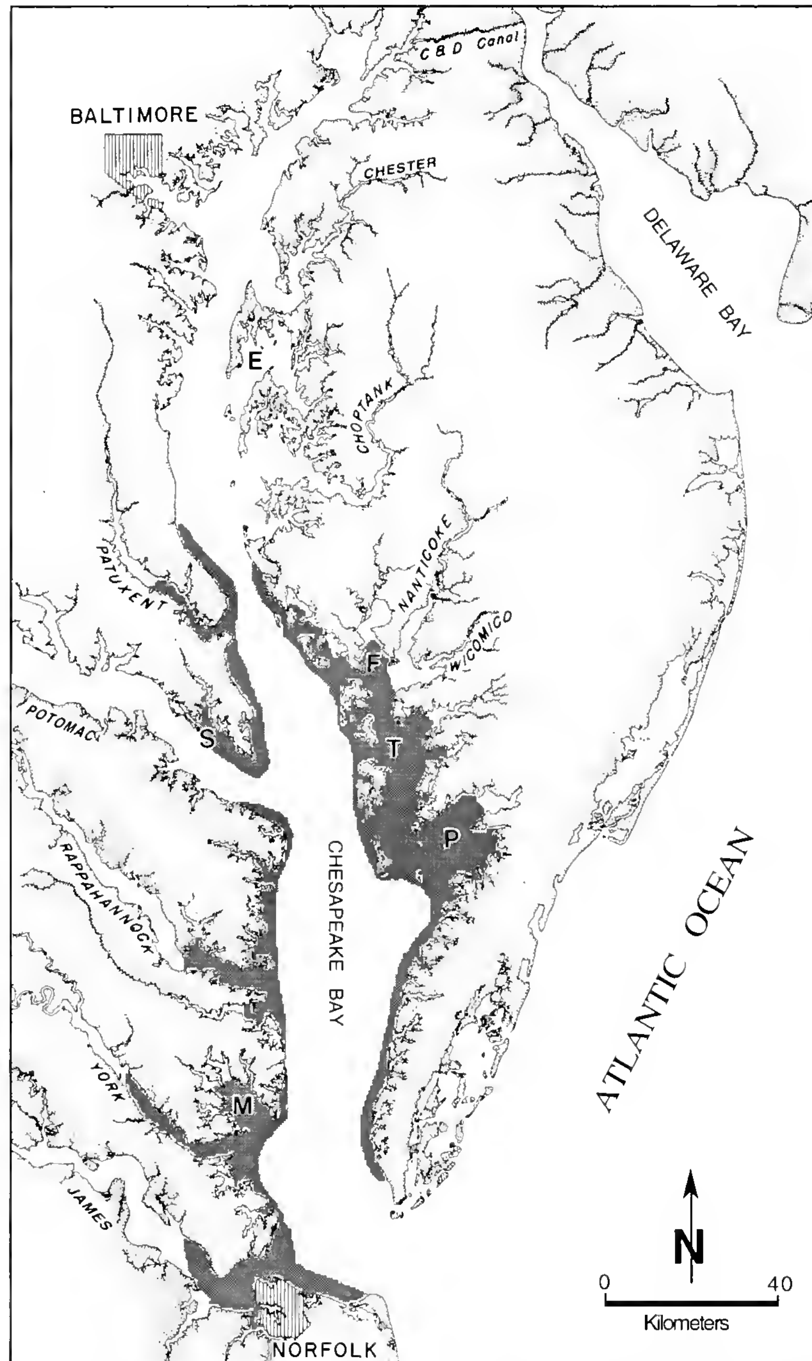

Figure 1. Distribution of *P. marinus* in Chesapeake and Delaware Bays prior to its spread during the 1980s. E = Eastern Bay, F = Fishing Bay, M = Mobjack Bay, P = Pocomoke Sound, S = mouth of St. Mary's River, T = Tangier Sound. Data from Andrews (1981) and Krantz and Otto (1981).

P. marinus epizootiology in Chesapeake Bay prior to the late 1980s was provided by Andrews (1988).

It has long been known that the distribution and local abundance of *P. marinus* are controlled by environmental conditions (Andrews 1988). During the late 1980s and early 1990s the distribution and epizootiology of *P. marinus* in the Chesapeake Bay deviated from historical patterns as the result of four consecutive drought years and concomitant warm winters from 1985 to 1988. During that period, *P. marinus* spread to all productive oyster grounds in Chesapeake Bay either by natural processes or by movement of infected oysters. Elevated salinities and warm winters allowed the pathogen to survive in areas that historically were disease-free. Although drought conditions have abated and rainfall patterns have returned to more or less typical conditions, with wet winters and springs, especially during 1993 and 1994, *P. marinus* continues to persist tenaciously in most areas of the Chesapeake Bay. The presence of the pathogen throughout the James River seed area in Virginia has been especially troublesome because infections develop to lethal levels when seed oysters are transplanted to high salinity growout areas.

The purpose of this review is to summarize the current distribution of *P. marinus*, to discuss the current understanding of the epizootiology of the pathogen in Chesapeake Bay, with emphasis on changes since 1985, and to discuss the impact of this pathogen on the oyster resource of the Chesapeake Bay. We will focus on environmental controlling factors, as they are particularly important in Chesapeake Bay, and we will attempt to identify areas where data are especially lacking and where research needs to be focused.

PRESENT DISTRIBUTION OF *P. MARINUS*

As of late 1994, *P. marinus* is known from as far north as Wellfleet Harbor, Cape Cod Bay, MA (Ford 1996), south throughout the bays and estuaries along the east coast of the United States, including virtually all oyster beds in Delaware and Chesapeake Bay, and throughout the Gulf of Mexico as far south as Tabasco, Mexico (Burreson et al. 1994a, Soniat 1996). In the mid-1980s, *P. marinus* had not been reported north of Chesapeake Bay; thus, the present distribution represents either a major northward expansion of *P. marinus* or a significant increase in abundance of the parasite in areas where it may have been present but was undetectable.

Although *P. marinus* was reported periodically from native oysters in Delaware Bay in the mid-1950s, probably as a result of importing infected oysters from Chesapeake Bay or other southern areas, it never became established and has never been responsible for significant oyster mortality (Ford 1992). This situation changed in 1990 when *P. marinus* became abundant in Delaware Bay and was also found in Great Bay along the Atlantic Coast. Abundance and distribution within Delaware Bay increased during 1991 and significant oyster mortality occurred then and in subsequent years. Prevalence of *P. marinus* in New Jersey coastal bays during 1991 ranged from 30% in Dry Bay, Manasquan and Tuckerton, 50% in Raritan Bay and 85% in Great Bay. As of 1994, the parasite is abundant on all oyster beds on the north shore of Delaware Bay, including the seed beds (Fig. 2); it seems to be much less abundant along the southern, Delaware shore (Ford 1996).

In Delaware Bay, it appears that *P. marinus* spread from undetected localized foci as a result of unusually warm winters during the period (Ford 1992), although effluent into the Maurice River from shucking houses processing *P. marinus*–infected oysters from the Gulf of Mexico may have also contributed to the spread. Because of the drought conditions and concomitant warm winters, the pathogen was able to become established and it has now replaced *Haplosporidium nelsoni* (MSX), although perhaps temporarily, as the most important oyster pathogen in Delaware Bay (Ford 1996).

The spread of *P. marinus* northward into Long Island Sound was probably also facilitated by warm winters. The pathogen may have spread from undetected localized foci of infection established in the past by importation of infected oysters from southern areas, but possibly also by recent movement of infected oysters, although recent movements have not been documented. Prevalence and intensity of *P. marinus* are high in oyster samples from some areas of Long Island Sound and the south shore of Cape Cod, for example Cotuit, MA, and oyster mortality attributed to this pathogen has been relatively high in some areas (Ford 1996).

In the Chesapeake Bay, *P. marinus* spread into historically low salinity areas during the prolonged drought of the late 1980s and it is now present on all public oyster beds in both Virginia and Maryland (Figs. 1 and 2), although significant oyster mortality is restricted to those areas where salinity is above about 12 ppt for most of the summer and fall. The parasite is also now present in the bays along the seaside of the eastern shore of Virginia and Maryland, probably as a result of moving infected oysters to those locations from Chesapeake Bay.

Unfortunately, Virginia scientists were not aware of the spread of *P. marinus* during 1985 and 1986. Dr. Jay Andrews had retired in 1984 and the oyster disease monitoring program that had been underway since 1959 was terminated. The first indication of the spread was very high mortality in September 1986 in oysters transplanted from the James River seed area to three tributaries along the south shore of the Potomac River, the Coan and Yeocomico Rivers and Machodoc Creek. Disease analyses revealed high levels of *P. marinus* in all three areas (>90% prevalence, 2.6–3.4 weighted prevalence). The source of the seed was revealed as Miles ground in the lower portion of the James River seed area (Fig. 3) and subsequent analyses of oysters from that site revealed high prevalence (96%) of *P. marinus* although most infections were light (weighted prevalence = 1.36) (Burreson 1987). It became clear that the parasite had spread into the lower seed areas and had been moved to the growout areas in infected seed oysters. The drought conditions allowed *P. marinus* to spread into the seed area and also allowed it to flourish in the growout areas because salinity was favorable (>12 ppt) in those areas as well. During the seven year period from 1985 through 1991, only 1989 was considered a wet year. The growout tributaries off the south shore of the Potomac River had previously been free of significant mortality caused by *P. marinus* although the parasite was observed in these areas during some years (Andrews 1981). Oysters in the lower portion of the James River seed area were known to harbor *P. marinus* periodically (Andrews and Hewatt 1957), but prevalence and intensity were always low.

By 1988, intensity of *P. marinus* infections in endemic areas had increased dramatically and oyster mortality was high, especially during 1987 and 1988. In addition, favorable salinities allowed the pathogen to spread into new areas and by 1991 *P. marinus* had spread to most oyster growing areas of the Chesapeake Bay including Maryland either by natural processes or by movement of infected oysters (Table 1). Oysters in previously non-enzootic areas were highly susceptible to *P. marinus*, infection prevalence and intensity were unusually high, and mortality was high on both planted grounds and public beds in favorable salinity. The parasite was present at Wreck Shoal (WS) (Fig. 3) in the middle of the James River seed area in 1986 and had spread to Deepwater Shoal (DWS), the uppermost oyster bed in the James River by 1988. Prevalence and intensity of *P. marinus* continued to increase in the James River through 1991. Similarly, the pathogen spread throughout the Rappahannock River and was present at Ross Rock, the uppermost oyster bed by 1992, although both prevalence and intensity were very low at that site.

A similar up-bay spread of *P. marinus* occurred in Maryland through the 1980s and early 1990s (Figs. 1 and 2) from foci of infection in Tangier Sound, Holland Strait, Tar Bay and near the mouth of the St. Mary's River. By 1987 the parasite had spread up the main stem of the Bay to Swan Point north of the mouth of the Chester River and throughout Fishing Bay and the mouth of the Choptank River. In the Potomac River the parasite spread to the mouth of Clements Bay during 1987. During 1988 *P. marinus* spread throughout the Choptank and Little Choptank Rivers and further up the Potomac River to the mouth of the Wicomico River. By 1992 the pathogen had spread throughout the Chester River and

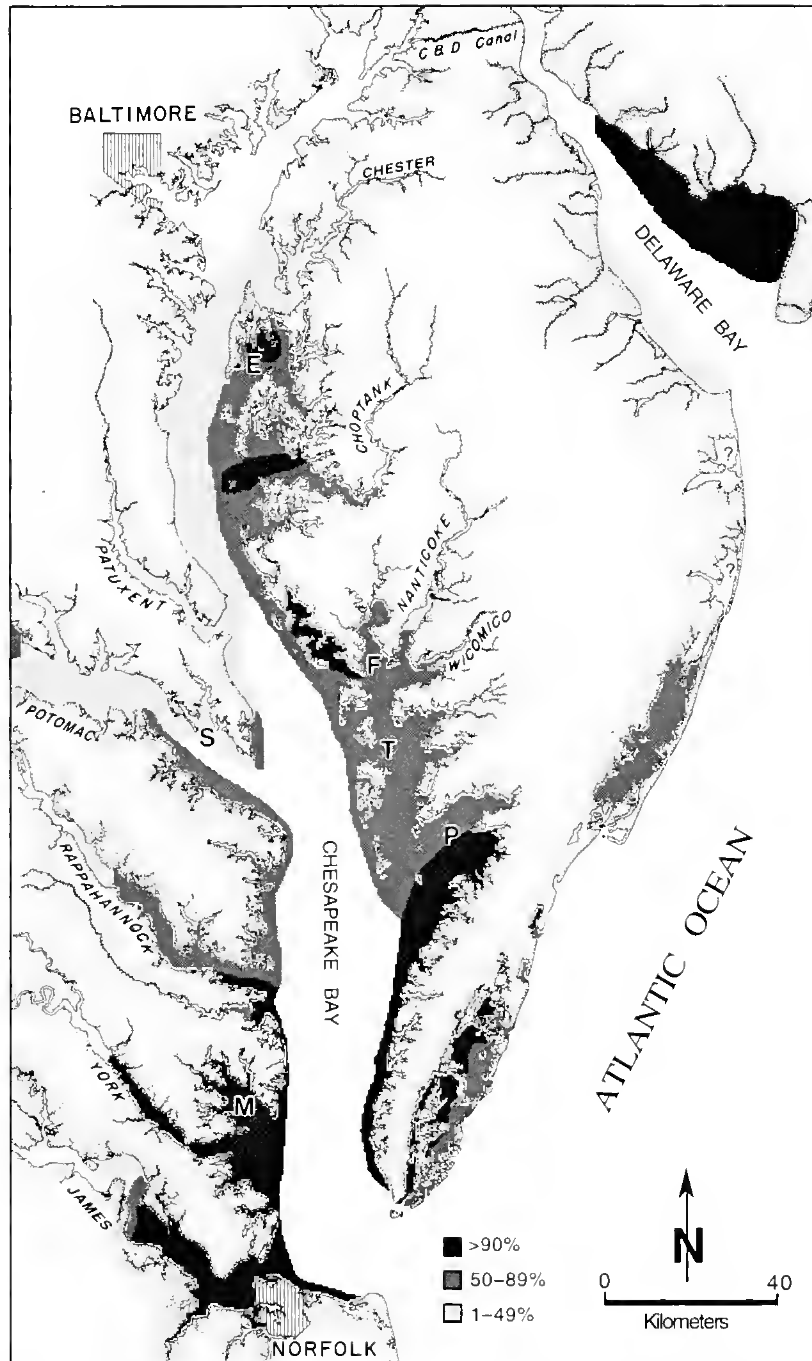

Figure 2. Present distribution of *P. marinus* in Chesapeake and Delaware Bays. Shading indicates maximum annual prevalence. E = Eastern Bay, F = Fishing Bay, M = Mobjack Bay, P = Pocomoke Sound, S = mouth of St. Mary's River, T = Tangier Sound. Data from Ragone Calvo and Burreson (1995), G. E. Krantz (personal communication) and S. E. Ford (personal communication).

was present on every productive oyster bar in Maryland (Krantz 1993). Intensity of infections during summer and fall increased each year in previously invaded areas and oyster mortality was greater than 50% in areas with favorable salinity including most areas south of Kent Point (Krantz 1990, Krantz 1992, Krantz 1993).

The spread of *P. marinus* into areas in the lower Chesapeake Bay where it was historically absent seems to have been a long-term acquisition. Unusually high spring runoff during 1993 and 1994, a very wet July in 1994 and a cold winter in 1993–94 had little effect on the subsequent fall prevalence of *P. marinus* in the James River, VA (Table 1), although intensity of infections declined somewhat from a peak in 1991. Prevalence and intensity of *P. marinus* infections did decline to a greater extent in the upper Bay in Maryland during 1994 (Table 1). The historical absence of *P. marinus* in the upper Bay and upper reaches of the major tributaries suggests that the pathogen will eventually be eliminated from these areas if normal environmental conditions of cold win-

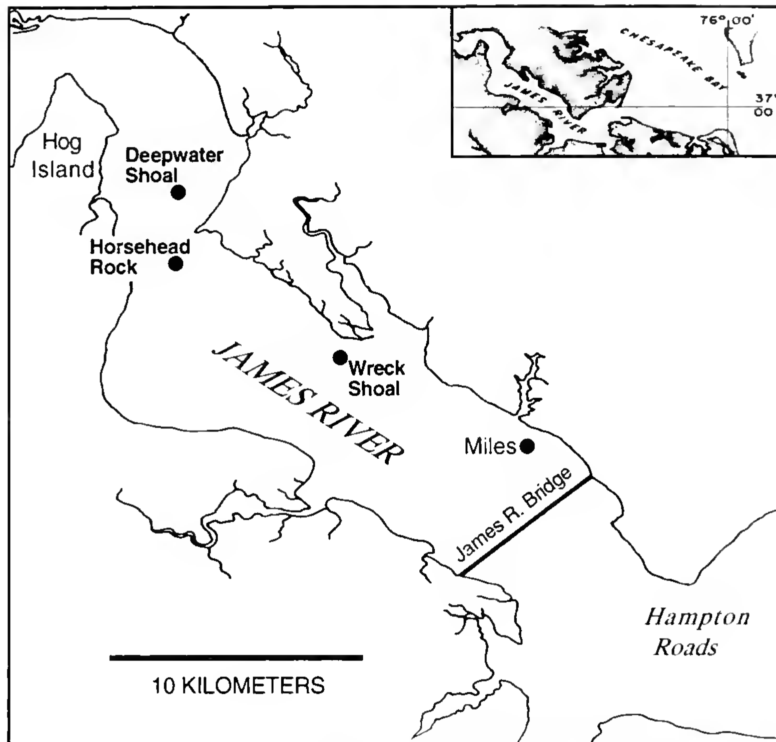

Figure 3. James River, VA, showing locations of various monitoring stations.

ters and wet springs continue, but monthly monitoring in Virginia during the 1990s has demonstrated that the decline will be slow and may take a decade or more. Unfortunately, with the present widespread distribution of *P. marinus*, any drought period will allow the pathogen to increase in abundance and will only prolong the problem.

South of Chesapeake Bay, *P. marinus* has always been present in bays and estuaries including intertidal oyster beds. The drought conditions of the late 1980s also caused a dramatic increase in abundance of *P. marinus* in North Carolina. Oyster mortality attributable to *P. marinus* was first documented in the fall of 1988 in southern North Carolina. From 1988 through 1992 the pathogen

TABLE 1.

Prevalence (% infected) of *P. marinus* at various locations in Chesapeake Bay[a]

Location	1980	1986	1989	1991-92[b]	1994
Virginia					
James River, Wreck Shoal	0	0	100	100	100
James River, Horsehead Rock	0	0	48	100	96
James River, Deepwater Shoal	0	0	8	88	56
Rappahannock River, Broad Creek	04	84	44	100	64
Rappahannock River, Smokey Point	0	04	44	100	46
Rappahannock River, Bowlers Rock	nd	0	40	88	16
Rappahannock River, Ross Rock	0	0	0	24	0
Maryland					
Swan Point	0	0	03	23	03
Chester River, Old Field	0	0	10	37	20
Eastern Bay, Bugby	0	0	100	100	63
Choptank River, Cooks Point	0	0	23	100	90
Choptank River, Sandy Hill	0	0	53	100	83
Patuxent River, Broomes Island	50	50	57	100	40
Potomac River, Cornfield Harbor	75	nd	nd	100	77
Potomac Rover, Ragged Point	0	nd	93	90	10
Potomac River, Lower Cedar Point	0	0	03	10	83
Holland Straits	80	nd	nd	100	57
Tangier Sound, Old Woman's Leg	80	nd	23	100	73

[a] Data from Andrews (1981), Burreson (1987, 1990, 1992, 1993), Krantz (1990, 1992, personal communication), Krantz and Otto (1981) and Ragone Calvo and Burreson (1995). nd = no data.

[b] For most locations, either 1991 or 1992 was the year of highest prevalence.

spread northward along the eastern edge of Pamlico Sound and then across to the western side, eventually infecting all oyster beds and causing high mortality. Oyster mortality from *P. marinus* continued during 1993 and 1994 in Pamlico Sound, but mortality seems to have declined in southern areas near Bogue Sound (M. Marshall, personal communication).

The status of *P. marinus* in South Carolina and more southern states does not seem to have changed significantly from historical levels although there have been few data published on distribution and intensity in these areas (Burrell et al. 1984, Crosby and Roberts 1990) and extensive disease monitoring is lacking. The pathogen is present and causes some oyster mortality in most areas.

ANNUAL CYCLE OF *P. MARINUS* PREVALENCE AND INTENSITY IN CHESAPEAKE BAY

Samples of non-spat oysters from natural oyster beds exhibit a pronounced seasonal cycle in both prevalence and intensity (expressed as weighted prevalence) of *P. marinus* infections when diagnosed with the fluid thioglycollate technique (Ray 1952, Ray 1966) of mantle, gill and rectal tissue (Fig. 4). Typically, prevalence and weighted prevalence of *P. marinus* infections begin to increase in June. On average, maximum values of both parameters are reached in September; prevalence at WS (Fig. 4) reaches 100% every year and weighted prevalence reaches 2.0 with some years above 3.0. These values contrast with 1954 when prevalence of 60% and weighted prevalence of 1.5 were considered intense infections (Andrews and Hewatt 1957). The relative contribution of multiplying overwintering infections and acquisition of new infections to this increase is not clear, but based on timing of *P. marinus* transmission (see below) it appears that the early summer increase is primarily the result of proliferation of overwintering infections. Prevalence may remain high through January, but intensity, measured as weighted prevalence, usually declines sharply in October if peak values are above 2.5. This decline is probably due, at least in part, to death of heavily infected oysters. Prevalence and weighted prevalence values decline through the winter and reach minimum values in late spring, typically April or May (Fig. 4). Since 1988 some infections have been found throughout the winter and spring in Virginia except at locations where salinity becomes less than about 5 ppt for extended periods. Detectable overwintering infections are contrary to the situation prior to 1985 when *P. marinus* infections were either absent or undetectable during winter (Andrews 1988), and are probably the result of the much higher abundance of the parasite since 1985. However, the winter/spring decline in prevalence is in part an artifact of the low sensitivity of the standard fluid thioglycollate medium (FTM) technique (see Fig. 5). Recently, Ragone Calvo and Burreson (1994) using antibody detection and Bushek et al. (1994) using total body burden fluid thioglycollate analyses have also shown that prevalence does not decline as dramatically during winter as routine FTM assay would suggest. However, intensity does decline during late winter and spring and all infections during that period are of very low intensity (Fig. 5) (see also Bushek et al. 1994). The decline in intensity is partly the result of mortality of moderately and heavily infected oysters during winter, but intensity values decline even in areas where infection intensity is relatively low and where no mortality occurs. It is not known if this

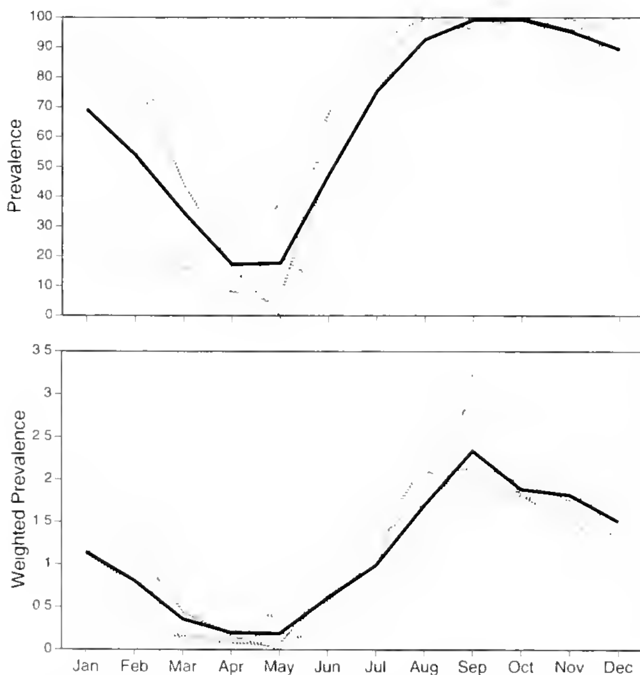

Figure 4. Annual cycle of *P. marinus* prevalence (top) and infection intensity, expressed as weighted prevalence (bottom), in Chesapeake Bay oysters. Dotted lines demonstrate year-to-year variability for years 1988–94. Bold line represents the average of all years, 1988–94. Prevalence and intensity were determined using the FTM method described by Ray (1966). Oysters (n = 25) were sampled monthly from Wreck Shoal, James River, VA.

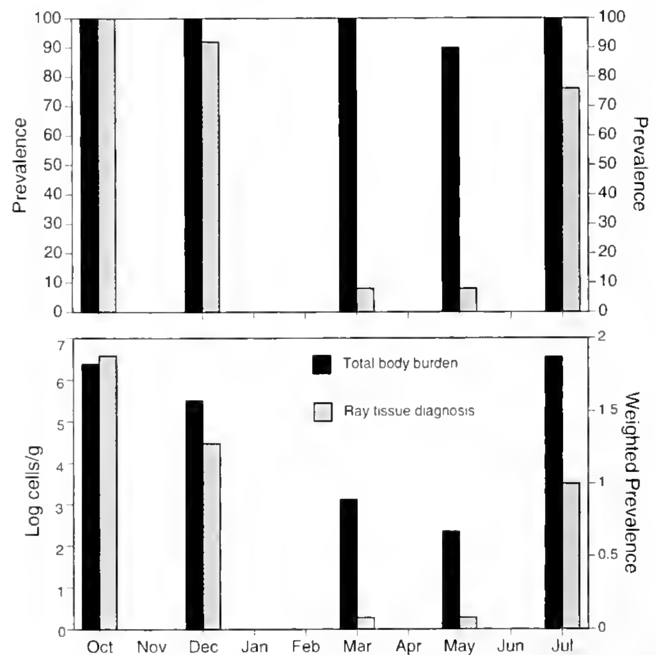

Figure 5. Seasonal prevalence (top) and intensity (bottom) of *P. marinus* as determined by standard Ray tissue FTM assays and by total body burden estimations. Intensity is expressed as weighted prevalence for standard FTM assays (right axis) and as log10-transformed cells per gram wet tissue weight for body burden estimations (left axis). Oysters were sampled from Wreck Shoal, James River, VA. Sample size was 25 for standard FTM assays and 20 for total body burden assays.

decline is the result of active defense processes by the oyster or passive processes related to tolerance of *P. marinus* to temperature and salinity although previous researchers have suggested that parasite cells are actively eliminated (Andrews 1988).

Maximum and minimum prevalence and weighted prevalence values during the annual cycle and the timing of increases and decreases are affected by the local temperature and salinity regimes (see next section) although the general pattern remains consistent in all areas. For example, while prevalence values have reached 100% at Wreck Shoal in the James River every fall since 1988, they reached 100% at Horsehead Rock, an upriver station in lower salinity, only during 1991. In addition, annual prevalence values usually peak one or two months later and begin to decline one to two months earlier in these low salinity areas.

There seems to be an oyster size threshold required for *P. marinus* infection in nature. Spat less than about 30 mm in shell height are rarely found infected using routine FTM assay (Burreson 1991). This apparent size threshold may be the result of low sensitivity of FTM assay, but more likely is due to reduced filtering capacity of small oysters. It does not appear to be the result of innate resistance to the parasite, as Andrews and Hewatt (1957) have demonstrated that small oysters acquire infections when dose is high. Infections with *P. marinus* are known to be dose dependent and small oysters probably don't filter enough water to acquire sufficient infective stages of the parasite in nature.

ANNUAL CYCLE OF *P. MARINUS*-INDUCED OYSTER MORTALITY

In areas of favorable salinity (>12 ppt) oyster mortality resulting from *P. marinus* infections usually begins about the first of August and continues through early winter although most oysters die in late August and September. The proliferation of *P. marinus* is temperature dependent and abundance within an oyster increases so long as temperature is above about 20°C; thus, an unusually warm spring or fall will prolong the development period of the pathogen and result in greater oyster mortality.

The mortality pattern of oysters placed into salinity regimes conducive to parasite development depends on the prior history of *P. marinus* infection. Uninfected oysters larger than about 30 mm shell height usually acquire *P. marinus* infections during mid to late summer of the first year. Mortality is usually low because declining water temperature during fall prohibits development of most infections to lethal levels, but mortality as high as 40% may occur if oysters are about 50–60 mm shell height. High mortality, often greater than 90%, will occur in these oysters during the second summer if environmental conditions are favorable for *P. marinus* development (Fig. 6). This mortality pattern is drastically different than that prior to 1985 when significant oyster mortality from *P. marinus* did not occur until the third summer after initial infection. Management strategies proposed by Andrews and Ray (1988) to harvest oysters after two summers of growout were successful prior to the 1980s, but have not been as effective since 1986 because high mortality occurs during the second summer of exposure.

Spat that are less than about 30 mm shell height during late summer/early fall will usually not acquire *P. marinus* that summer and they can often be grown to market size before significant mortality from *P. marinus* occurs. Aquaculturists can reduce mortality caused by *P. marinus* by spawning oysters late and delaying placing them in *P. marinus*-enzootic waters until late September. In this situation most spat avoid infection by *P. marinus* but still grow well until winter. They will acquire *P. marinus* infections during the next summer, but mortality will be low. Experience has shown that oysters can reach market size by the following spring and can be harvested before high mortality results the following summer (M. Luckenbach, Virginia Institute of Marine Science [VIMS], personal communication).

Because *P. marinus* is present on all seed-oyster bars in the Chesapeake Bay, oysters should not be moved from seed areas to high salinity growout areas. Light infections will intensify and high mortality will almost certainly occur the first summer after transplantation. Nor should *P. marinus*-infected oysters be moved to low salinity with the expectation that the pathogen will be eradicated. Monthly monitoring in the upper James River, VA (Ragone Calvo and Burreson 1994), has clearly shown that *P. marinus* can survive long periods (weeks to months) of salinity below 5 ppt and days to weeks in fresh water.

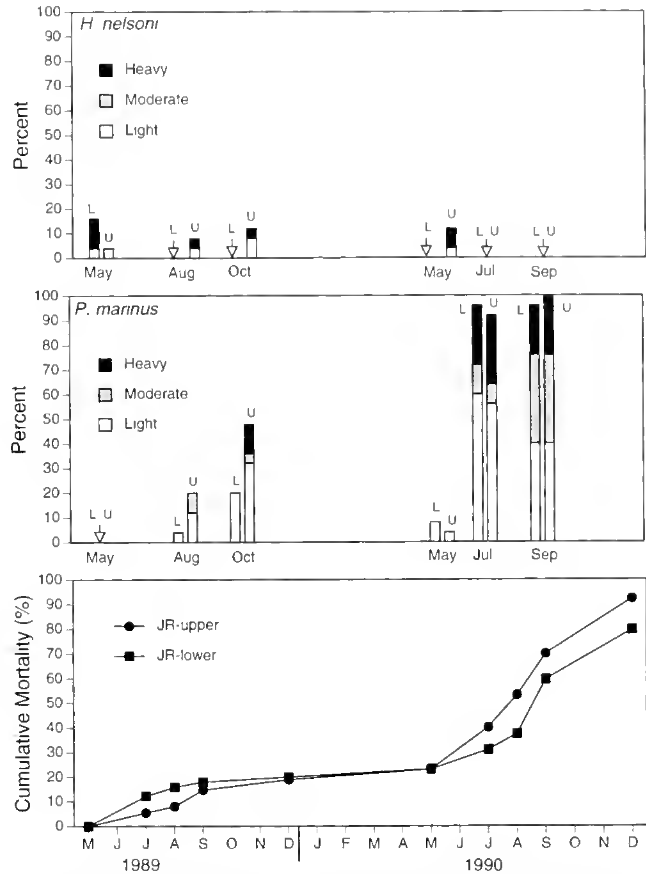

Figure 6. Disease-associated cumulative oyster mortality (bottom) and prevalence and intensity of *H. nelsoni* (MSX) (top) and *P. marinus* (middle) in hatchery-reared juvenile oysters deployed in the lower York River, VA. Prevalence of *H. nelsoni* was very low, especially during 1990, so mortality can be attributed to *P. marinus*. For parasite data, prevalence is indicated by total bar height and percentage of sample in each intensity category by shading. Sample size = 25. Arrows indicate samples examined but no infections found. Two oyster stocks are compared in each graph—upper James River (U) and lower James River (L). Mean shell height in July 1989 was 42 mm for both groups.

ENVIRONMENTAL CONTROL OF *P. MARINUS* INFECTIONS

Salinity

Clearly, salinity is an important environmental control of *P. marinus* because prevalence and intensity of the pathogen within an estuary increase with increasing salinity (Andrews 1988, Craig et al. 1989, Soniat and Gauthier 1989). Historically, *P. marinus* was absent from Chesapeake Bay waters with summer salinities of about 15 ppt or less and a large proportion of oyster grounds located in the upper reaches of Chesapeake Bay tributaries were disease-free. As a consequence of four consecutive drought years 1985–88 the abundance and distribution of *P. marinus* increased dramatically and the parasite became present on all oyster grounds in Virginia. The historical restriction of *P. marinus* to high salinity areas (>12–15 ppt) suggests that over the long term the parasite cannot tolerate the low salinities of the upper Bay or upper tributaries; however, since its spread in the late 1980s the parasite has persisted in most of these lower salinity areas despite the return to normal and even below normal salinities.

In 1987, VIMS initiated an intensive survey program to monitor *P. marinus* prevalence and intensity at three oyster bars in the upper James River, VA, which, prior to the drought years of 1985–88, were free of *P. marinus*. Since 1987, oysters (n = 25) have been sampled monthly from Wreck Shoal (WS), Horsehead Rock (HH), and Deepwater Shoal (DWS) (Fig. 3). These bars are located along a salinity gradient with average salinities for the years 1987–94 of 14 ppt (±4.3, n = 318) at WS, 9 ppt (±4.1, n = 166) at HH, and 7 ppt (±4.0, n = 245) at DWS. As a consequence of abnormally high salinities associated with below average streamflows, *P. marinus* invaded WS in the summer of 1986 and within a year prevalence at the site was 100%. The parasite spread upriver to HH during the summer of 1987 and was first observed at DWS in the summer of 1988. *P. marinus* spread through HH and DWS more slowly than at WS, but since 1990 peak fall prevalences have ranged from 40 to 88% at DWS and from 88 to 100% at HH (Fig. 7).

In addition to affecting the local distribution and abundance of *P. marinus*, salinity also has a significant effect on *P. marinus* infection acquisition and intensity. Paynter and Burreson (1991) found that juvenile cultured oysters deployed at a low salinity site (8–10 ppt) did not acquire infections while those at moderate (12–15 ppt) and high (16–20 ppt) salinity sites did acquire infections. Furthermore, infection intensity at the moderate salinity site was lower than that at the high salinity site. Similarly, the limiting effect of low salinity on *P. marinus* prevalence and intensity is observed in native James River oyster populations. At WS, the area having the highest salinity, infections overwinter at a higher prevalence and intensity and increase as the water temperature warms at a much faster rate than at the lower salinity areas, HH and DWS (Fig. 8). During the summer months infections in WS oysters generally progress to moderate and heavy intensity in response to high temperatures and salinities and disease-associated mortality results. For instance, during the late summer and fall months of 1994 salinity at WS ranged from 12 to 20 ppt and moderate to heavy *P. marinus* infections were observed in 12–30% of the oysters sampled each month (Fig. 8). Prevalences and infection intensities decrease in an upriver direction from WS indicative of the limiting effect of low salinity on *P. marinus*. Generally, only a few moderate to heavy infections are observed at HH and infections at DWS rarely progress to moderate and heavy intensity. This was apparent in 1994 (Fig. 8) when summer and fall salinities ranged from 8 to 15 ppt at HH and from 5 to 12 ppt at DWS. While environmental fluctuations may alter the severity of *P. marinus* epizootics from year to year, the general trend of increasing prevalence and intensity in a downriver direction persists.

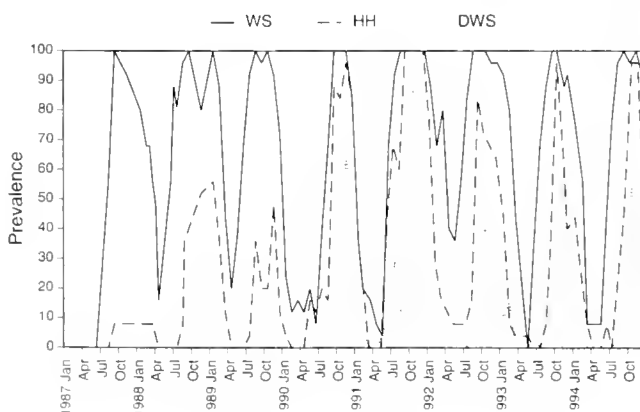

Figure 7. Prevalence of *P. marinus* in oysters sampled along a salinity gradient in the upper James River, VA. Oysters (n = 25) were sampled monthly from WS, HH and DWS. Diagnoses were made using standard FTM assays. Average salinities for period 1987–94 at WS, HH and DWS were, respectively, 14, 9 and 7 ppt.

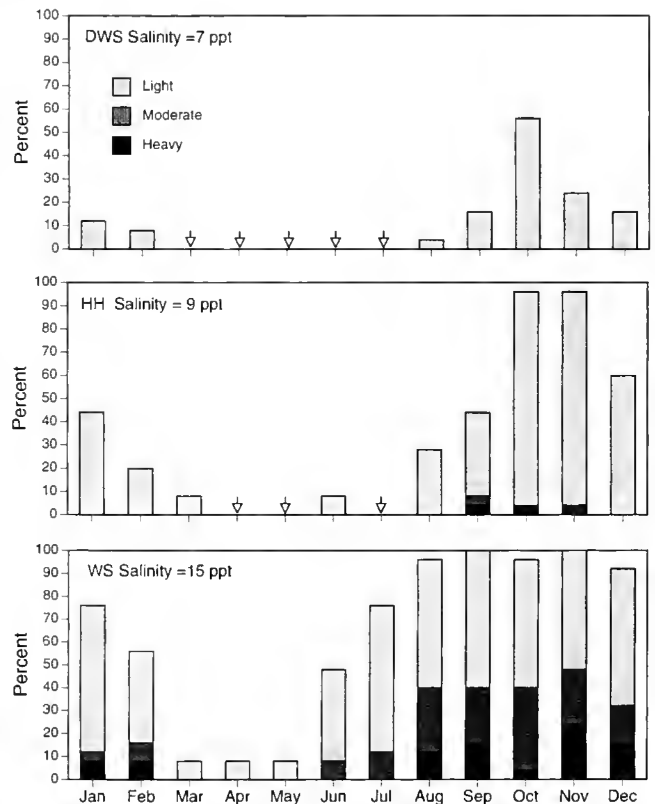

Figure 8. *P. marinus* prevalence (total bar height) and percentage of sample in each intensity category (shading) in oysters sampled along a salinity gradient in the upper James River, VA, in 1994. Oysters (n = 25) were sampled monthly from WS, HH and DWS. Diagnoses were made using standard Ray tissue FTM assays and infection intensity was categorized as light, moderate and heavy. Arrows indicate samples examined but no infections found. Salinity is the average for 1994 based on two to three observations per months.

Based on these studies of *P. marinus* infection patterns along the James River salinity gradient and in other Chesapeake Bay tributaries, critical salinity regimes for *P. marinus* activity can be defined. These studies indicate that: 1) if summer and fall salinities are consistently less than 9 ppt, *P. marinus* may persist but infections are limited to light intensity and no oyster mortality results; 2) if summer and fall salinities vary from 9 to 15 ppt, some infections may progress to moderate and heavy intensity, but associated oyster mortality is relatively low; and 3) if summer and fall salinities are consistently greater than 15 ppt, moderate to heavy infections may be numerous and oyster mortality may be relatively high.

Preliminary statistical analysis of the relationship of salinity and *P. marinus* infection intensity and prevalence in James River oysters was conducted using a Spearman rank correlation analysis. The analysis was based on 180 observations which included monthly determinations of prevalence for a five year period, 1990–94, at three oyster beds, WS, HH, and DWS (Fig. 3). Twenty-five oysters were collected from each site each month and examined for *P. marinus* by culturing rectal, gill, and mantle tissue in FTM following the method described by Ray (1966). Infection intensities were ranked as light, moderate, and heavy and assigned numerical values of 1, 3 and 5 according to the scale of Mackin (1962). The numerical intensity values, which included 0 for negative diagnoses, were then averaged for the determination of weighted prevalence. Salinity was recorded at each site 1–3 times each month and monthly means were determined.

The results of the correlation analysis demonstrated a highly significant ($p < 0.0001$) and strong correlation between salinity and *P. marinus* prevalence and intensity in James River oysters (Spearman rank corrected rho = 0.729 and 0.727, respectively) (Fig. 9). A subsequent linear regression analysis indicated that salinity accounts for 51% of the variability in prevalence. Only a limited number of field studies employing statistical analyses of data have been conducted. Significant positive correlations between salinity and *P. marinus* prevalence and intensity have been observed in Gulf Coast oysters (Soniat 1985, Craig et al. 1989, Soniat and Gauthier 1989) and in South Carolina oysters (Crosby and Roberts 1990). In the Gulf of Mexico, salinity (0–34 ppt) was observed to account for only 20% of the site-to-site variability in *P. marinus* infection (Craig et al. 1989) and in South Carolina salinity (29 to 35 ppt) was only weakly correlated with infection (Kendall rank tau = 0.094) (Crosby and Roberts 1990). The present analysis of the relationship between salinity and *P. marinus* activity in the James River suggests that salinity may play a more significant role in regulating *P. marinus* in the Chesapeake Bay than in more southern waters, although differences in the correlation results may also be attributed to differences in salinity regime and in experimental design, particularly sampling frequency. More rigorous statistical analysis of James River data should help to further our understanding of the role of salinity in regulating *P. marinus* prevalence and intensity.

The association of salinity with *P. marinus* prevalence and intensity has been addressed by several researchers. Mackin (1951) suggested that high flushing rates, typical in the upper reaches of estuaries, dilute infective pathogen cells thereby limiting the ability of water-borne infective stages to infect oysters. Thus, the absence of *P. marinus* from low salinity areas was attributed to the absence or scarcity of infective cells (Ray and Mackin 1954, Mackin 1956, Andrews and Hewatt 1957). Andrews (1988) related the Chesapeake Bay distribution of *P. marinus* to the physical circulation dynamics of specific Chesapeake Bay tributaries. He asserted that large flushing-type rivers, such as the James River, are not as favorable to the pathogen as small coastal plain tributaries, such as the Choptank River, because large discharges of fresh water during the winter and spring reduce the period of salinities favorable to *P. marinus* and dilute the concentration of infective cells.

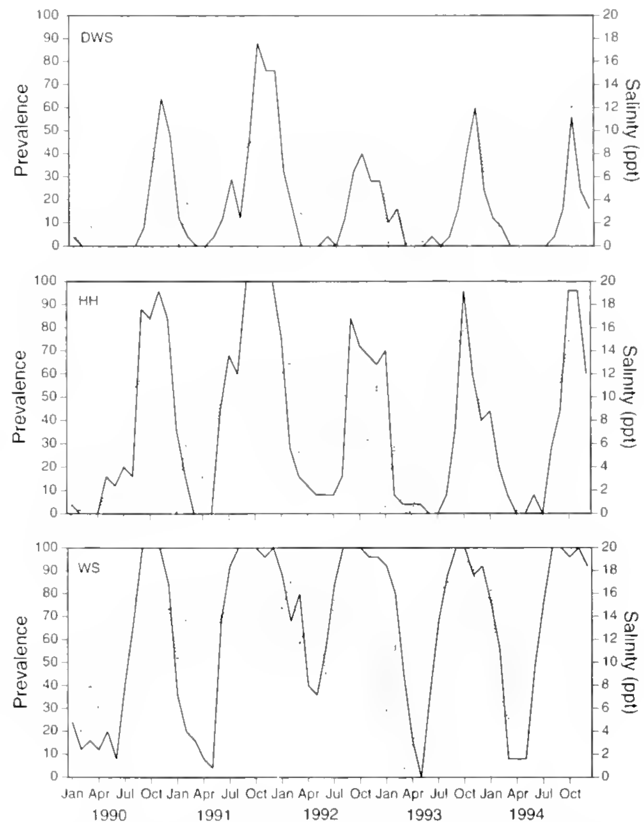

Figure 9. *P. marinus* prevalence (solid line) and mean monthly salinity (dotted line) at DWS, HH and WS, James River, VA. Oysters (n = 25) were sampled monthly and prevalence was determined using standard Ray tissue FTM assays. Mean monthly salinity was calculated from measurements recorded one to three times each month.

While dilution of infective particles by fresh water discharge may be an important factor influencing the distribution of *P. marinus* it is not entirely responsible for the reduced disease levels observed in low salinity areas. In recent years many laboratory investigations have documented an inhibitory effect of low salinity on various aspects of *P. marinus* epizootiology. Scott et al. (1985) found lower mortality in infected oysters held in the laboratory at 8–10 ppt than in oysters held at 21–25 ppt. Their results support the work of Ray (1954) in which *P. marinus* in artificially infected oysters tolerated low salinity (10–13.5 ppt) exposure but development of infections and subsequent mortalities of oysters were delayed relative to the high salinity (26–28 ppt) control group. Ragone and Burreson (1993) exposed naturally infected oysters from the upper James River, VA, to three low salinity treatments, 6, 9 and 12 ppt, and found no reduction in *P. marinus* prevalence at any of the treatments after eight weeks of exposure. However, development of infection was retarded at 12 ppt compared to the high salinity (20 ppt) control and infection intensity did not increase at 6 and 9 ppt. Oyster mortalities after eight weeks were

significantly less at 6 and 9 ppt than at 12 or 20 ppt, which were nearly equivalent. This study suggests that 9–12 ppt is a critical range for *P. marinus* activity supporting recent field observations. Although *P. marinus* infection progression may be limited by low salinity, *P. marinus* is quite tolerant of low salinities, unlike *H. nelsoni* which is intolerant of salinities less than 10 ppt (Ford 1985). Chu et al. (1993) succeeded in artificially establishing infections in oysters maintained in the laboratory at salinities as low as 3 ppt. Prevalences of *P. marinus* five weeks after challenge by mantle cavity injection with 10^6 meronts were 50, 70 and 82% at 3, 10 and 20 ppt, respectively. All infections observed at 3 ppt and most found at 10 ppt were of low intensity, suggesting that parasite proliferation within the host was limited relative to the high salinity control.

Differences in oyster mortality and infection progression between high and low salinity environments may be attributed to the direct effect of salinity on host and/or parasite physiology. Several in vitro investigations have helped us gain a better understanding of the direct effect of salinity on *P. marinus*. Perkins (1966) and Chu and Greene (1989) found that low salinity inhibited sporulation of prezoosporangia isolated from oyster tissue cultured in FTM. The recent, successful culture of *P. marinus* (Gauthier and Vasta 1993, Kleinschuster and Swink 1993, La Peyre et al. 1993) has allowed a more rigorous examination of the salinity tolerance of *P. marinus* in the absence of host influences. In vitro, *P. marinus* is tolerant of a wide range of salinities and has been reported to proliferate at osmolalities from 340 to 1930 mOsm (10–60 ppt) (Dungan and Hamilton 1995). Osmolalities below 340 mOsm were not tested. Maximal proliferation was observed at 790 mOsm (25 ppt) and near-maximal proliferation occurred within the range of 475–960 mOsm (15–30 ppt) (Dungan and Hamilton 1995). While cultured *P. marinus* cells exhibit growth at salinities as low as 10 ppt they are relatively intolerant of acute hypoosmotic shock. Burreson et al. (1994b) exposed *P. marinus* meronts harvested from 22 ppt culture media to 0, 3, 6, 9, 12 and 20 ppt artificial seawater. After a 24 hour exposure period at 28°C, viability was assessed using the vital stain neutral red. Percent mortality was 99% at 0 ppt, 90% at 3 ppt, 70% at 6 ppt, 43% at 9 ppt, 20% at 12 ppt and <5% at the 22 ppt control treatment. The effect of salinity on percent mortality was highly significant. When the osmotic concentration of the various seawater treatments was adjusted with sucrose to the equivalent of 22 ppt, percent mortality was low in all treatments and no different than the optimal control condition demonstrating that low salinity–induced mortality was caused by a decrease in osmotic pressure, not a decrease in sodium or another important ion. The low survival of cultured *P. marinus* cells at 6 ppt is surprising considering the documented ability of the parasite to survive in oysters at 6 ppt and 20°C for a period of eight weeks (Ragone and Burreson 1993) and may not be relevant to natural conditions in which changes in osmotic condition are likely to be more gradual and mediated by host responses.

In summary, within the Chesapeake Bay region *P. marinus* activity is greatly influenced by salinity. Prevalence and intensity of the pathogen intensify during drought years during which low stream flows cause above average salinities in upper tributary waters. In general, prevalence and intensity of *P. marinus* increase in a downriver direction. Infections are restricted to low intensity in areas consistently having salinities of less than 9 ppt, while high intensity infections and associated oyster mortality often occur during the summer and fall in areas with salinities greater than 12–15 ppt. Once established in a low salinity area the parasite tenaciously persists and has been observed to tolerate salinities <5 ppt for a period of at least three months and to quickly respond to exposure to favorable salinities as evidenced by increases in prevalence and intensity. Laboratory studies have demonstrated that *P. marinus* survival, infection progression and pathogenicity are salinity limited, supporting recent field observations.

Temperature

Temperature appears to be the most important environmental factor affecting the large scale geographic distribution of *P. marinus* (Ray and Mackin 1954, Andrews and Hewatt 1957, Quick and Mackin 1971). The northern limit of *P. marinus* is believed to be controlled by minimum winter temperature (Andrews 1988). Maximum summer temperatures and/or the duration of temperatures above 20–25°C are probably also important, but the role of minimum or maximum temperature on the geographic distribution has not been rigorously investigated.

Within the Chesapeake Bay, seasonal temperature changes are largely responsible for the seasonal periodicity of the annual *P. marinus* cycle. Winter temperatures, which on average (1947–90) are below 5°C for eight weeks, are associated with a regression in tissue infection levels resulting in spring minimums in infection intensity and prevalence. Infections begin to intensify in late spring as water temperature exceeds about 20°C and parasite proliferation occurs (Andrews 1988). In Chesapeake Bay, temperatures favorable to parasite proliferation, >20°C, occur for about 20 weeks and temperature may exceed 25°C for a period of 10 weeks. The highest parasite prevalences and intensities are observed in September immediately following maximal summer temperatures. The occurrence of high prevalences and intensities at high temperature most likely reflects temperature associated increases in parasite multiplication rate but may also relate to temperature associated depressions in host defense capabilities and physiological condition. In high salinity environments, infections intensify to lethal levels and mortality usually occurs from August through October. Infection intensity declines as temperatures decline in winter (Figs. 10 and 11), however, the parasite is known to persist patently at temperatures as low as 0–5°C (Andrews 1988). Winter water temperature in the Bay typically averages 4–5°C, but may be as low as 1°C or less for extended periods during unusually cold winters. Body burden analysis allowed the documentation of a remarkable decline from December to May in number of meronts per gram wet weight of oyster tissue; however, prevalence remained at 90–100% (Fig. 5). These residual infections rapidly proliferate as temperatures rise in late spring.

Numerous field and laboratory experiments have focused on the relationship between temperature and *P. marinus* infection intensity and prevalence. *P. marinus* infection in South Carolina oysters was significantly but weakly correlated with temperature (Kendall rank correlation coefficient = 0.283) (Crosby and Roberts 1990); temperature explained 16.7% of the variability in infection intensity. This result contrasts with those of Burrell et al. (1984) and Craig et al. (1989) in which no statistically significant relationship between temperature and intensity was found in South Carolina and Gulf of Mexico oysters, respectively. Differences in these results have been attributed to differences in frequency and interpolation of temperature measures (Crosby and Roberts 1990).

In the James River tributary of the Chesapeake Bay, *P. marinus* intensity and prevalence clearly follow seasonal fluctuations in water temperature (Fig. 10). Preliminary statistical examination of

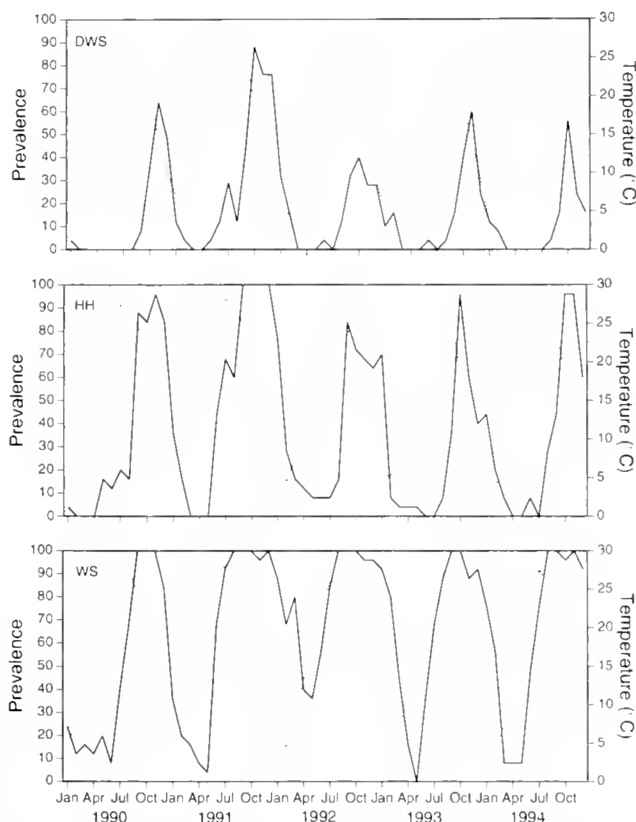

Figure 10. *P. marinus* prevalence (solid line) and mean monthly water temperature (dotted line) at DWS, HH and WS, James River, VA. Oysters (n = 25) were sampled monthly and prevalence was determined using standard FTM assays. Mean monthly temperature was calculated from temperature measurements recorded at six minute intervals at the VIMS York River monitoring station.

this relationship was conducted using a Spearman rank correlation analysis. Data for *P. marinus* are the same as described for the statistical analyses of salinity relationships. Mean monthly water temperature was calculated from water temperatures recorded at six minute intervals by a continuous metering system at the VIMS York River monitoring station.

The initial correlation analysis failed to find a statistically significant relationship between mean monthly water temperature and *P. marinus* prevalence or intensity ($p > 0.05$). This result agrees with the findings of Soniat (1985) and Craig et al. (1989) and contrasts with the findings of Crosby and Roberts (1990) which indicated a statistically significant but weak correlation between water temperature and *P. marinus* intensity. However, when the James River data set was reanalyzed with temperature lagged by two to four months, a significant correlation between water temperature and *P. marinus* prevalence and intensity was found. The relationship was strongest when the temperature was lagged three months (eg. April prevalence and January temperature); water temperature was strongly and significantly correlated with both prevalence (Spearman rho = 0.704, $p < 0.001$) and weighted prevalence (Spearman rho = 0.706, $p < 0.001$). Regression analysis indicated that when lagged three months temperature explained 39% of the variability in prevalence and 46% of the variability in weighted prevalence.

The contribution of temperature to the year-to-year variability in *P. marinus* activity is not well understood. Minimum winter temperature, while thought to control the geographic distribution of the pathogen, is not clearly associated with year-to-year variability of *P. marinus* epizootics in the Chesapeake Bay. During an eight year (1987–94) monthly parasite survey of James River oysters (described above, see salinity discussion) extreme above and below average fluctuations in winter temperature were observed. The relationship of these temperature fluctuations to the subsequent summer epizootics is somewhat obscure, in part because it is difficult to separate the effects of salinity fluctuations from temperature fluctuations. The coldest winters in terms of average winter temperature and duration of weekly average temperatures below 5°C were the winters of 1987–88 and 1993–94 (Fig. 11). Regardless of the cold winter, subsequent summer prevalences and intensities in 1988 were among the highest recorded, but this was also an abnormally dry year. In 1994 winter water temperatures were below 5°C for a period of eight weeks and 1–2°C below the long term average for six of the eight weeks. This unusually cold temperature regime seemed to have little negative impact on *P. marinus* as 1994 had the third highest average summer intensity and prevalence was still greater than 96% at WS for a five month period from August through December (Fig. 11). During the winter of 1989–90 record low temperatures were observed in December but after the first week of January water temperatures were generally above average. The low December temperatures may have contributed to the relatively early decline of overwintering infections and to the relatively slow rise in prevalence during the summer; however, salinity was also relatively low during the period.

Abnormally warm winter temperatures may have a more significant impact on *P. marinus* activity than abnormally cold temperatures. The winter of 1991 was the warmest winter during our survey period (Fig. 11). Mean weekly temperature never went below 5–6°C during the winter and temperatures were 1–3°C above the long term average throughout the year. Overwintering prevalences were low, but prevalence rapidly increased with the onset of warm summer temperatures and remained above 90% for six months at WS (Fig. 11), making 1991 the worst year in terms of average summer prevalence. The cool fall of 1990 and low 1990 prevalences may be responsible for the low 1990–91 overwintering levels, while the abnormally warm winter water temperatures combined with dry conditions probably caused high summer prevalences in 1991. However, 1991 summer temperatures were also above average and probably also contributed to the high summer parasite level (Fig. 11).

The association between temperature and *P. marinus* infection has been the focus of several laboratory investigations. Andrews and Hewatt (1957) reported that at 15°C the development of established infections was retarded and new infections did not appear. Similarly Fisher et al. (1992) found infection progression and oyster mortality were reduced in oysters held at 18°C compared to those held at 27°C. More recently, Chu and La Peyre (1993) exposed oysters held at 10, 15, 20 and 25°C to *P. marinus* through mantle cavity injections of 10^6 meronts obtained from infected oyster tissue. Infections were observed in oysters from all treatment groups; however, prevalence declined with decreasing temperature and moderate and heavy infections were only observed in oysters at 20 and 25°C. Forty-six days after challenge *P. marinus* prevalence was 23% at 10°C, 46% at 15°C, 91% at 20°C and 100% at 25°C.

The influence of temperature on *P. marinus* infection intensity and prevalence may relate to host and/or parasite activity. Both

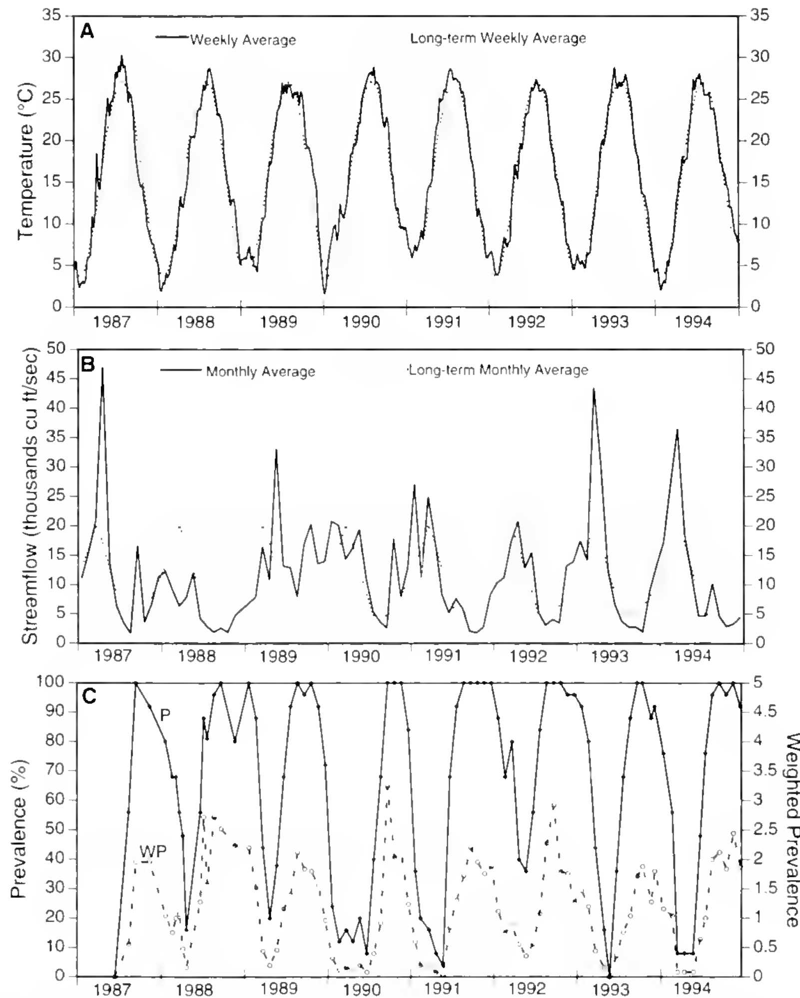

Figure 11. Year-to-year variability of water temperature (A), James River streamflow (B), and prevalence (P) and weighted prevalence (WP) of *P. marinus* (C). In Panel A, weekly temperature averages for each year are contrasted with the long term mean weekly temperature for the period 1947–94. In Panel B, monthly streamflow averages for each year are contrasted with the long term mean monthly streamflow for the period 1951–95. Water temperature is from the continuous record of the VIMS York River Monitoring Program; streamflow data were obtained from the U.S. Geological Survey.

cellular and humoral oyster defense activities have been shown to be affected by environmental temperature (Fisher 1988, Chu and La Peyre 1989, Chu and La Peyre 1993). Unfortunately, the role of these putative oyster defense activities in combating *P. marinus* remains speculative. In vitro culture of *P. marinus* has afforded an opportunity for analysis of temperature effects on the parasite in the absence of host influences. Proliferation of *P. marinus* in culture was near maximal at room temperatures between 15 and 35°C and optimal at 35°C. Minimal proliferation occurred at 10 and 40°C, and no proliferation occurred at 4°C (Dungan and Hamilton 1995). In a study conducted by Burreson et al. (1994b) cultured *P. marinus* cells were quite tolerant of a 24 hour exposure to temperatures as low as 1°C at high salinity (22 ppt). Survival at 1 and 5°C treatments was greater than 90% based on a vital stain assay and did not significantly differ from that at 10, 15 and 28°C treatments (Burreson et al. 1994b). Temperature appeared to have a greater effect when exposed cells were in lower salinity conditions. A general trend of higher mortality at lower temperature was observed at 6, 9 and 12 ppt, however, the temperature effect was only significant at 9 ppt. It was suggested that cold temperatures may inhibit metabolic processes such as free amino acid release which may enable some cells to survive at lower salinities. Prior to the culture of *P. marinus*, in vitro studies on the effect of temperature were limited to the assessment of sporulation of presporangia isolated from thioglycollate-incubated infected tissue. Viability was determined by the presence of motile spores within sporangia. Sporulation was optimal at 25–35°C (22 ppt) (Perkins 1966). Both the maximum percent sporulation and the rate of sporulation were greatly reduced at 20°C (60% of optimal) and no sporulation occurred at temperatures less than 18°C (Perkins 1966). In a similar experiment Chu and Greene (1989) observed that prezoosporangia survived at 4°C for up to four days but did not survive below 0°C for one day.

In summary, it appears that in the Chesapeake Bay region *P. marinus* activity and annual periodicity are largely controlled by seasonal temperature fluctuations. This conclusion is supported by a strong statistically significant correlation between temperature and *P. marinus* prevalence and intensity. It is difficult to precisely define the effect of temperature on year-to-year variability of *P. marinus* infections based on analyses conducted to date. However some trends are apparent. Abnormally cold winter temperatures may hasten the decline in infection intensity during the winter

months and delay the rise in prevalence during the summer months, but they have little impact in reducing the severity of summer epizootics. Conversely, abnormally warm winter temperatures probably increase the severity of summer epizootics.

The interaction of temperature and salinity is probably more important than either factor acting alone. Recent evidence suggests that, in Chesapeake Bay, the prevalence and intensity of *P. marinus* decline much more rapidly during winter in low salinity areas (<10 ppt) than in high salinity areas (>18 ppt) (Ragone Calvo and Burreson 1994). Laboratory investigations are needed to determine the synergistic effect of temperature and salinity fluctuations on the progression and/or regression of established *P. marinus* infections.

TRANSMISSION DYNAMICS

Although it is well documented that transmission of *P. marinus* is direct from oyster to oyster and that any life cycle stage of *P. marinus* seems capable of initiating infections in the laboratory (Ray 1954, Andrews 1988), the natural dynamics of transmission are poorly understood. Transmission is dose dependent and it seems to take unusually high concentrations of any life cycle stage to initiate infections (Andrews 1988). Transmission is thought to occur through the digestive tract because initial foci of infection occur in the gut epithelium (Mackin 1951), although this observation needs confirmation with careful laboratory studies. In any case, the cell type that actually initiates infection and the mechanism of infection are poorly understood. The role of flagellated, free-swimming zoospores in initiating infections in nature is especially problematic. Zoospores certainly don't seem to be required for initiation of infections, as infections result from exposure to isolated meronts or even minced, infected oyster tissue. However, the transformation that may occur after merozoites or minced tissue are added to an aquarium or injected into the mantle cavity of an oyster are unknown. The occurrence of early infections in the stomach epithelium suggests ingestion of infective stages, not penetration of gill or mantle by zoospores. But perhaps zoosporulation occurs in the gut lumen and released zoospores penetrate in localized areas of the gut epithelium. It appears that a very high dosage of zoospores, on the order of 1×10^5, is required to initiate an infection (Andrews 1988). This dose seems high for an efficient parasite but may be an artifact of the experimental designs employed. Zoospores must have some function or their production wouldn't have evolved. Maybe they are a dispersal mechanism and are only produced in nature under certain conditions that are not presently understood.

Inoculation of non-zoospore stages into the mantle cavity of oysters has demonstrated that meronts produce higher prevalences and higher intensities of infection than prezoosporangia (Volety and Chu 1994), but the pattern and process of infection were not followed in these studies. A high proportion of *P. marinus* cells in an oyster occur within host hemocytes and it has been proposed that hemocytes that scavenge the epithelial surface of the oyster gut lumen phagocytose ingested *P. marinus* cells and then migrated through the epithelial layer and into the oyster carrying the parasite with them. An innovative study with intubated fluorescent polystyrene beads has demonstrated that such events do occur (Alvarez et al. 1992). Once inside an oyster and under favorable environmental conditions, *P. marinus* multiplies within hemocytes, eventually killing the hemocyte and releasing the *P. marinus* cells. These cells are phagocytosed by other hemocytes and the cycle repeats; eventually pathogen cells are carried throughout the oyster. The developmental cycle of *P. marinus* within oysters is relatively well understood and recently has been reviewed by Perkins (1991, 1993), but studies that examine the initial infection process in oysters are critically needed.

Even though it is known that transmission is direct, in nature there is a poor understanding of the source of infective stages, the dose required to initiate infections and the duration of the infection window. The prevailing conceptual model is that transmission occurs during periods of high oyster mortality in summer and early fall as infective *P. marinus* cells are disseminated upon death and decomposition of infected oysters (Andrews 1988). However, dead, gaping oysters are consumed rapidly by scavengers (Hoese 1964) and probably don't decompose naturally and release *P. marinus* cells into the water. The parasite can survive passage through the gut of scavengers (Hoese 1964), but the role of scavengers in spreading infections is unclear. In the Gulf of Mexico, transmission of *P. marinus* can occur via the ectoparasitic snail *Boonea impressa* (White et al. 1987), but for the Chesapeake Bay no vectors have been identified. Dissemination of *P. marinus* in fecal matter from live oysters seems likely, given the destruction of gut epithelium observed in live, heavily infected oysters, but is poorly documented. Mackin (1962) proposed that *P. marinus* overwinters as a free hypnospore in the sediment and that annual epizootics are initiated by release of infective cells in the spring. Andrews (1988) countered that if this were true, imported uninfected oysters should develop infections in June or early July rather than late July or August as he observed. Nevertheless, the presence of a saprobic stage should not be ruled out. Recently, flow cytometric techniques have been developed that may allow quantification of disseminated *P. marinus* cells in the water column (Roberson et al. 1993). Such data should provide insight into seasonality of infection pressure.

Field experiments to assess the timing of infections are underway at VIMS. Separate groups of uninfected oysters are being exposed in the lower York River for two week periods throughout the year and are then warmed in the laboratory for four weeks to allow infections to develop to detectable levels. Results for 1994 indicate that the highest infection pressure occurs during the last two weeks of August and the first two weeks of September (Fig. 12), a period that corresponds closely with maximum oyster mortality; however, some infections were acquired as early as late June.

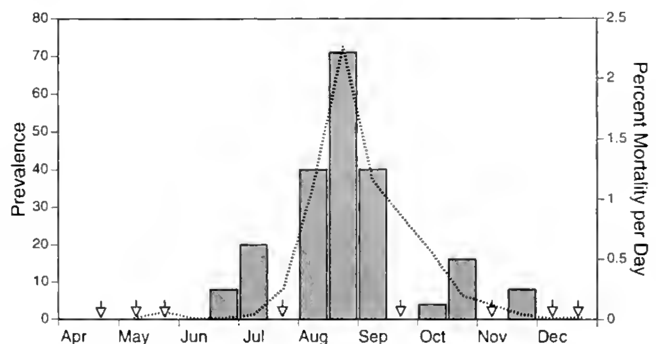

Figure 12. *P. marinus* infection acquisition (bars) in uninfected sentinel oysters deployed for two week periods in the lower York River, VA, and percent mortality per day of local infected oysters (line). Bars represent the prevalence of infection in sentinel oysters as determined by total body burden assays. Arrows indicate no new infections during the period.

There has not been much laboratory research conducted on the effect of environmental variables on transmission dynamics, but meaningful experiments are difficult to perform in the laboratory because of the difficulty of simulating natural conditions and the artificial nature of the challenge used in most experiments. In experiments where *P. marinus* meronts were injected into the mantle cavity of oysters held at various temperatures and salinities, transmission did occur at temperatures as low as 10°C (salinity = 17.5 ppt) and salinities as low as 3 ppt (temperature = 21.0°C) (Chu and La Peyre 1993, Chu et al. 1993). These results clearly show that infection by *P. marinus* is possible at low temperature and low salinity conditions. However, there is little evidence that infections occur under these conditions in nature, probably because of an absence of infective cells in the water or low oyster filtration rates. Andrews and Hewatt (1957) found that new infections were not acquired in the field at salinities ranging from 1 to 13 ppt and Paynter and Burreson (1991) found no infection acquisition in the field at salinities ranging from 8 to 12 ppt. These results suggest that although it is possible for *P. marinus* to infect oysters at relatively low temperature and salinity conditions, such transmission probably does not occur in nature. However, it must be remembered that diagnosis during these studies was by routine FTM assay; more sensitive diagnostic techniques may yield different conclusions.

Movement of *P. marinus* into historically low salinity areas occurred during drought periods when salinities were elevated. Now that the pathogen is present and persisting on all oyster beds it is important to determine if transmission is occurring in areas where salinity has returned to more normal conditions. Field studies using uninfected sentinel oysters in low salinity areas are probably the best method to determine whether transmission is occurring in low salinity areas. Other critical research areas for transmission dynamics include elucidation of the early infection process including cell type and infection site, timing of infections in nature and the role of environmental variables.

THE ROLE OF OYSTER DEFENSE MECHANISMS

Considering all of the research that has been conducted on the role of oyster defense mechanisms in controlling *P. marinus* infections it is perhaps surprising how little is known about the topic. If oyster defense capabilities do control levels of *P. marinus* in oysters, the components and mechanisms involved have not been identified so it is impossible to assess the role of defense mechanisms in the epizootiology of *P. marinus* disease. Unfortunately, most studies have been correlative studies where some putative defense mechanism such as lysozyme or agglutinin is measured and correlated with intensity of *P. marinus* infections. These studies have produced much useful data on components of the defense mechanisms in oysters and their relation to environmental parameters, but they have not demonstrated any direct link between pathogen levels and serum or cellular components, perhaps because of the high variability of measured parameters and because correlation analysis, even when it is statistically significant, doesn't necessarily demonstrate cause and effect. Because of typical highly variable results, investigators have been reluctant to rule out any component, but recently Chintala et al. (1994) have demonstrated, and clearly stated, that the particular serum agglutinins studied play no role in defense against *P. marinus*.

More innovative, directed studies are needed to determine the role of the various hemolymph components that have been postulated as important in defense against *P. marinus*. Experimental manipulation of the defense components and subsequent monitoring of infection progression, compared to untreated control oysters, should shed light on the role of specific hemolymph components. For example, employing monoclonal antibodies as blocking agents of putative defense components, passive transfer of purified components into oysters or incubation of *P. marinus* with serum components prior to injection into oysters may enable determination of the roles of these components. Unfortunately, much preliminary research may have to be done before meaningful studies can be conducted.

One critical need is to determine how *P. marinus* avoids intracellular killing by hemocytes. Although *P. marinus* is not an obligate intracellular parasite, most cells in oysters are found within hemocytes. Obviously, hemocytes recognize the parasite as foreign and phagocytose individual cells. However, there seems to be no intracellular killing of the parasite or at least the multiplication ability of the parasite during summer far outweighs any killing. Rather, the parasite multiplies within hemocytes and eventually bursts the cell membrane releasing more individual cells that get phagocytosed by other hemocytes and carried throughout the oyster in hemolymph. Oyster hemocytes are known to produce the typical free oxygen radicals (ROIs) involved in intracellular killing by vertebrate phagocytes (Anderson et al. 1992, Anderson 1994) but they seem to be ineffective against *P. marinus*, at least during periods of active parasite multiplication, possibly because *P. marinus* suppresses ROI production (Volety and Chu 1995).

THE ROLE OF POLLUTION AND WATER QUALITY IN *P. MARINUS* ABUNDANCE

One of the questions most often asked of oyster disease researchers concerns the role of declining water quality in the increase of oyster diseases during the recent past. Most oystermen and many of the lay public consider pollution to be a critical factor in disease processes and blame it solely for the increase in oyster diseases. However, there is little evidence to support their claim. In the Chesapeake Bay there is no correlation between water quality or the level of pollution and disease abundance. Abundance of *P. marinus* is just as high in relatively unpolluted areas as in polluted areas of equivalent salinity. For example, Tangier Sound is one of the areas in Maryland hardest hit by oyster diseases and it was characterized by the EPA Chesapeake Bay Study as having the best water quality in Maryland. Similarly in Virginia the abundance of oyster pathogens is high wherever salinity is favorable and many of the areas where oysters were decimated by disease, such as Pocomoke Sound, are relatively pristine.

Pathogen abundance clearly correlates with salinity levels and the dramatic increase in abundance in the late 1980s can be explained by drought conditions and resulting increased salinity with concomitant warm winters as a secondary factor. As discussed in earlier portions of this review, a variety of laboratory studies and field observations support the primary role of salinity and temperature in modulating *P. marinus* abundance.

Nonetheless, even relatively unpolluted areas today are not as pristine as they were even 20 years ago so some subtle effects of pollution or water quality cannot be completely ruled out. Pollution effects are known to modulate host defense mechanisms in aquatic vertebrates (Anderson 1990), but since the role of oyster defense mechanisms, if any, in controlling *P. marinus* infections is not understood, it cannot be concluded that pollution suppresses the oyster's ability to inhibit the pathogen.

Although pollution clearly is not one of the primary factors responsible for recent increases of oyster diseases, there has been very little research on the potential subtle effects of toxicants on *P. marinus* disease progression. Only recently have studies been completed that suggest some effect of toxicants on *P. marinus* disease development. Winstead and Couch (1988) reported rapid proliferation of *P. marinus* in oysters exposed to high concentrations (600 mgl^{-1}) of the carcinogen n-nitrosodiethylamine when compared with unexposed control oysters. Chu and Hale (1994) found elevated *P. marinus* prevalence in oysters exposed to water soluble fractions derived from estuarine sediments grossly contaminated with polycyclic aromatic hydrocarbons and then challenged with *P. marinus*. These results suggest some effect of these chemicals on either pathogen multiplication or host condition or defense mechanisms, but it is difficult to determine the environmental relevance of these studies because it is unclear how the concentrations utilized in the experiments compare to actual levels of these compounds found in the water column in nature.

Tributyltin (TBT) has also been shown to enhance *P. marinus* disease progression and increase cumulative oyster mortality during experiments using environmentally relevant levels of TBT. Maximum prevalence and intensity levels of *P. marinus* occurred sooner in oysters exposed to 100 pptr TBT for five months and exposed to *P. marinus* after one month than in oysters not exposed to TBT and infected with *P. marinus* similarly (Anderson et al. 1995). In addition, mortality was higher in oysters that were both exposed to TBT and also infected with *P. marinus* than in oysters either infected but unexposed or exposed but uninfected. Similar results were obtained in experiments using 30 and 80 pptr TBT (Fisher et al. 1995) although the experimental design was somewhat different from that of Anderson et al. (1995)

These studies suggest that environmental toxicants may have some effect on disease development in highly polluted areas, but as the authors emphasize, there is no evidence that the dramatic increase in abundance of *P. marinus* since 1985 is the result of increased environmental pollution. Undoubtedly, further research will better clarify the subtle interactions among oysters, disease agents and environmental contaminants.

OTHER FACTORS INFLUENCING THE EPIZOOTIOLOGY OF *P. MARINUS*

There are other factors that potentially may influence the epizootiology of *P. marinus* in the Chesapeake Bay, but little research has been done that is specific to the Bay. For example, recent modelling studies in the Gulf of Mexico (Powell et al. 1996) suggest that timing of food availability is important to enable oysters to outgrow the parasite (Soniat 1996). Because of the longer growing season in the Gulf of Mexico than in the Chesapeake Bay, nutrition may be more critical in the Gulf of Mexico than in the Bay. Another factor that potentially may influence the epizootiology of *P. marinus* disease in Chesapeake Bay is the well-documented summer hypoxia, but no research has been done on this interaction.

It is well known that many other species of molluscs in Chesapeake Bay harbor cells of *Perkinsus* sp. (Andrews 1954). The taxonomic status of *Perkinsus* in these other hosts has not been clarified, but if they are *P. marinus* then these other molluscs could serve as reservoir hosts for the pathogen. The significance of putative reservoir hosts in the epizootiology of *P. marinus* disease is unknown. Studies are needed to determine if lethal *P. marinus* infections can be induced in oysters using *Perkinsus* cells isolated from other mollusc species.

EFFECT OF *P. MARINUS* ON THE OYSTER RESOURCE OF CHESAPEAKE BAY

Prior to 1985 *P. marinus* had little significant impact on the Maryland oyster industry because the pathogen was uncommon in Maryland. There were localized foci of infected oysters in the St. Mary's River in the 1960s and high mortality had decimated the local population by the late 1970s. The parasite was reported in Fishing Bay in the 1970s and in Eastern Bay in 1981. In Virginia, where *P. marinus* historically was restricted to the lower Bay and mouths of major tributaries, significant, but tolerable, oyster mortality occurred in these areas, especially in high salinity areas or during dry years. Nonetheless, harvest in Maryland and Virginia varied between 2 and 3.5 million bushels annually during the 1930s, 1940s and 1950s (Fig. 13). In Maryland the harvest was primarily from public oyster beds, but in Virginia over 80% of the harvest came from private planters who planted disease-free seed oysters from the Upper James River to growout grounds in the lower Bay areas of Mobjack Bay and Hampton Roads. Productive public beds occurred in the York River and Rappahannock River, Pocomoe Sound and various small tributaries along the western shore of the Bay (Andrews 1988). Typically, private planters in Virginia held seed oysters for three years on growout grounds, but as knowledge of *P. marinus* epizootiology increased and it was learned that most mortality occurred during the third year, planters began harvesting after only two years of growout (Andrews 1988). This early-harvest disease-avoidance strategy worked well during the late 1950s and annual harvest in Virginia varied from 3 to 4 million bushels during that period.

The sudden epizootic of *H. nelsoni* (MSX) in Mobjack Bay beginning in 1959 (Haskin and Andrews 1988) and the resulting high oyster mortality caused private planters to eventually abandon the traditional growout areas in the lower Bay by the mid-1960s and move operations to lower salinity areas in the Rappahannock River and small tributaries along the south shore of the Potomac River such as the Coan and Yeocomico Rivers. The 1970s were generally wet and levels of both *H. nelsoni* and *P. marinus* were reduced: from about 1967 through 1981 oyster harvest in Virginia was more or less stable at about 1 million bushels annually (Fig. 13)—greatly reduced from pre-1960 levels because of reduced growout acreage resulting from abandonment of the traditional growout areas in the lower Bay. In Maryland, good spat sets coupled with low pathogen abundance because of reduced salini-

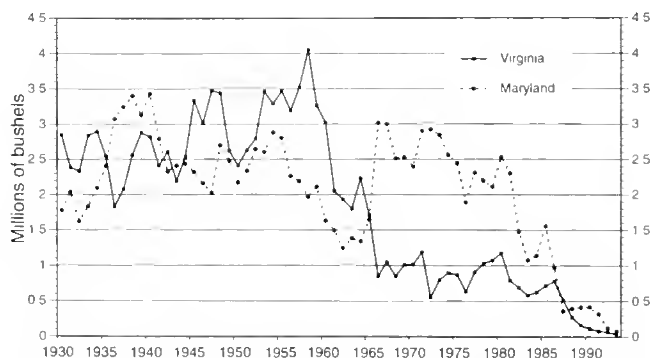

Figure 13. Annual market oyster landings in Chesapeake Bay from 1930 to 1994.

ties during the 1970s produced harvests between 2 and 3 million bushels annually during the 1970s (Fig. 13).

Both 1980 and 1981 were dry years; *P. marinus* intensified and *H. nelsoni* spread into Maryland for only the second time since it first appeared in the Bay in 1959. Mortality was high in areas where salinity was favorable for the pathogens and oyster landings declined in both states from 1982 through 1984.

The four consecutive drought years from 1985 through 1988 were catastrophic for oyster resources in both Maryland and Virginia. As outlined above, *P. marinus* spread to most oyster growing areas of the Chesapeake Bay including Maryland either by natural processes or by movement of infected oysters. Oysters were highly susceptible to *P. marinus* and mortality was high on planted grounds in Virginia and on public beds in both Virginia and Maryland. *H. nelsoni* also intensified during 1987 and 1988 and contributed substantially to the mortality in both states. The end result in Virginia was the virtual elimination of oysters from public beds in the lower Bay and from all but the uppermost reaches of the major tributaries. Current estimates are that less than 5% of traditional public oyster beds in Virginia are productive and these are all in the upper James River. Public beds were depleted by disease, and private planters, fearing losses from planting infected seed oysters, were (and still are) reluctant to transplant oysters for growout. Because of the absence of oysters in other areas of the lower Bay, harvesting pressure increased significantly in the upper James River, VA, beginning in 1986. Annual harvest increased in Virginia during 1986 and 1987 (Fig. 13) because of th large numbers of oysters in the upper James River, but the stocks were rapidly fished out and harvest plummeted in subsequent years. Since 1988, over 95% of the public market-oyster harvest in Virginia has come from the James River. Although there have been some restrictions placed on harvesting in Virginia, no quotas have been adhered to and remaining critical broodstocks in Virginia are being fished heavily. In Maryland, approximately 79% of the harvest during 1993–94 came from areas north of the Bay Bridge and 66% of the harvest came from the Chester River. As a comparison, during the 1973–74 season in Maryland, only 2.1% of the harvest came from the Chester River. These harvest figures from low salinity areas in both Virginia and Maryland demonstrate the impact of disease on the oyster resource in high salinity areas of the Chesapeake Bay.

The oyster resource rebounded in Maryland during 1994–95 and harvest increased for the first time in three years. Wet springs during 1993–94 resulted in lower salinity in Maryland, reduced levels of *P. marinus* and increased oyster survival in high salinity. In Virginia, no improvement was observed during 1994–95.

EPIZOOTIOLOGY GENERALIZED MODEL

A generalized model of *P. marinus* epizootiology in Chesapeake Bay is shown in Fig. 14. The model, based on data from the last decade, represents average timing of events that vary annually depending on temperature and salinity regimes. Infection regression begins in November and continues through May when minimum prevalence and intensity values are reached. Minimum infection parameters are reached approximately three months after minimum winter temperature and about one month after minimum salinity, although timing of minimum salinity varies much more than minimum temperature. Infection regression seems to be caused by the direct effects of temperature and salinity on *P. marinus* survival. The role of oyster defense mechanisms in infection regression is unknown but cannot be ruled out.

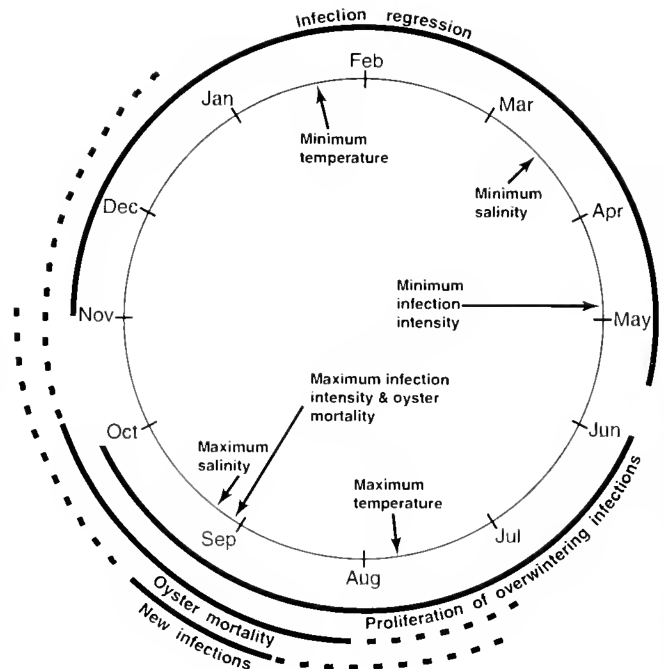

Figure 14. Generalized summary of *P. marinus* epizootiology in Chesapeake Bay. Dashed lines represent months of reduced activity compared to months represented by solid lines.

Infection prevalence and intensity begin to increase in June as water temperature increases above 20°C and overwintering infections begin to proliferate. Increase in prevalence and intensity from June through most of August seems to be due almost entirely to the proliferation of overwintering infections. Infection proliferation probably continues until October in oysters that don't die from the disease. Maximum prevalence and intensity occur in September, approximately six weeks after maximum water temperature, if salinity is greater than about 15 ppt, but values may peak one or two months later in lower salinity. Maximum values reached depend on the salinity regime.

A warm spring allows early proliferation of overwintering infections and oyster mortality may begin by early July, but under typical conditions most oysters die in August and September. Some mortality may continue at a reduced level until January or even later depending on fall temperatures. Total oyster mortality depends on the temperature and salinity regime and on the infection history.

New *P. marinus* infections are acquired by oysters shortly after oyster mortality commences and the correlation between new infections and oyster mortality suggests that the dying oysters are the source of infective stages. Most new infections are acquired during the last two weeks of August and the first two weeks of September, corresponding with the period of greatest oyster mortality. Because temperatures are greater than 25°C during this period and infections develop rapidly, there may be one or more cycles of oyster mortality/new infections/proliferation between late July and early October.

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EPIZOOTIOLOGY OF *PERKINSUS MARINUS* DISEASE OF EASTERN OYSTERS IN THE GULF OF MEXICO

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ABSTRACT *Perkinsus marinus* (Mackin, Owen and Collier) is a major cause of mortality of eastern oysters, *Crassostrea virginica* (Gmelin), along the Gulf of Mexico. The parasite is discontinuously distributed in estuaries from Tabasco, Mexico, to the Everglades of Florida. Its distribution is essentially coincident with its oyster host, although oysters survive well at salinities slightly lower than those tolerated by the parasite. Besides a low-salinity refuge, Gulf oysters apparently adapt to parasitic challenge with high recruitment and fast growth, which allows them to respond to natural estuarine variability more quickly than the parasite can. Temperature and salinity, but particularly their interaction, are important environmental influences on levels of *Perkinsus* in oysters. Most of the variation in levels of infection, however, are not explained by these factors. Other environmental and biological factors must affect the levels of parasitism observed in the field. These likely include, but are not limited to, pollution and other human influences, host nutrition and growth, spawning and reproduction, age and resistance, oyster density and distribution, and disease vectors.

KEY WORDS: *Perkinsus marinus, Crassostrea virginica*, epizootiology, Gulf of Mexico

INTRODUCTION

Epizootiology is that branch of biology that deals with the nature, ecology, and causes of outbreaks of animal diseases. Its particular focus therefore is on the sum of factors, including their interactions, which control the occurrence, distribution, and intensity of an animal disease or pathogen. Its sister science is epidemiology, which treats similar aspects of human diseases—of which we, not surprisingly, have much greater knowledge. Even within the field of epizootiology, our knowledge of diseases of aquatic organisms generally lags far behind that of insects and domesticated species. Among aquatic pathogens, however, *Perkinsus marinus* (Mackin, Owen and Collier), which parasitizes the eastern oyster *Crassostrea virginica* (Gmelin), is one of the most intensively studied and best known.

P. marinus was first described by Mackin, Owen, and Collier (1950) as *Dermocystidium marinum*, because of its apparent similarity to the freshwater parasitic fungus *Dermocystidium salmonis* (Davis). Later observations suggested that *D. marinum* gave rise to gliding cells on "mucoid tracks" similar to slime molds and was reclassified as *Labyrinthomyxa marina* (Mackin and Ray 1966). Although ultrastructural studies by Perkins (1969) revealed a likeness of the parasite to the fungi, no cytoplasmic extensions or rhizoids were observed. Levine (1978) renamed the parasite *P. marinus* on the basis of electron microscope work by Perkins (1976), which revealed the presence of an apical complex in a motile zoospore stage. The organism now resides in the protozoan phylum Apicomplexa, which it shares with the sporozoans (see Perkins 1996).

The discovery of *P.* (= *Dermocystidium*) *marinus* is the legacy of Texas A&M University Project 9, a multi-institutional effort funded by a consortium of oil companies, which investigated the causes of oyster mortalities in Louisiana oil fields (Mackin and Hopkins 1958, Ray 1996, S. H. Hopkins, unpublished observation). Extensive studies were conducted on the effects of crude oil, bleedwater, natural gas, drilling mud, and seismographic surveys on oysters. The general conclusion of these studies was that none of these pollutants or activities could explain the widespread oyster mortalities observed, but that high mortalities were caused by a parasite associated with high temperature and high salinity (Mackin and Hopkins 1958, 1962; see also Ray 1996). Numerous studies (e.g., Ray et al. 1953, Mackin 1956, Quick and Mackin 1971, Ogle and Flurry 1980, Soniat 1985, Craig et al. 1989) have documented that *P. marinus*, commonly called "Dermo," is most prevalent during the warm months in high-salinity areas and is widely distributed along the Gulf Coast. (See Table 1 for a chronology of selected studies.) The purpose of this paper is to review the 45 years of progress regarding our knowledge of the epizootiology of *P. marinus* in the Gulf of Mexico.

DISTRIBUTION ON THE GULF COAST

P. marinus is discontinuously distributed in estuaries of the Gulf of Mexico from Tabasco, Mexico (Burreson et al. 1994) to the Everglades of Florida (Craig et al. 1989; Fig. 1). Its distribution in the Gulf of Mexico is essentially coincident with that of its oyster host, although the host grows well at salinities slightly lower than that tolerated by the parasite (Mackin 1956). It is unlikely that any mesohaline Gulf estuary with even modest oyster populations is free of the parasite; indeed, the long-term absence of the parasite from an oyster population is more noteworthy than its presence.

A recent southern limit of the parasite has been established by Burreson et al. (1994), who found it in the Carmen, Machona, and Mecoacan Lagoons of Tabasco, Mexico. Prevalence (or percent infection, PI) and disease intensity (or weighted incidence, WI) ranged from 60% and 0.6 at Rio San Felipe (Carmen Lagoon, 15 ppt) to 100% infection and a WI of 3.1 at Los Jimenez (Machona Lagoon, 32 ppt). (Reports on prevalence in oyster populations tend to be underestimates due to the possibility of false negatives using the standard thiogycollate test.)

Mackin (1962) reported "Dermo" from Tampico Bay, Mexico, which until recently was the southernmost published record for the parasite. *Perkinsus* has not been extensively sampled from the lagoons north of Tampico Bay to the Rio Grande estuary near Port Isabel, Texas. Hildebrand (personal communication), however, indicates that C. E. Dawson found 100% infections and "high intensities" of the parasite in oyster populations from Laguna Tamiahau and that the parasite is likely found throughout the oyster-producing lagoons of the region.

TABLE 1.

A chronology of selected epizootiological and related studies on *P. marinus* from the Gulf of Mexico.

Reference	Area	Comments
Mackin et al. 1950	Barataria Bay, LA	Mackin, Owen and Collier describe *D. marinum* from histological sections of Gulf oysters, type locality is Sugar House Bend in southern Barataria Bay
Mackin 1951	Barataria Bay, LA Pensacola, FL Gulfport, MS Aransas Bay, TX	The histological consequences of infection corroborates "Dermo" as a major cause of oyster mortality
Ray 1952	LA, FL	Ray develops a culture technique which greatly aids epizootiological studies
Mackin 1953	Barataria Bay, LA	Heavy infections of "Dermo" are found in 86% of gapers and only 3% of live controls.
Ray 1953	Grand Isle, LA	Young oysters are shown to be less susceptible to "Dermo" than older ones
Ray 1954a	LA, TX	"Dermo" is transmitted to uninfected oysters by proximity and feeding.
Ray 1954b	LA, TX	Culture, transmission, pathogenicity, distribution, epizootiological, and host-specificity studies are reported
Dawson 1955	Apalachicola Bay, FL	More than 50% of oysters from 19 stations infected, 11% show heavy infections, "Dermo" is widespread in the Bay
Mackin 1956	Barataria & Terrebonne Basins, LA	The salinity tolerance of "Dermo" is nearly as great as the host, freshwater dilution of infective elements and higher concentrations of dissolved materials are hypothesized as important
Mackin & Boswell 1956		A detailed life cycle is presented
Mackin 1962	Tampico, Mexico Aransas Bay, TX Galveston Bay, TX Barataria & Terrebonne Basins, LA Biloxi, MS Mobile Bay, AL Pensacola, FL Cedar Key, FL	"Dermo" is reported from Tampico, Mexico—no records southward. "Dermo" is not found in Copano Bay, Lower Laguna Madre, Matagorda Bay, and Atchafalaya Bay. No information available south of Cedar Key, FL
Mackin & Sparks 1962	Lower Barataria Bay, LA	Mortalities of oysters in trays are associated with "Dermo" intensity
Ray 1966a	Freeport, TX Galveston Bay, TX Terrebonne Basin, LA Barataria Basin, LA E. of Miss. River, LA Apalachicola Bay, FL Santa Rosa Sound, FL Tampa Bay, FL	Uninfected samples found only at Smith's Point (Galveston Bay) and St. Vincent's Reef (Apalachicola Bay)
Ray 1966b	TX, LA, FL	An apparent absence of "Dermo" prompts a modification of the Ray (1952) technique
Perkins & Menzel 1966	Galveston Bay, TX Apalachicola Bay, FL	A motile planont (zoospore) stage is described. Planonts are apparently infective
Quick & Mackin 1971	FL	Eighty-six stations are sampled. The parasite was found "concomitantly with the host throughout the year and at salinities from 6 ppt to 36 ppt."
Beckert et al. 1972	AL	"Dermo" is widespread in Mobile Bay
Hofstetter 1977	Galveston Bay, TX	Sampling of 17 of the major reefs shows "Dermo" to be widespread
Ogle & Flurry 1980	Mississippi Sound, Biloxi Bay, Horn Island, MS	Four reefs sampled monthly for 2 years show typical late summer maxima and winter minima at nonepizootic levels
Soniat 1985	Galveston Bay, TX	One reef (April Fool) is sampled monthly for 2 years. WI is more closely correlated with the interaction of temperature (T) and salinity (S) than T or S alone.
White et al. 1987	Lake Borgne, LA Galveston Bay, Port Aransas, TX	The snail *B. impressa* transmits "Dermo" to uninfected LA oysters
Soniat & Gauthier 1989	Galveston Bay, TX Lake Calcasieu, Vermillion Bay, Terrebonne Bay, Barataria Bay, Adams Bay, E. of Miss River, Lake Borgne, LA Biloxi Bay, MS	May to January sampling shows a correlation of S, but not T, with WI. "Dermo" not found in Vermillion Bay (Southwest Pass)
Soniat et al. 1989	Galveston Bay, TX	May and June sampling of 16 reefs shows "Dermo" absent from some reefs in Trinity and East Bays
Craig et al. 1989	Gulf of Mexico	"Dermo" is found at all 49 sites, from South Bay (near Brownsville, TX) to Faka Union Bay (Everglades, FL); three regional foci of infection are identified, north central TX, central LA, and S.W. FL.
Gauthier et al. 1990	Lake Calcasieu, Terrebonne Basin, Barataria Basin, E. of Miss. River, LA	"Dermo" is sampled in four watersheds and is absent at only one low-S site; WI is correlated with S, digestive gland atrophy, and the occurrence of *Nematopsis* spp
Wilson et al. 1990	Gulf of Mexico	"Dermo" distribution is linked to land use and pollution patterns.
Gauthier & Fisher 1990	W. Galveston Bay, S. Padre Island, TX, Lake Borgne, LA	A quantitative hemolymph assay is developed.
Powell et al. 1992	Gulf of Mexico	A 4-year Gulf-wide study shows that WI is strongly correlated with long-range weather patterns.
Burreson et al. 1994	Tabasco, Mexico	"Dermo" is found in Carmen, Machona, and Mecoacan lagoons, thus establishing Tabasco, Mexico, as the southern limit
Powell et al. 1994	Galveston Bay, TX	An energy-flow model produces realistic simulations of disease progression and greatly aids understanding of the relationships among disease, oyster condition, and environmental factors.

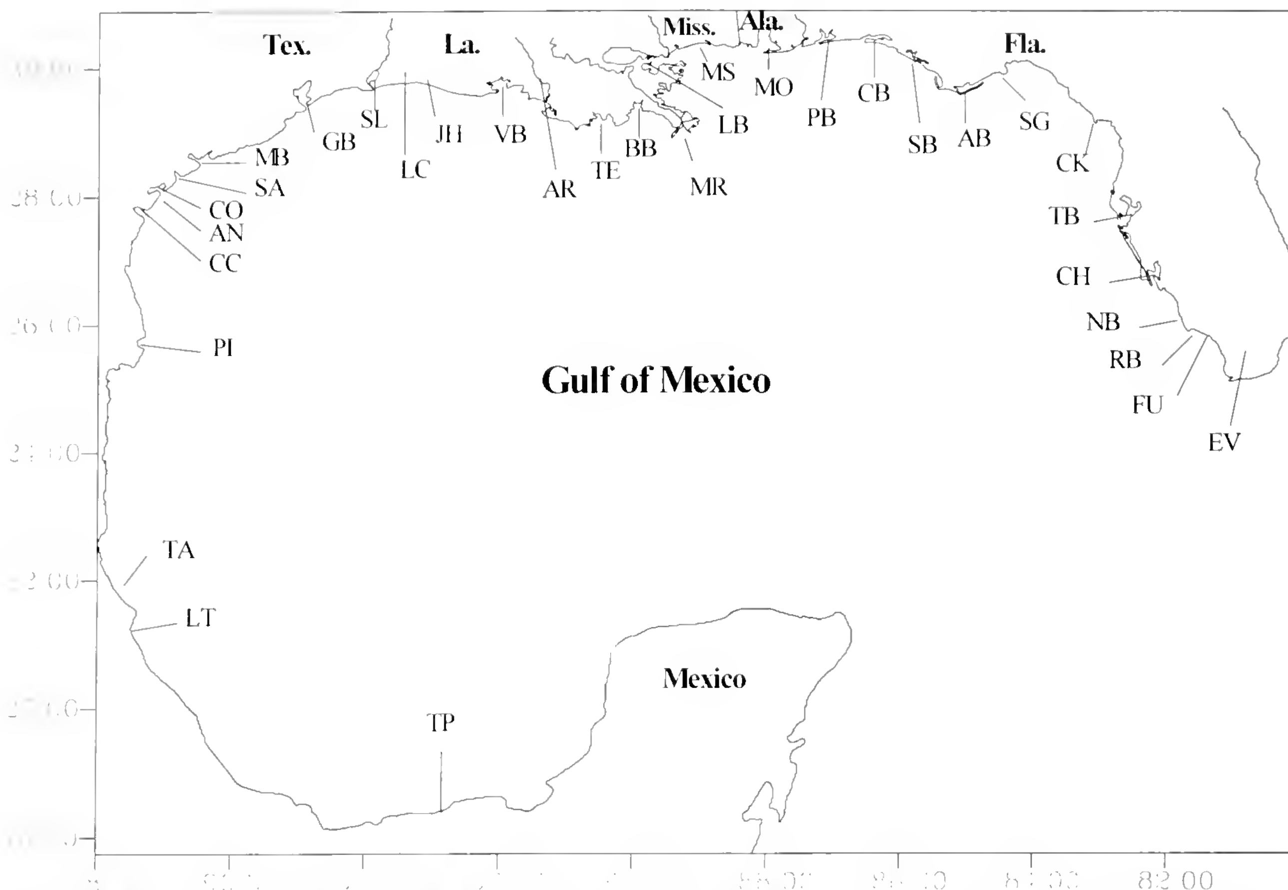

Figure 1. Map of the Gulf of Mexico showing selected geographical features of significance to the known distribution of *P. marinus*. TP = Tabasco Province, LT = Laguna Tamiahua, TA = Tampico Bay, PI = Port Isabel, CC = Corpus Christi Bay, AN = Aransas Bay, CO = Copano Bay, SA = San Antonio Bay, MB = Matagorda Bay, GB = Galveston Bay, SL = Sabine Lake, LC = Lake Calcasieu, JH = Joseph Harbor Bayou, VB = Vermillion Bay, AR = Atchafalaya River, TE = Terrebonne Bay, BB = Barataria Bay, MR = Mississippi River, LB = Lake Borgne, MS = Mississippi Sound, MO = Mobile Bay, PB = Pensacola Bay, CB = Choctawatchee Bay, SB = St. Andrews Bay, AB = Apalachicola Bay, SG = St. George Sound, CK = Cedar Key, TB = Tampa Bay, CH = Charlotte Harbor, NB = Naples Bay, RB = Rookery Bay, FU = Faka Union Bay, and EV = Everglades. See text for discussion.

The parasite is present in the Port Isabel area (South Bay, lower Laguna Madre), where Craig et al. (1989) reported prevalences of 82% and light infections (WI = 0.33). Oysters of the lower Laguna Madre are separated by a distance of 400 km from the nearest *C. virginica* populations in Redfish Bay, near Corpus Christi—due to the hypersaline conditions of the Laguna Madre (Groue and Lester 1982).

The Corpus Christi Bay area was recently sampled by Craig et al. (1989). Winter samples from Nueces Bay (northern Corpus Christi Bay) had prevalences of 78% and were lightly infected (WI = 0.33), whereas winter samples from Ingleside Cove (southern Corpus Christi Bay) had a PI of 98% and a WI of 0.33. The presence of the parasite at the northern and southern extremes suggests that it is distributed throughout the Bay.

Some disagreement exists in the literature as to the occurrence of *Perkinsus* in the Aransas Bay area. For example, Hoese (1963) speculated on factors which might explain the absence of "Dermo" from the region, yet later surveys (e.g., Craig et al. 1989) confirm its presence.

Mackin (1962) reported that the few samples taken from Copano Bay and Matagorda Bay were negative. *Perkinsus* is, however, present in both Bays (Ray 1966a, Craig et al. 1989); in fact, Craig et al. (1989) suggest that the Matagorda Bay–Galveston Bay area is one apparent regional focus of infection in the Gulf of Mexico. Sufficient sampling with typically positive results has accumulated which suggests that *Perkinsus* is likely continuously distributed from Corpus Christi Bay to Matagorda Bay.

The distribution of *Perkinsus* in the Galveston Bay complex (West Bay, Trinity Bay, East Bay, Galveston Bay proper) is well known (Mackin 1962, Ray 1966a, Hofstetter 1977, Soniat and Gauthier 1989, Soniat et al. 1989, Craig et al. 1989). Although occasional uninfected samples are found there (e.g., Ray 1966a, Soniat et al. 1989), no reefs, not even those in the low-salinity waters of Trinity Bay, are consistently disease-free.

Perkinsus is present in Sabine Lake, which borders Texas and Louisiana. The estuary has not been intensively sampled, and thus, the extent of its occurrence is not known. Sabine Lake is a relatively polluted, low-salinity estuary where the parasite is found at unexpectedly high prevalences, based on prevailing temperatures and salinities (Powell, personal communication). Craig et al. (1989), for example, found 100% prevalence (WI = 1.0) in a sample from lower Sabine Lake (Blue Buck Point) where the salinity was 8 ppt and when the temperature was 18°C.

Gauthier et al. (1990) reported relatively intense infections (WI = 1.93–3.03) and high prevalences (93–100%) at three stations along a north-south transect in Lake Calcasieu. Most of the sam-

pling effort has been in the southern portion of the Bay, where Ray (1982) and Craig et al. (1989) also reported high prevalences. Levels of infection were particularly high in East Cove (PI = 67–100%, WI = 1.5–3.8); Ray (1982) states that infection levels were "among the highest I have seen in more than thirty years' study in the Gulf of Mexico."

Between Lake Calcasieu and the Terrebonne Basin oyster habitat is scarce, and the parasite, like its host, is discontinuously distributed. Craig et al. (1989) report it from Joseph Harbor Bayou (PI = 73%, WI = 1.50) as well as Southwest Pass, which connects Vermillion Bay to the Gulf of Mexico (PI = 52%, WI = 0.33, Salinity (S) = 8 ppt). Soniat and Gauthier (1989) did not find the parasite in Southwest Pass but sampled when salinity was lower (S = 4 ppt). It is unlikely that *Perkinsus* extends far if at all into Vermillion Bay. Vermillion and Atchafalaya Bays are freshwater to oliogohaline bays that support few if any oyster populations. Indeed, the salinity of the water is so depressed in this area that oyster reefs are found 5 to 6 miles offshore (Price 1954); apparently, these offshore reefs have never been sampled for "Dermo." *Perkinsus* has only been found near the eastern boundary of the Atchafalaya Basin (Craig et al. 1989), in Oyster Bayou (PI = 90%, WI = 0.33, S = 11 ppt), and Melancon et al. (1995) did not find it there when the salinity was only 1.2 ppt.

Perkinsus is widespread throughout the Terrebonne/Barataria estuary. The Terrebonne Basin extends from Point au Fer to Bayou Lafourche, whereas the Barataria Basin encompasses the region from Bayou Lafourche to the Mississippi River. Melancon et al. (1995) recently mapped the oyster resources of the basins and sampled for *Perkinsus*. The distribution of oyster and parasite in the interdistributary estuaries of Louisiana is unlike that of typical Gulf gradient estuaries. In interdistributary estuaries, most of the freshwater input is at the boundaries, with some freshwater input at the head of the estuary. In contrast, gradient estuaries receive most of their freshwater from up-estuary. As a consequence, the zone of greatest abundance of oysters in interdistributary estuaries takes the shape of an inverted "u." Lower levels of parasitism, therefore, are found not only up-estuary, but also along the perimeters of the basins. In fact, one of the few populations of oysters in the Gulf of Mexico that appears to be consistently disease-free is one at Tiger Pass, an outlet from the Mississippi River (Powell, personal communication). Mackin (1962) states "areas of the lower Barataria are never free of disease" and this is probably true of the adjacent lower Terrebonne Basin as well. There are, however, populations that are temporarily disease-free. For example, Melancon et al. (1995) recorded a number of uninfected samples from summer during a low-salinity period. (The highest salinity at which a negative sample was found was 11.3 ppt.) Enough studies have been conducted in the area (Mackin et al. 1950; Mackin 1951, 1953, 1956, 1962; Ray 1953, 1954a, 1954b, 1966b; Soniat and Gauthier 1989; Craig et al. 1989; Gauthier et al. 1990; Melancon et al. 1995) to conclude that *Perkinsus* is ubiquitous in and continuously distributed across the Terrebonne/Barataria Basin. In fact, Craig et al. (1989) list Barataria Bay as one of the apparent Gulf-wide foci of infection.

The Mississippi River is a natural barrier to oyster distribution; however, seed oysters from public grounds east of the Mississippi River are routinely transported to and bedded on private leases in the Terrebonne/Barataria Basin. Thus, man transports oysters and possibly infective elements from east of the Mississippi River to west of the River on an annual basis (see Ray 1996).

Soniat and Gauthier (1989) found WI values from 0.90 to 1.90 in seven samples taken in Louisiana east of the Mississippi River—higher than those of Mackin (1962), who reported a range of 0 to 1.90. Lake Borgne is an area that supports a few low-salinity reefs (Mackin and Hopkins 1962), and populations there are intermittently infected (White et al. 1987, Soniat and Gauthier 1989, Gauthier et al. 1990).

The Pearl River forms a boundary between Louisiana and Mississippi. Freshwater outflow from the Pearl depresses salinities in the northern part of Lake Borgne and no oyster reefs are found there. Mackin and Hopkins (1962) indicate that there is good oyster production near Grande Isle (the one near the Louisiana and Mississippi border), but there are no records of samples taken for "Dermo."

The most extensive study of *Perkinsus* parasitism in Mississippi was that of Ogle and Flurry (1980). They sampled four reefs over a 25-month period (S = 0–35 ppt) and found relatively low prevalences (PI = 0–60%) and intensities (WI = 0.88). Soniat and Gauthier (1989) report higher intensities from a single station in lower Biloxi Bay (WI = 2.00–2.27, S = 5–25 ppt). The parasite is distributed throughout the oyster-producing area of Mississippi Sound and extends *at least* into the lower reaches of the coastal bays (Ogle and Flurry 1980, Soniat and Gauthier 1989, Craig et al. 1989). Insufficient sampling in Mississippi makes a determination of the northern limits of the parasite in the bays difficult, but it probably coincides with the upper distribution of oyster populations.

Most of the sampling effort for *Perkinsus* in Alabama has been in Mobile Bay, especially the southwestern portion of the Bay where the greatest concentration of reefs is found. Mackin (1962) reported very heavy WI values from several reefs, whereas Craig et al. (1989) found light infections at a single site. The most extensive sampling in the state is reported by Beckert et al. (1972). They sampled seven reefs in Mobile Bay and found the classic pattern of light infections (e.g., PI = 26.5%, WI = 0.27) in the upper part of the Bay and heavier infections in the lower Bay (e.g., PI = 84.4%, WI = 2.03). There are no records of uninfected populations of oysters in Mobile Bay.

The distribution of *Perkinsus* in Florida is well known. The most extensive study was that of Quick and Mackin (1971) who sampled 91 stations, 86 of which were from the northern and western coasts, and found the parasite "concomitantly with the host throughout the year and at salinities from 6 ppt to 36 ppt."

A number of studies have verified the presence of the parasite in the Pensacola Bay area (Mackin 1951, 1962; Ray 1952; Quick and Mackin 1971; Little and Quick 1976; Craig et al. 1989). Little and Quick (1976) found *Perkinsus* in both Escambia Bay and East Bay of the Pensacola Bay estuary. Levels of disease were especially high in Escambia Bay (PI = 50–100%, WI = 1.2–2.5), where Little and Quick (1976) ascribed the "over 90%" mortality of commercial-sized oysters in September 1971 to "dermo disease." The parasite extends into Santa Rosa Sound (Sabine Island) where it was found by Ray (1966a) at high prevalence (PI = 80%) and intensities (WI = 2.27). Quick and Mackin (1971) and Craig et al. (1989) reported *Perkinsus* from Choctawhatchee Bay, where the parasite is probably widespread; for example, Craig et al. (1989) found it prevalent (but not intense) at a high-salinity (S = 13 ppt, PI = 92%, WI = 0.33) site and a low-salinity (S = 0 ppt, PI = 98%, WI = 0.67) site. Data from Quick and Mackin (1971) and Craig et al. (1989) likewise suggest that "Dermo" is prevalent throughout the oyster-growing areas of the St. Andrews estuary (St. Andrews Bay, West Bay, East Bay).

The Apalachicola Bay area typically supplies about 90% of the oysters produced in Florida (Ingle 1982), and substantial sampling for *Perkinsus* has been conducted there. Dawson (1955) sampled 401 oysters from 19 stations and demonstrated that the disease was widespread. Half of the oysters were infected; light infections occurred in 28.2%, whereas moderate to heavy infections were found in 11.0% of the oysters. Annual mean salinities at the stations ranged from 7.6 to 29.3 ppt, with no difference in intensity of infection in oysters from low-salinity (<17.7 ppt) and high-salinity (>17.7 ppt) reefs. Ray (1966a) sampled three sites in the Bay—St. Vincents' Bar (PI = 0%), Cat Point Bar (PI = 100%, WI = 2.75), and Green Point Flat (PI = 77%, WI = 1.88). Quick and Mackin (1971) found the parasite widely distributed in Apalachicola Bay and St. George Sound. Craig et al. (1989) found light infections (WI = 0.33) at the eastern (Dry Bar, S = 13 ppt, WI = 63%) and western (Cat Point Bar, S = 7 ppt, WI = 92%) extremes of the area. Sufficient sampling has been conducted in the region to conclude that *Perkinsus* is continuously distributed across Apalachicola Bay and St. George Sound.

From St. George Sound to Tampa Bay, the parasite is found associated with localized oyster populations sustained by the outflows of the Aucilla, Suwannee, Waccasassa, Crystal, and other smaller rivers (Quick and Mackin 1971). It has been reported from Cedar Key area by a number of investigators (Ingle and Dawson 1953, Quick and Mackin 1971, Craig et al. 1989). For example, Craig et al. (1989) found prevalences of 100% in oysters from Black Point (S = 14 ppt, WI = 2.00). They suggest that the area east of the Mississippi River to Cedar Key, FL, has the Gulf-wide minimum for infection levels.

Perkinsus is prevalent and widespread in Tampa Bay. Ray (1966a) reported it from three populations with PI values ranging from 27 to 93% and WI values ranging from 0.40 to 1.65. Quick and Mackin (1971) sampled 53 stations and found it throughout the Tampa Bay estuary. Craig et al. (1989) sampled four stations and found all populations 100% infected, with WI values from 1.67 to 2.33 (S range = 28–34 ppt). The parasite has also been reported from the Charlotte Harbor area by Quick and Mackin (1971) and Craig et al. (1989). In fact, Craig et al. (1989) indicate that the Tampa Bay–Charlotte Harbor area has much higher than average levels of infection and is a Gulf-wide focus of infection.

Quick and Mackin (1971) report that the parasite is present near Ft. Myers (mouth of the Caloosahatchee River and behind Estero Island). Mackin (1962) indicates that a "few oysters" are produced as far south as Lee County (south of Ft. Myers) and populations of *C. virginica* become scarce and sparse southward. Craig et al. (1989) found the parasite in Naples Bay (S = 28 ppt, PI = 100%, WI = 2.33), Rookery Bay (S = 34 ppt, PI = 100%, WI = 0.67), and Faka Union Bay (S = 27 ppt, PI = 100%, WI = 1.00). Quick and Mackin (1971) established sample stations for *Perkinsus* in the Florida Keys, but it is uncertain if the oysters sampled were *C. virginica*. Until further sampling extends the range, the southeastern limit of *P. marinus* in the Gulf of Mexico is considered to be Faka Union Bay near the Everglades of Florida (Craig et al. 1989); however, Powell (personal communication) has found *Perkinsus* in Bahia de Jobos, Bahia de Boqueron, and Bahia Montalva in Puerto Rico.

TRANSMISSION AND PREVALENCE

Environmental Factors

Temperature and salinity are the main environmental factors considered in epizootiological studies of *P. marinus*. Although both factors and, in particular, their *interaction* are important (Soniat 1985), most of the variation in levels of parasitism is *not* explained by variations in temperature and salinity. Other environmental and biological factors, of which we know little or nothing, must significantly affect the levels of parasitism observed in the field.

Temperature and Salinity

Water temperature is one of the major environmental factors determining the prevalence and intensity of *P. marinus*. In some field studies (e.g., Dawson 1955) a significant positive correlation has been established between temperature and levels of parasitism, but the relationship does not hold in all cases (e.g., Soniat 1985). In most cases the disparity is understandable. For example, if a field survey is conducted over a short period of time, temperature varies little and may not appear to be important. Likewise, in areas where there is significant variation in salinity, salinity appears to be more important (e.g., Soniat 1985). Furthermore, in some estuaries temperature and salinity may be inversely correlated (e.g., Soniat 1985) and thus the two factors have opposing effects on the parasite.

Recent *in vitro* laboratory studies (Chu and Greene 1989) have demonstrated that *P. marinus* is especially proliferative at temperatures above 20°C, which corresponds well with field observations. Hofmann et al. (1995) modeled the response of the parasite by assuming the effect of temperature on cell division to be a standard temperature dependency on growth. The equation takes the form

$$r_d[T] = r_d[T_o]\, e^{(0.06931(T(t) - T_o))} \tag{1}$$

where T_o = 20°C, $r_d[T]$ = the specific rate of cell division (day^{-1}), $r_d[T_o]$ is the rate at 20°C, and the exponential corresponds to a Q_{10} of 2.0. At T_o = 20°C (and S = 20 ppt), $r_d[T_o]$ = 0.555 day^{-1}; that is, the population can double in size every 2 days.

At 10 ppt and less, the rate of cell division is modified by salinity (Chu and Greene 1989). For salinities below 10 ppt, equation (1) takes the form

$$r_d[T,S] = r_d[T_o,S_o]\, (S/10)\, e^{0.06931(T(t) - T_o)} \tag{2}$$

where S_o = 20 ppt and $r_d[T_o] = r_d[T_o,S_o]$ = 0.555 day^{-1} (Hofmann et al. 1995). Thus, at salinities below 10 ppt the specific rate of cell division is decreased. The above equation, which quantifies the response to the interaction of temperature and salinity, closely tracks field data (Soniat and Powell 1994, Hofmann et al. 1995).

Climatic patterns, which in turn affect local rainfall and the severity of the local winter, are related to levels of parasitism along the Gulf (Powell et al. 1992). Recent data indicate that the Gulf-wide mean infection intensity follows the El Nino cycle fairly closely (Powell, personal communication).

Other Environmental Factors

It has been hypothesized that pollution may affect the susceptibility and resistance of oysters to *P. marinus* (Winstead and Couch 1988, Chu and Hale 1994). Pollutants might increase disease susceptibility by increasing the number or virulence of infective elements. Alternatively, pollutants could reduce disease resistance by a physiological stress and associated energy drain on the host (see Paynter 1996) or by a suppression of the host immune system (see Anderson 1996).

Winstead and Couch (1988) exposed eastern oysters to high concentrations (600 mg/l) of n-nitrosodiethylamine, resulting in significantly increased levels of *P. marinus*. Chu and Hale (1994) demonstrated that water-soluble pollutants from sediments enhanced preexisting infections and increased the oysters' susceptibility to infection.

Field studies also suggest a link between human activity and parasite distribution. Craig et al. (1989) found that agricultural and urban land use affected the Gulf-wide distribution of *P. marinus*. Salinity and agricultural use yielded the best two-variable regression model (maximum r-squared improvement), whereas the best three-variable model additionally included urban land use. (Seven variables were considered: salinity, temperature, condition index, length, residential use, industrial use, and agricultural use.) Correlation coefficients never exceeded 0.30, indicating that much of the variation in levels of parasitism remained unexplained. Wilson et al. (1990) developed a similar model to investigate the geographical distribution of *P. marinus* on a bay scale. A seven-variable model (mean latitude for each bay, agricultural land use, industrial land use, mean oyster length, total polynuclear aromatic hydrocarbons concentration [PAH], total pesticide concentration, and salinity) explained 49% of the variation in *P. marinus* intensity. (Length is a surrogate parameter for age, which relates to pollution body burdens and levels of infection. Gulf-wide, latitude is an adequate surrogate for a temperature/salinity interaction, since the northern Gulf of Mexico is cooler and wetter than the southern Gulf.) Total PAH concentrations and industrial land use were positively correlated with prevalence, whereas latitude and agricultural land use were negatively correlated with prevalence.

Biological Factors

Numerous biological factors of the host potentially affect the transmission or prevalence of *P. marinus*. These factors include, but are not limited to, oyster nutrition and growth, spawning and reproduction, age and resistance, and density, distribution and interactions with vectors.

Nutrition and Growth

Recent modeling studies (Powell et al. 1996) suggest that the timing of the spring bloom of phytoplankton is critical in the initiation of epizootics. If the spring bloom occurs early, more energy is shunted into somatic growth than into reproduction and oysters quickly accrue biomass and outgrow the parasite. The oyster and the parasite are thus in a kind of race. If the oyster can grow fast enough, the parasite concentration is effectively diluted and the oyster wins. If the growth of the oyster is slow, the parasite will win the race. An early spring bloom produces less mortality in an oyster population because more of the net production goes into somatic growth and less goes into reproduction, and one of the oyster's primary defenses is to outgrow the disease (Hofmann et al. 1992). A drop in food supply favors the parasite, since reduced food supplies do not affect the division rate of *P. marinus* (Powell et al. 1996) but do reduce oyster growth and fecundity (Soniat and Ray 1985). Consequently, other factors that diminish oyster growth, especially if they are operative in the summer when the parasite is rapidly proliferating, favor the initiation of epizootics in modeling simulations. Thus, high turbidity that decreases feeding efficiency, low current flow that delivers food at a suboptimal level, and dense populations of filter feeders that outcompete the oyster for food, all reduce oyster growth rates and, *in modeling scenarios* if not nature herself, favor the parasite over the host (Powell et al. 1996).

Spawning and Reproduction

Early observations suggested that heavy mortalities of oysters often followed spawning. It was hypothesized that the stress of spawning weakened host oysters such that they were more easily infected and had less resistance to the progression of disease. Mackin (1953) tested this hypothesis, and although he found no relationship between levels of infection in pre- and postspawning oysters, he observed a degeneration of the gonads if the disease struck in an early stage of gonadal development (see also Mackin 1962). Subsequent studies by Wilson et al. (1988) and Choi et al. (1993, 1994) suggest that *P. marinus* can decrease oyster fecundity. Choi et al. (1994), for example, found a relationship between the rate of gamete production and infection intensity in certain months. Heavy mortalities of oysters following spawning have an alternate explanation. Spawning reduces oyster biomass more than "Dermo" biomass so that the number of cells per gram of oyster increases, possibly to lethal levels.

The effect of spawning success on disease progression in an oyster population may be of even more significance than the effect of level of disease on oyster fecundity. For example, Powell et al. (1996) suggest that recruitment failure is likely a principal mechanism increasing population infection intensity and initiating an epizootic. Likewise, one way in which an epizootic can be terminated is by a massive, successful spawning event. New and uninfected biomass thus replaces old, infected oysters that have died from the disease. Infection intensity in juvenile oysters is reduced, and if enough food is present, juvenile oysters grow fast enough to dilute *P. marinus* in the population and maintain population fecundity (Powell et al. 1996).

Age and Resistance

Mackin (1951) reported that Louisiana oysters less than 1 year of age are not infected as extensively as are market-sized oysters. Ray (1953), also working in Louisiana, found that spat held in an area of high endemism appeared to be highly refractive to infections. Andrews and Hewatt (1957), however, fed a tissue mince of heavily infected gapers to young oysters which became infected and died as quickly as older control oysters. Subsequent studies in Texas (Hofstetter 1977, Ray 1987) suggested that young oysters under natural conditions could become heavily infected at an early age.

Apparently then, there is no inherently greater immunity in young as compared to old oysters. The observation that young oysters often have lower levels of infection can be explained by the fact that they must be exposed to a sufficient number of infective elements to initiate an infection. Furthermore, young oysters are growing rapidly when the parasite is in its initial "lag phase" of growth before *Perkinsus* population growth is greatly accelerated. Thus, if enough infective elements are present in the environment, young oysters can become heavily infected, especially if the proliferation of the parasite population is rapid relative to the growth of the oyster host. When oysters are young, their cell division rate approximates the cell division rate of "Dermo." Thus, under optimal conditions of growth for the host, the parasite cannot grow fast enough to reach a high intensity until the oyster reaches adulthood, when its growth rate declines.

Density, Distribution, and Vectors

The close proximity of infected oysters to uninfected oysters appears to be an important factor in the spread of *P. marinus* (Ray

1987). The potential for an epizootic is thus related to the distance of the uninfected population from a source of infection, the prevailing water currents, and possibly the efficacy of transmission by vectors—all of which determine the number of infective elements available to the susceptible population. Transmission, however, includes two distinct processes which operate on different spatial and temporal scales—reef-to-reef transmission which occurs over relatively greater distances and times, versus oyster-to-oyster transmission *on reefs* which acts over shorter distances and requires less time.

Andrews (1988) reviewed the results of a number of tray experiments designed to test the effectiveness of transmission over varying distances. His conclusion was that transmission of *P. marinus* tends to be localized and that isolation of beds is a useful management strategy for controlling the disease during normal years (see also Andrews and Ray 1988). The effects of isolation in retarding the spread of the disease are also supported by studies of Ray (1987) in Galveston Bay, TX. Of particular importance is the occurrence of "killing floods" which destroy oyster populations, diminish infection levels, and ultimately decrease the number of infective elements in the estuary. Such events provide "natural experiments" in which the temporal and spatial response of the system can be observed. Freshet mortalities remove diseased oysters from the estuary, provide new, clean shell for spat settlement, and "reset" the system at a lower level of infection. The success of the system response (again) depends initially upon the success of oyster recruitment and subsequently upon the ability of the oysters to outgrow the disease (Powell et al. 1996). High recruitment and fast growth help explain why Gulf oyster populations thrive at temperatures and salinities that would cause the demise of their northern counterparts.

Although the evidence clearly suggests that the direct transmission of waterborne infective elements from oyster to oyster is the primary mechanism for spreading the disease (Ray 1954a, 1954b; Mackin 1962; Perkins and Menzel 1966; Andrews 1988), scavengers may also be important as disease vectors (Ray 1954b, Hoese 1962, White et al. 1987). Numerous scavengers live on Gulf oyster reefs and consume dead and dying oysters. They include, among others, blue crabs, mud or xanthid crabs, nereid polychaetes, and fishes such as gobies and blennies (Andrews 1988). Hoese (1962), for example, found live *Perkinsus* in the intestinal tracts of oyster drills (*Urosalpinx cinerea*) and fishes (*Gobiosoma bosci*, *Chasmodes bosquianus*, *Opsanus tau*) and on the bodies of xanthid crabs (*Neopanope texana*, *Rithropanopeus harrisii*). Gapers (dead oysters with the meat intact) are rarely observed in the field, and it is likely that many of the *Perkinsus* cells from dead oysters pass through the digestive systems of scavengers before the oysters decay (Hoese 1962, Ray 1987).

White et al. (1987) demonstrated that the ectoparasitic snail, *Boonea* (=*Odostomia*) *impressa*, directly transfers *Perkinsus* from uninfected to infected oysters. These ectoparasites puncture the mantle edge of oysters to suck body fluids and transfer viable *Perkinsus* cells as they move from oyster to oyster.

SUMMARY AND CONCLUSIONS

Although *P. marinus* is one of the most studied marine pathogens, much remains to be learned. Its distribution in the Gulf of Mexico is fairly well known; exceptions are Mexico where it has not been extensively sampled and some bays where its upper limit has not been established. Its relationship with temperature and salinity is well documented, yet these factors do not explain most of the variation in levels of parasitism observed in the field. A more complete accounting for observed patterns will require a more complex explanation. Of likely importance will be the *interaction* of temperature and salinity, the *timing* of the spring bloom in relation to the increase in temperature in the spring, and the *interplay* of physical factors (especially temperature and salinity), nutritional factors, and host health and condition. To achieve such an understanding requires a model of the disease process, field and laboratory verification of model responses, and ongoing iterative interactions among model, field, and laboratory studies.

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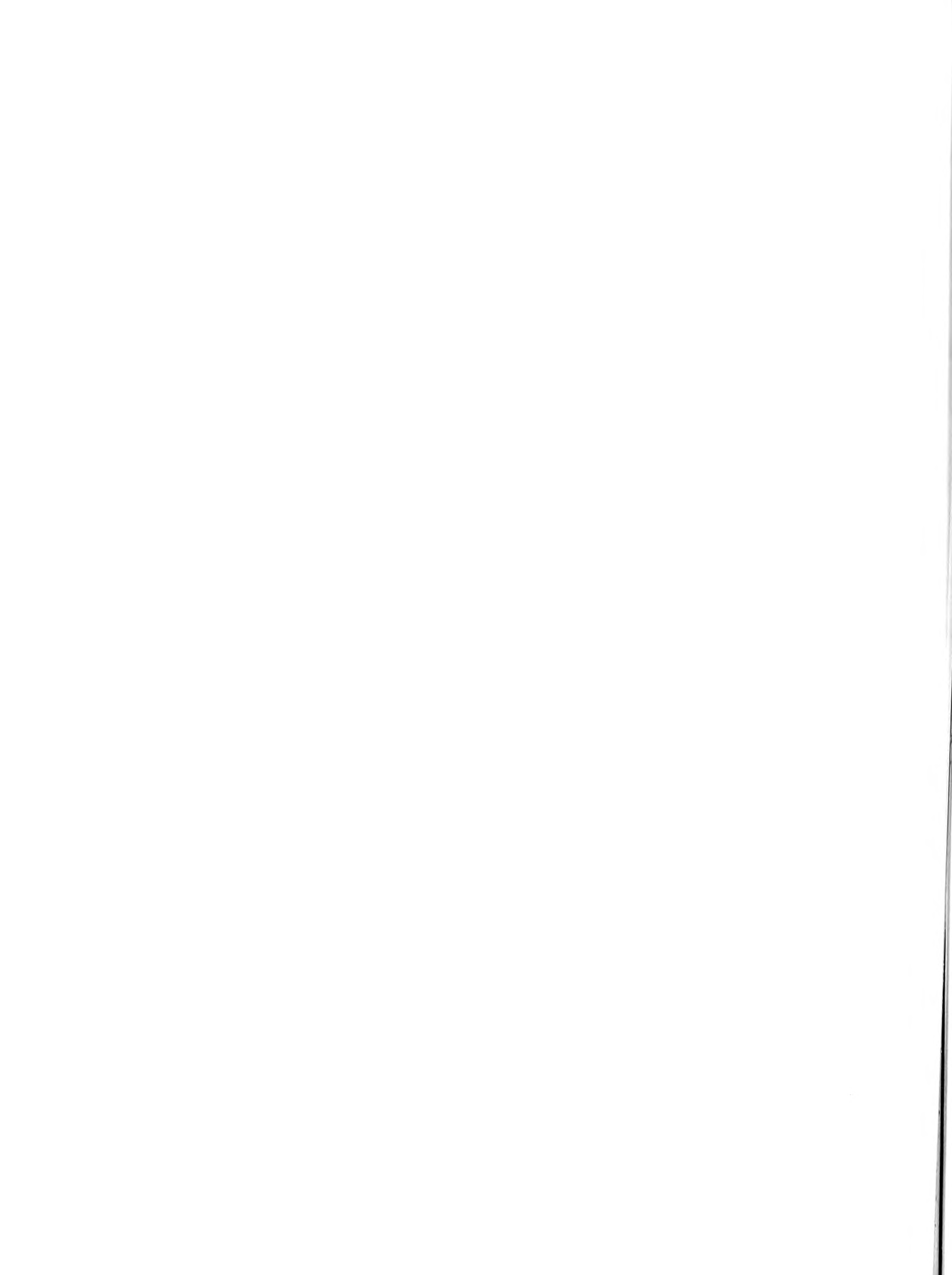

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RANGE EXTENSION BY THE OYSTER PARASITE *PERKINSUS MARINUS* INTO THE NORTHEASTERN UNITED STATES: RESPONSE TO CLIMATE CHANGE?

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ABSTRACT From its discovery in 1949 until 1990, the oyster parasite *Perkinsus marinus*, cause of Dermo disease in the eastern oyster *Crassostrea virginica*, was found primarily from Chesapeake Bay south along the Atlantic Coast of the United States and into the Gulf of Mexico. In 1990 and 1991, the parasite suddenly appeared in locations from Delaware Bay, NJ, to Cape Cod, MA, a range extending more than 500 km north of Chesapeake Bay. An earlier incursion of the parasite into Delaware Bay in the 1950s, associated with importation of large numbers of infected oysters from Chesapeake Bay, did not cause detectable mortalities or result in the establishment of a significant parasite population. The parasite was no longer detected after imports of infected oysters ceased. In contrast, the epizootic that began in Delaware Bay in 1990 resulted in high disease prevalence and intensity, and caused heavy mortalities, but was not linked to similar imports. Several hypotheses for the sudden appearance of the parasite in the northeastern United States are considered: 1) the parasite was transmitted via infected oysters introduced from enzootic southern areas into northern waters; 2) a change in the genetic structure of either host or parasite increased the parasite's ability to invade and proliferate in the northeast; 3) the environment in the northeastern United States became more favorable for parasite activity; or 4) some combination of these three. The *simplest* explanation consistent with available data is that the pathogen was repeatedly introduced, by many means over many years, into various northeast locations where it remained undetected and was stimulated to proliferate into an epizootic by a recent extreme warming trend. Above average winter, rather than summer, temperatures were associated with the 1990s epizootic. Also, cold winters, not cool summers, were correlated with the disappearance of *P. marinus* from Delaware Bay in the 1950s. Stopping or materially slowing the epizootic will probably require a series of consecutive cold (i.e., average or below average temperatures) winters and cool springs that will delay and restrict the proliferation of parasites during the following summer. Eliminating the parasite from its new range may be difficult even with cooler temperatures, however, as the development of low temperature–adapted parasites could occur now that large populations are established in a region where selection pressure exists for this trait.

KEY WORDS: Dermo disease, *Crassostrea virginica*, temperature, genetic change, parasite introduction, environmental change

HISTORICAL DISTRIBUTION OF *PERKINSUS MARINUS*

In 1940, oyster growers in Louisiana began reporting unusual mortalities of oysters and blamed oil pollution caused by companies drilling in the Gulf of Mexico (Mackin and Sparks 1962). Scientists hired by the oil companies in 1947 soon identified a protozoan, *Perkinsus* (= *Dermocystidium*, = *Labyrinthomyxa*) *marinus*, as the cause (Mackin et al. 1950, Mackin and Hopkins 1962). At about the same time, researchers in Virginia found the same parasite in oysters that had survived a 1949 mortality in the Rappahannock River (Andrews 1955). Over the next few years, *P. marinus* was found in oysters throughout the southeastern United States and Gulf of Mexico (Ray 1954). The disease caused by *P. marinus* is commonly called "Dermo" disease, a reference to the genus (*Dermocystidium*) in which the parasite was originally placed.

Although *P. marinus* was found over a wide area during a relatively brief period by scientists looking for it, the pathogen had likely been present for many years. Mackin and Sparks (1962) searched published records of the oyster industry in Louisiana dating back to the early 1900s and found that reported mortalities had the characteristics later found to describe *P. marinus* activity (see Andrews 1988). The mortalities occurred during warm, dry periods; they selectively affected older oysters; and they did not affect other members of the oyster community. *P. marinus* was also identified in archived tissue sections of Louisiana oysters fixed around 1930 (Owen pers. comm. in Ray 1954). Andrews and Hewatt (1957) considered it probable that the parasite had existed in Chesapeake Bay before they first recorded it in 1949. This belief was supported by the fact that the Virginia industry harvested annually as many oysters between 1950 and 1959, years of high disease levels, as it had during the two decades before *P. marinus* was discovered (Haven et al. 1978).

CHANGES IN THE DISTRIBUTION OF *P. MARINUS* IN THE 1980s AND 1990s

With the exception of a localized and nondestructive incursion into Delaware Bay, described below, and findings in the upper Chesapeake Bay in the mid-1970s (Otto and Krantz 1977), detectable *P. marinus* activity remained limited to waters from the lower Chesapeake Bay south. Reports of the parasite in Connecticut and Massachusetts (Sindermann 1970, Quick 1977) are given without documentation. In the 1980s, this observed distribution began to change (Fig. 1). From 1985 to 1988, *P. marinus* was detected, and caused mortalities, increasingly farther upestuary in the Maryland portion of Chesapeake Bay (Burreson and Ragone Calvo 1996). It appeared, about the same time, in the small estuaries along the Atlantic Coast from Virginia to southern New Jersey (Table 1; Burreson and Ragone Calvo 1996). In 1990, a major epizootic began on the New Jersey side of Delaware Bay. From 1991 to

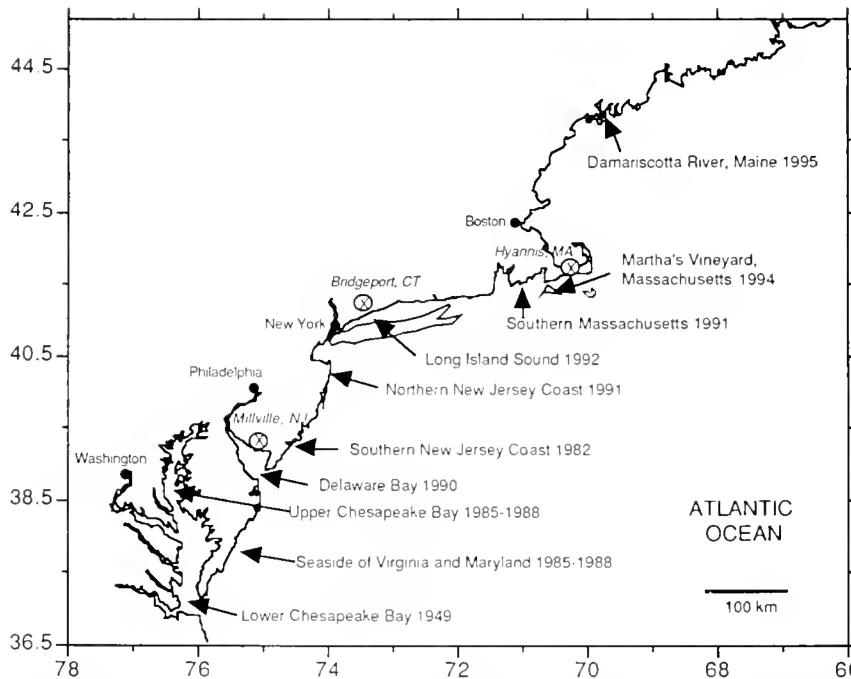

Figure 1. Map of the Atlantic Coast of the northeastern United States showing the dates when *P. marinus* was first detected. The three locations depicted with an encircled X are stations from which long-term air temperature deviation records were analyzed (see Fig. 6).

1992, the parasite was found progressively northward along the Atlantic Coast to Cape Cod, MA. In 1995, its presence was confirmed in Maine.

This paper compares and contrasts the 1950s' and 1990s' incursions of the parasite into Delaware Bay, documents the observed spread of *P. marinus* northward from Chesapeake Bay beginning in 1990, and discusses various hypotheses for its sudden range extension.

TABLE 1.

History of *P. marinus* in New Jersey oysters. Prevalences are the maxima recorded, usually in autumn. Note that the extent of sampling and the type of diagnosis varied from year to year.

Year	% Prevalence	Comments
1952		Delaware Bay planters begin importing oyster seed from lower Chesapeake Bay
1953–54	4	A few infected oysters detected in lower Delaware Bay (Andrews & Hewatt 1957)
1955	40–60	Regular RFTM monitoring in Delaware Bay initiated by Rutgers Oyster Research Lab
1956	10–15	Regular RFTM monitoring in Delaware Bay
1957	25–35	First outbreak of MSX disease on New Jersey leased grounds
1958	20–25	Mortalities from MSX disease spread throughout bay
1959	8	Embargo on all oyster imports into and exports from Delaware Bay
1960	8	Regular RFTM monitoring of Delaware Bay
1961	0	Regular RFTM monitoring of Delaware Bay
1962	4	Regular RFTM monitoring of Delaware Bay
1963	0	Regular RFTM monitoring of Delaware Bay
1964–73		Regular RFTM monitoring discontinued except at Cape Shore, where *P. marinus* still present
1975	52	Infections found in tissue sections from Maurice River Cove grounds and followed up with RFTM culture; some gapers heavy; nothing found at other locations tested
1976		Heavy infections found in early July in lower seed bed oysters after <1 month at Cape Shore
1975–80		No regular RFTM monitoring except at Cape Shore, where *P. marinus* still present
1981		Infected oysters no longer found at Cape Shore
1982–87	10–14	Infections found in tissue sections of oysters from coastal New Jersey bays and rivers (Great Bay, Tuckahoe River, Great Egg Harbor Bay)
1985	5	One infected oyster found in tissue sections of 20 oysters from lower New Jersey seed beds
1988	5	Very light infections found on Delaware Bay natural oyster beds off Kelly Island, DE, and Egg Island Point, NJ (Hofmann et al. 1995)
1990	90	Atypical mortalities in Maurice River and at Cape Shore signal onset of epizootic and resumption of RFTM sampling
1991–95	100	Intensification and spread on New Jersey side of Delaware Bay; few or no infections on the Delaware side

Incursions of *P. marinus* into Delaware Bay: 1950s and 1990s

The 1950s Epizootic

The oyster industries of both Delaware and New Jersey developed and now operate in similar fashions (Ford 1996). Seed oysters are transplanted each year from the public beds in the upper bay onto private leased grounds in the lower bay (Fig. 2), where they grow and fatten until they reach market quality. Historically, when native seed was insufficient to supply the needs of planters, oysters were brought in from other areas. Seed oysters from Chesapeake Bay were imported into Delaware Bay for decades beginning in the early 1880s (Goode 1887 quoted in Andrews and Hewatt 1957, Ford 1996), but the practice appears to have expanded around 1950 when the native seed supply was severely depleted (H. Bickings, Sr., Bivalve Packing Co., pers. comm. 1989). Originally, most of the seed came from the James River, where *P. marinus* was not present, but in 1952–53 it became illegal to sell James River oysters for direct shipment out of Virginia (Andrews and Hewatt 1957). Thereafter, many Delaware Bay planters bought "seed" oysters from private leases in the Hampton Roads area and other higher salinity regions of Chesapeake Bay where *P. marinus* was present and causing heavy losses (Andrews 1988). Infected oysters were brought by the shipload for planting on the leased grounds of lower Delaware Bay.

From the studies of Mackin (1962) and Ray (1954) in the Gulf of Mexico, and Andrews and Hewatt (1957) in Chesapeake Bay, it was already clear that *P. marinus* could be transmitted directly from oyster to oyster. Limited surveys had detected a few infected oysters in Delaware Bay in 1953 and 1954, and some planters had reported losses in 1954 and 1955 (G. Christensen 1956, unpublished observation). To determine whether the disease had spread from the imported oysters, a survey was begun by the Rutgers University Oyster (now Haskin Shellfish) Research Laboratory, under the direction of Dr. Harold Haskin. Extensive sampling was performed throughout the year over a 4-year period from 1955 through 1958, first by Ms. Greta Christensen and later by Mr. Donald Kunkle. Collections were made from all regions of the New Jersey side of Delaware Bay, including both leased grounds and seed beds. Less extensive sampling, concentrated on the leased grounds in late summer and autumn, was continued until 1963. From 1955 to 1958, particular attention was paid to sampling the imported Virginia seed and adjacent native oysters. Oysters were processed using the standard Ray's fluid thioglycollate medium (RFTM) assay to detect *P. marinus* and the Ray/Mackin rating system to estimate sample weighted prevalence on a scale from 0 to 5 (Ray 1954).

Infected oysters were found almost exclusively from late summer into early winter; detectable infections were few or nonexistent from late winter through early summary (Fig. 3). Peak prevalence, recorded during the late summer and autumn, reached 50–60% on a few grounds, but generally did not exceed 40%. Most infections were in oysters brought from Virginia and in the native oysters planted nearby (Fig. 4A); however, disease levels were not consistently different between the two groups. Infections were negligible on the seed beds and in the far eastern edge of the leased grounds where oysters were rarely planted. The highest weighted prevalences were between 1.0 and 1.3. These scores were low compared to those in Virginia and the Gulf of Mexico, but a rating of 1.0 or more is sufficient to cause some deaths in an affected population (Andrews and Hewatt 1957, Mackin 1962). Mortalities were not assessed during the Rutgers study, but based on weighted prevalences, it was estimated that losses due to *P. marinus* could not have exceeded 20% annually and were only about 5% in most areas (G. Christensen 1956, unpublished observation).

The localized distribution of *P. marinus* and the proximity of infected native oysters to imported stocks were highly suggestive of transmission from the Virginia oysters. Lack of preintroduction monitoring, however, prevented unequivocal determination of the parasite's origin. A few years after the introductions had begun, another event occurred that provided additional evidence that the Virginia oysters had introduced *P. marinus* into Delaware Bay. In the spring of 1957, heavy mortalities of oysters began on the New Jersey leased grounds. By 1959, they had spread throughout the

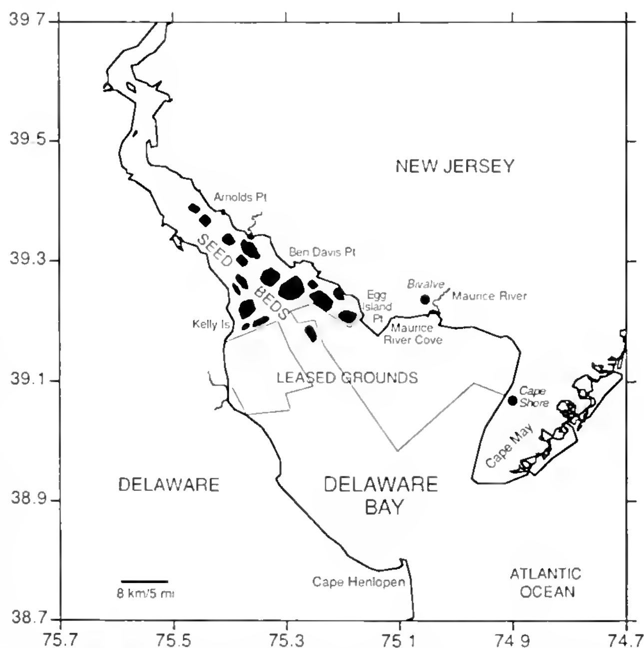

Figure 2. Map of Delaware Bay showing locations of major seed oyster beds and the leased oyster grounds.

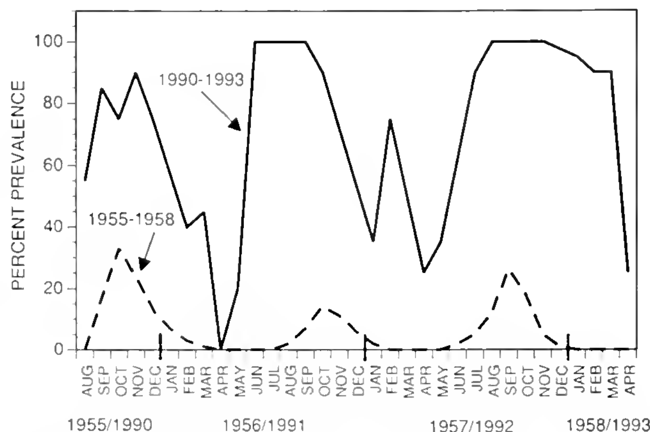

Figure 3. Prevalence of *P. marinus* in planted oysters on the New Jersey leased grounds during 3-year periods after initial disease outbreaks in 1955 and 1990. Samples of 20 oysters were collected approximately monthly and diagnosed using the standard Ray/Mackin RFTM assay. Data from 1955 to 1958 taken from G. Christensen (1956, unpublished observation).

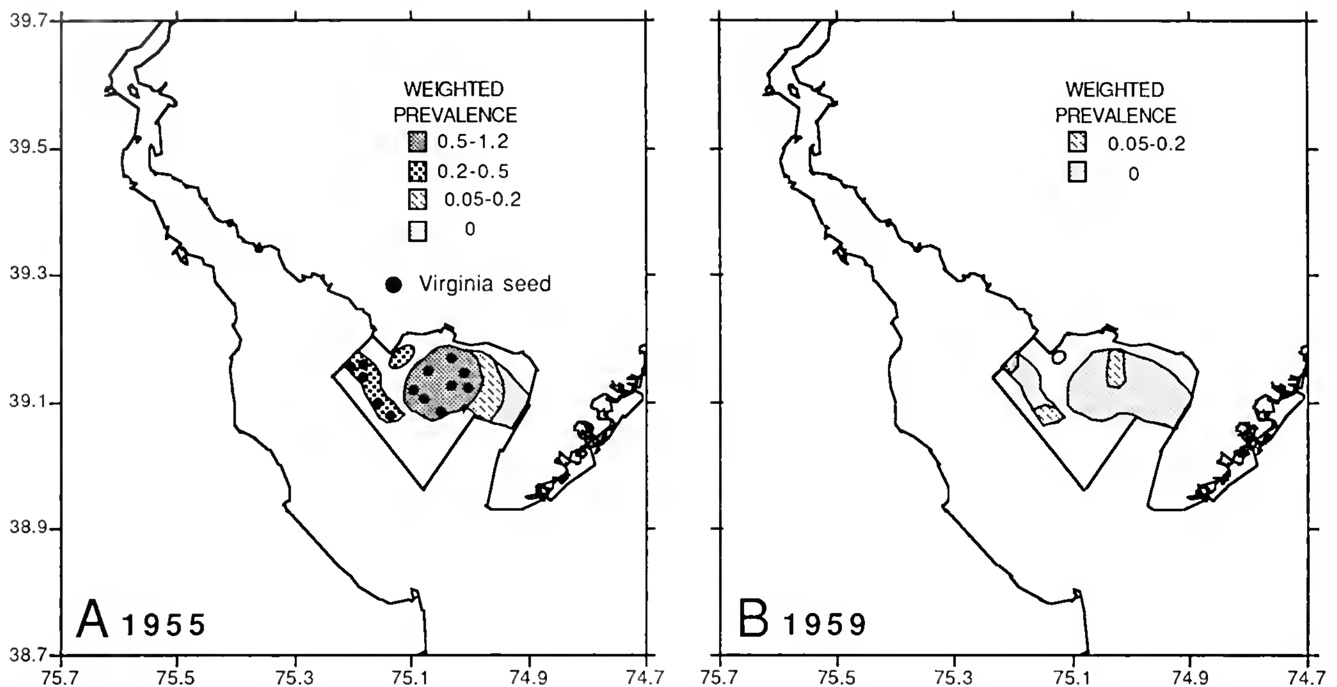

Figure 4. Maps of Delaware Bay showing the distribution of *P. marinus* on the New Jersey side of the bay in 1955 (A), when the parasite first became widespread, and in 1959 (B) after the MSX epizootic. A few infected oysters were found on the lower seed beds. No oysters were collected from the Delaware side of the bay. Data from G. Christensen (1956, unpublished observation).

bay. Intensive sampling failed to show significant presence of *P. marinus* (Fig. 4B; Table 1). Instead, another parasite, *Haplosporidium nelsoni*, was identified as the etiological agent of what is now known as MSX disease (Haskin et al. 1966). In 1959, all imports and exports into and out of Delaware Bay were embargoed. From 1957 through 1963, regular sampling of certain leased grounds showed a diminishing presence of *P. marinus* in Delaware Bay (Table 1). After 1963, routine diagnosis using RFTM was discontinued, although an intensive sampling program for *H. nelsoni*, which was diagnosed in stained tissue sections, was underway (Ford and Haskin 1982). Tissue section histology is much less sensitive a diagnostic method for *P. marinus* than is RFTM incubation, but well-developed infections can be detected.

The presence of *P. marinus* in Delaware Bay during the 1950s was interpreted as an introduction, started and maintained briefly by planting of infected oysters from lower Chesapeake Bay (Ford and Haskin 1982). When the importation stopped, it became evident that the parasite was not able to sustain itself without continued introduction of infected oysters. Andrews (1988) noted that *P. marinus* also disappeared from major planting areas in the lower Chesapeake after the *H. nelsoni*–caused epizootic of 1959–60 killed most of the oysters, greatly reducing the host population for *P. marinus*. The same occurrence in Delaware Bay between 1957 and 1959 undoubtedly contributed to the elimination of *P. marinus*. In contrast to the Chesapeake, however, *P. marinus* never reappeared to cause problems in Delaware Bay, even after the oyster population recovered from the *H. nelsoni* epizootic in the late 1960s and 1970s (Ford 1996).

Perkinsus marinus remained enzootic until 1980 in stocks of oysters undergoing selection for resistance to *H. nelsoni* at the Rutgers Cape Shore Laboratory on the Delaware Bay side of the Cape May Peninsula (Fig. 2) (Haskin and Ford 1979). Experimental oysters, kept in trays on intertidal flats in front of the laboratory, had apparently acquired parasites from native oysters that had been infected by Virginia oysters planted nearby in the 1950s (G. Christensen 1956, unpublished observation) and from southern stocks imported in the early 1960s for the breeding program (W. J. Canzonier, Maurice River Oyster Culture Foundation, pers. comm. 1995). The disease was perpetuated by the high density and relatively high (shallow water, intertidal) temperature regime under which the stocks were held. Maintaining distance between trays, especially those containing different year classes, successfully delayed *P. marinus*–caused mortalities for 2–3 years, although *H. nelsoni* began killing susceptible oysters within a year (Haskin and Ford 1979).

Occasional detection of *P. marinus* in tissue sections examined for *H. nelsoni* indicated that the parasite was never completely eliminated from the rest of Delaware Bay either (Table 1). The most obvious example occurred after several experimental plantings of 2–3-month-old spat from the Cape Shore intertidal location to a leased ground in the Maurice River Cove between 1964 and 1970 (see Fig. 2). The spat apparently had low levels of *P. marinus*, although no patent infections were discovered until 1975 when tissue sections of several oysters with *P. marinus* were found during sampling for *H. nelsoni*. Followup RFTM assays of oysters on this and adjacent leases were positive for *P. marinus* in early October 1975, with maximum prevalence of 55% and weighted prevalence of 1.1. Although *P. marinus* undoubtedly caused a few deaths at this time, mortality rates on grounds with the parasite were no different from those where it was undetected. Sampling outside of the immediate area failed to detect the pathogen and no further evidence was found in succeeding years. Subsequent findings, in the 1980s, were on natural seed beds southeast of Ben Davis Point (Table 1; Fig. 1).

The 1990s Epizootic

In August 1990, atypical mortalities began in MSX disease–resistant stocks at the Rutgers Cape Shore Laboratory (Fig. 2).

Perkinsus marinus was suspected when *H. nelsoni*, the resident pathogen, could not be detected in affected oysters. Dead and dying oysters incubated in RFTM, however, were found to have heavy *P. marinus* infections. At the same time, oysters in a tray suspended in the Maurice River from the dock of the Haskin Shellfish Research Laboratory at Bivalve (Fig. 2) began to die and were also found to be infected with *P. marinus*. Sampling of oysters on the river bottom showed that they, too, were heavily infected and beginning to die. At this point, a survey of all New Jersey oyster-growing areas was initiated to describe the spatial and temporal distribution of *P. marinus* and assess its impact on oysters. Samples were diagnosed for *P. marinus* using the standard RFTM method and for *H. nelsoni* using tissue sections. Predation- and nonpredation-caused mortalities were also estimated.

Sampling in August 1990 showed that high *P. marinus* infection prevalence and intensity (80–100% prevalence; 2.0–3.0 weighted prevalence) extended onto the leased grounds at the mouth of the Maurice River. Farther out into the bay, however, only a few (≤15%) lightly infected oysters were found. During the rest of the summer and into the autumn, infection prevalence and intensity increased in the Maurice River Cove and off Egg Island Point (Fig. 2). A general survey of the New Jersey oyster-growing areas in October 1990 found prevalences of 75–100% in the lower bay and detectable infections as far upbay as Arnolds Point, adjacent to the uppermost productive seed bed (Fig. 5A). Distribution on the seed beds was patchy and most upbay oysters remained free of patent infections. In addition to a center of heavily infected oysters in, and extending from, the Maurice River, a second apparent locus was present on the lower New Jersey seed beds. These were the only two areas of the bay where weighted prevalence exceeded 3.0 (Fig. 5A). No infections were reported on the Delaware side of the bay (J. Tinsman, Delaware Department of Natural Resources, pers. comm. 1990).

In contrast to the 1950s, prevalences in the 1990s rapidly reached 90–100% in the middle and lower reaches of the bay (Fig. 3). Detectable infections were found throughout most of the year, although levels were depressed in late winter and spring. Over the next 4 years (1991–94), *P. marinus* continued to intensify on the New Jersey beds wherever oysters were planted or growing naturally. The parasite spread upbay into the lower salinity regions on the New Jersey side of the estuary, and by the autumn of 1994, prevalences of 100% and weighted prevalences exceeding 3.0 were found as far upbay as Arnolds Point (Fig. 5B).

Oyster mortalities were only 15–20% on the leased grounds and less than 10% on the seed beds in 1990, but they increased markedly the following year. Between 1991 and 1993, estimated annual mortality rates associated with *P. marinus* were 30–50% on the leased grounds and seed beds as far upbay as Ben Davis Point. Between Ben Davis and Arnolds Points, mortalities decreased from 15 to 0%. The pathogen *H. nelsoni* was rare in Delaware Bay between 1989 and 1994; consequently, *P. marinus* was the major nonpredation cause of oyster deaths. In contrast, no mortalities associated with *P. marinus* were recorded on the Delaware side of the estuary through the spring of 1995, although scattered infections were found and may have been responsible for oyster deaths reported in late summer of that year (J. Tinsmann, pers. comm. 1995).

PRESENCE OF *P. MARINUS* IN COASTAL NEW JERSEY BAYS AND RIVERS—1980s

During the early surveys for *P. marinus*, Andrews and Hewatt (1957) failed to detect the pathogen on the seaside of Virginia although it was prevalent in the Chesapeake Bay. Similarly, no parasites were found in samples from coastal New Jersey in the mid-1950s (G. Christensen 1956, unpublished observation). From

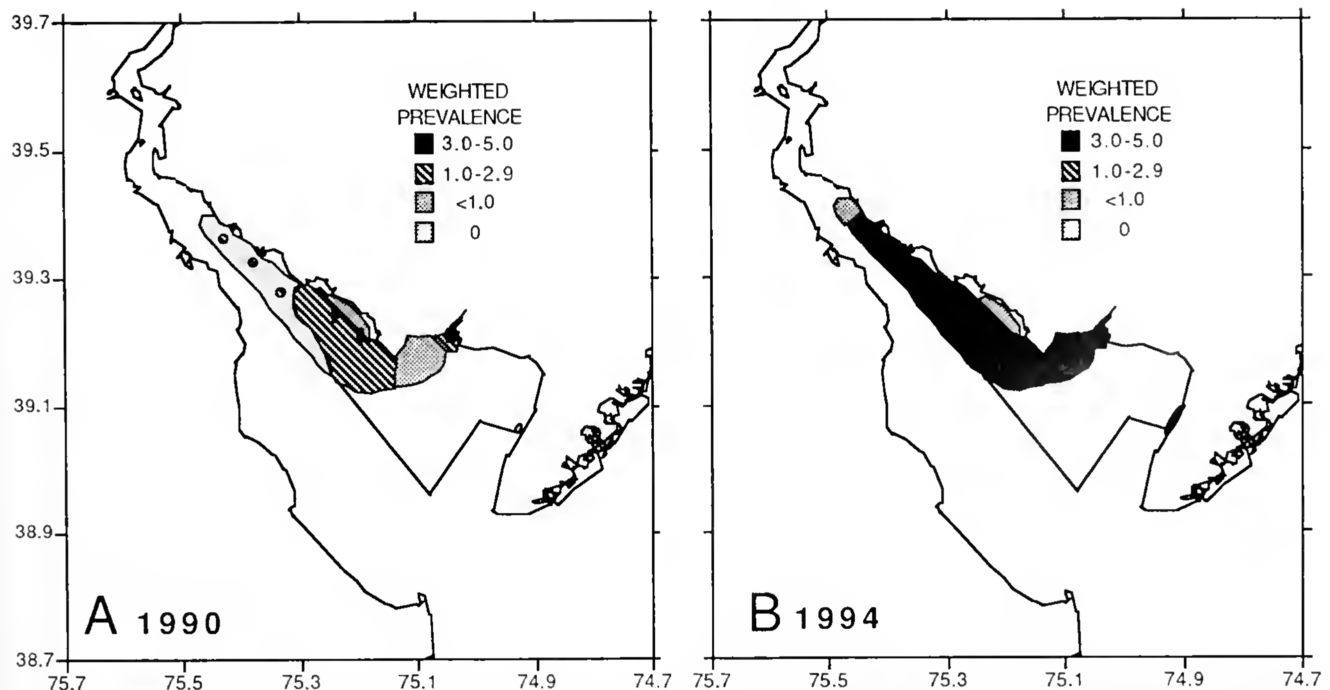

Figure 5. Maps of Delaware Bay showing the distribution of *P. marinus* in 1990 (A), the first year of the current epizootic, and in 1994, when the parasite had spread over most of the New Jersey side of the bay. No oysters were collected from the eastern and southeastern sections of the leased grounds because they were extremely sparse in this region. No infected oysters were detected on the Delaware side of the bay in 1990 and only scattered, light infections were found as late as 1994.

1982 through 1988, however, 10–15% of oysters collected in several southern New Jersey coastal locations and examined by tissue section for *H. nelsoni* were found to have *P. marinus* (Table 1). These were autumn and early winter collections, which maximized the detection of parasites, but given the relative insensitivity of tissue section diagnosis for light *P. marinus* infections, true prevalences were undoubtedly much higher. No unusual mortalities were reported by planters or state biologists collecting the samples; however, *H. nelsoni*–caused mortalities were high at the time and would have masked any caused by *P. marinus*. Regular disease monitoring in coastal New Jersey had not been performed before 1982, but no *P. marinus* was detected in occasional tissue sections collected in earlier years for *H. nelsoni* diagnosis. In the autumn of 1990, when the Delaware Bay epizootic was starting, light infections were found by RFTM assay in 5–25% of oysters in the southern New Jersey coastal bays. By the following year, prevalences ranged from 30 to 85%, although weighted prevalences were generally below 1.0. Since 1992, maximum prevalences have been close to 100%, with weighted prevalences of 2.0–3.5. In the mid-1980s, *P. marinus*–infected oysters were found for the first time on the seaside of Maryland and Virginia (Burreson and Ragone Calvo 1996).

RANGE EXTENSION OF *P. MARINUS* FROM NEW YORK TO MAINE—1991 TO 1995

As the *P. marinus* epizootic spread in Delaware Bay in 1991, oysters from more northern locations were examined (Fig. 1). Samples from the Manasquan River and Raritan Bay in northern New Jersey, collected in October 1991, had light *P. marinus* infections in 30–50% of the oysters. Two 1991 samples from Long Island Sound were negative, but in early May 1992, very light infections were detected in 65–70% of oysters from Oyster Bay, on the north shore of Long Island, NY, and from the Connecticut shore of Long Island Sound. Known infection patterns (Andrews 1988, Burreson and Ragone Calvo 1996) indicate that the pathogen must have been present in a substantial number of oysters the year before to have caused such high, early-season prevalence in 1992. In fact, highly infected oysters had been found in 1991 in several mainland Massachusetts locations (E. J. Lewis, Cooperative Oxford Laboratory, pers. comm. 1992). By late summer 1995, prevalences of 75–100% and weighted prevalences of 2.5 or more were common in many Long Island Sound locations as well as in some southern Massachusetts locations (e.g., Wareham River on eastern Buzzard's Bay and Cotuit Harbor on southern Cape Cod). At the same time in Wellfleet Harbor, on the northeast shore of Cape Cod, maximum prevalence reached 100%, but intensities were relatively low (weighted prevalence $\leq$1.5). In 1994, the parasite was detected in ponds on the island of Martha's Vineyard, south of Cape Cod, MA, and by summer 1995, prevalence had reached 85% with a weighted prevalence of 1.8 at one location in Edgartown Great Pond. In 1995, very light infections were detected in oysters from the Damariscotta River, ME, shipped to the Haskin Shellfish Research Laboratory for research purposes. A similar finding was recorded at the Virginia Institute of Marine Science (E. Burreson, Virginia Institute of Marine Science, pers. comm. 1995). At both institutions, the total body burden assay (Bushek et al. 1994) was being used to diagnose infections and the maximum number of parasites detected in any oyster was extremely small ($\leq$11). Heavy mortalities, comparable to those in Delaware Bay, have not been reported by growers in Long Island Sound and New England. In some locations, no effect at all has been noted. Nevertheless, the high prevalence and intensity levels currently found indicate a potential for epizootic mortalities.

In contrast to the early surveys for *P. marinus* in the southern United States, which detected the parasite immediately and suggested that its presence in the region was not new, the more recent findings from New Jersey to Maine (1990–95) represent a rapidly progressing change in its observed distribution, abundance, or both. A number of surveys, as well as occasional sampling by several laboratories, had never found conclusive evidence of the pathogen north of New Jersey before 1991 (Newman 1971, Meyers 1981, Cooper and Durfee 1982, E. J. Lewis, Cooperative Oxford Laboratory, pers. comm. 1992, Ford, unpublished). Oysters from Martha's Vineyard were sampled for 3 years before *P. marinus* was first found in 1994. Frequent and intensive diagnosis of Maine oysters, which were being used as uninfected controls in experimental studies of *P. marinus* by several laboratories, had been underway at least since 1991, although they typically used hemolymph or standard RFTM assays rather than the more sensitive body burden method (Bushek 1994, E. Burreson, Virginia Institute of Marine Science, pers. comm. 1995).

HYPOTHESES FOR THE RECENT RANGE EXTENSION BY *P. MARINUS*

Why was detectable *P. marinus* activity confined to the southern United States for so many years only to appear over a new range of more than 500 km within a year's span? Several hypotheses are offered:

1. The parasite was transmitted via infected oysters introduced from enzootic southern areas into "disease-free" northern waters.
2. A change in the genetic structure of either host or parasite increased the parasite's ability to invade and proliferate in a new environment.
3. The environment in the northeastern United States became more favorable for parasite activity.
4. Some combination of the above.

Evidence for and against each of these hypotheses is evaluated, and a conclusion reached, in the following sections.

1) POSSIBLE INTRODUCTION OF *P. MARINUS* VIA INFECTED OYSTERS

Perkinsus marinus can readily be transmitted from infected to uninfected oysters (Ray 1954), making it easy to conclude that it was spread through the transplantation of parasitized oysters. In fact, there is considerable evidence that this has happened in the past. For instance, as detailed earlier, the presence of *P. marinus* in Delaware Bay during the 1950s was closely associated with the planting of infected seed oysters, and its "disappearance" with cessation of this practice. A localized outbreak on the New Jersey leased grounds in 1975 was linked to the planting of seed from intertidal flats in the lower Delaware Bay, where the parasite remained locally enzootic. The spread of *P. marinus* upestuary in Chesapeake Bay is thought to have been enhanced by the movement of infected seed oysters into previously "disease-free" growout areas (Burreson and Ragone Calvo 1996).

Evidence From the 1990 Delaware Bay Epizootic

There is no similar evidence that the 1990 epizootic in Delaware Bay was associated with planting of infected oysters. The

outbreak appeared to have three loci: the Maurice River, the New Jersey seed beds between Egg Island and Ben Davis Points, and experimental stocks at the Rutgers Cape Shore Laboratory (Figs. 2 and 5A). The almost simultaneous outbreaks at the three sites argue against a single, recent locus of infection, especially for the Cape Shore and Maurice River sites, which are separated by a distance of 20 km largely devoid of oysters since the MSX disease epizootic in the late 1950s (Fig. 5).

Maurice River

The town of Bivalve on the lower Maurice River is home to the New Jersey oyster industry, including a number of shucking houses whose effluent is discharged into the river. Beginning in the early 1960s, as the supply of native oysters fell after the onset of MSX disease, oysters were imported from the Gulf of Mexico for shucking. The need for imported oysters declined during the 1970s and early 1980s as Delaware Bay harvests improved, but it resumed in the 1980s when MSX disease again depressed landings (Ford 1996). According to local planters, oysters too small to be shucked may have been planted on Delaware Bay leased grounds, although this was prohibited and cannot be verified. What is certain, however, is that oysters from regions where *P. marinus* was epizootic, including Chesapeake Bay and the Gulf of Mexico, were being shucked along the Maurice River during the mid-1980s. Given the ease with which the parasite is transmitted, it is possible that native oysters in the river became infected, over a number of years, by *P. marinus* discharged in shucking house effluent. The Haskin Shellfish Research Laboratory is also sited on the river at Bivalve. Limited numbers of oysters from the James River, VA (see below), were occasionally held here in a recirculating system that released small volumes of water into the river. The Maurice River has a dense population of oysters, which would have fostered the establishment and spread of the parasite. It is also a seed area from which small quantities of oysters have been transplanted onto leased grounds.

Lower Seed Beds

The apparent centers of high *P. marinus* activity on the lower seed beds in the autumn of 1990 were more puzzling because they occurred on natural beds where oysters were not planted. This pattern may have been nothing more than a manifestation of the well-known patchy nature of *P. marinus* infections (Andrews 1988). Yet, the occasional *P. marinus* findings in Delaware Bay in the 1980s had been in this region (Table 1). Shells from oysters shucked in Bivalve were planted on several lower seed beds from 1982 to 1984, when southern oysters were being processed (J. Dobarro, New Jersey Bureau of Shellfisheries, pers. comm. 1992). Transmission of *P. marinus* from shells discarded by shucking houses (including bits of adhering meat, dead or sick-looking oysters, and live oysters too small to be shucked) must be considered, even though it has never been documented and would certainly depend on the time between shucking and planting (Ford 1992). In 1981, a new section of leased grounds that extended upbay between the lower seed beds (Fig. 2) was opened; however, there is no documentation that any oysters other than native seed from farther upbay were ever planted in this region.

Cape Shore Laboratory

Perkinsus marinus was diagnosed (using the RFTM assay) and continued to kill old oysters in experimental stocks at the Rutgers Cape Shore Laboratory until 1980, after which it was no longer detected. Nevertheless, it is possible that small numbers of parasites remained in subpatent infections and provided a source for the 1990 outbreak at this location. Alternatively, oysters from the upper James River may have reintroduced the parasite to the Cape Shore site. Representatives of this stock, free of detectable *H. nelsoni* and *P. marinus* infections, had been brought to the Laboratory every year since 1959 as susceptible controls in a long-term project to develop oyster strains resistant to MSX disease (Haskin and Andrews 1988). The spread of *P. marinus* up the James River had begun by the late 1980s (Burreson and Ragone Calvo 1996) and it is possible that these oysters contained undetected infections. The highest disease levels found at the start of the outbreak in the late summer of 1990, however, were in stocks that had been resident on the Cape Shore tidal flats for 3 years or more and that were isolated by distance from new imports. Whatever its ultimate origin, the immediate source for the 1990 Cape Shore outbreak must have been these old stocks. Nearby native oysters and younger experimental stocks were free of patent infections or only lightly infected.

Evidence from North of Delaware Bay

Documented evidence of recent oyster transplants into waters north of Delaware Bay from more southern waters is scarce. Seed oysters from Delaware Bay, including the Cape Shore, were occasionally moved into coastal bays of southern New Jersey for growout, but some locations, such as the Manasquan River and Raritan Bay, in northern New Jersey do not have commercial shellfisheries. Small (several hundred animals) groups of hatchery-reared spat produced at the Rutgers Cape Shore Laboratory in 1987, 1988, and 1989 were deployed in Cotuit and Wellfleet Harbors, MA, from 1988 to 1990. Around the same time, the industry moved oysters from the Long Island Sound region into Massachusetts for growout, a relatively common practice. Because *P. marinus* was not known to be present in either Delaware Bay or Long Island Sound at the time, testing for these parasites was not included in the "certification" required for these transplants. Nevertheless, *P. marinus* may well have been present at low levels in both locations.

These few known cases cannot, however, explain the almost simultaneous appearance of *P. marinus* in many locations over a range of more than 500 km (Fig. 1). On the other hand, for a century or more, oysters have been transplanted from southern regions, where *P. marinus* has existed for decades at least, to the north as supplements or replacements for depleted seed stocks (Ingersoll 1881, Kochiss 1974, Ford 1996). Other mechanisms for introduction are also not new: transit of boats with infected oysters growing on the hull; overboard discards from processors, dealers, restaurants, and consumers; planting of shells; movements of possible nonoyster vectors; and water currents). For instance, the ponds on Martha's Vineyard are enclosed saltwater ponds, separated by sand beaches from the Atlantic Ocean. No outside oysters have been planted in the ponds, but the sand barriers are mechanically breached about three times each year to release accumulated freshwater (P. Bagnall, Edgartown Shellfish Office, Edgartown, MA, pers. comm. 1995). The cuts stay open allowing water exchange with the Atlantic Ocean for 2 to 6 weeks before they are healed. Given the evidence of the long-term presence of *P. marinus* in southern waters, introduction of the parasite into the northeast through various transport mechanisms may have occurred for decades. Why, then, was the parasite not detected until recently?

2) A CHANGE IN THE GENETIC STRUCTURE OF EITHER HOST OR PARASITE INCREASED THE PARASITE'S ABILITY TO INVADE AND PROLIFERATE IN A NEW ENVIRONMENT

Race-specific host-parasite interactions in Dermo disease were investigated in a recent study by Bushek (1994). He isolated and cultured parasites infecting oysters at four locations from Texas to New Jersey and used these four isolates to infect offspring of oysters from four geographic locations and with different histories of exposure to *P. marinus*. The offspring were the same age and had been reared in a common environment to eliminate nongenetic responses. Parasites from New Jersey and Virginia, the two northernmost locations tested, caused significantly heavier infections than did those from Louisiana and Texas, and oysters from Maine and New Jersey developed significantly heavier infections than those from Virginia or Texas (Bushek and Allen 1996). There was no statistical interaction between host populations and parasite isolate. This suggested that parasites from the mid-Atlantic might be more virulent than those from the Gulf of Mexico. It also indicated that oysters from sites in the northeast where *P. marinus* has not been historically prevalent are more susceptible than those from southern locations where it has been active. Given this evidence, it is not necessary to postulate a change in susceptibility of northern oysters to account for a change in the distribution of *P. marinus*. Oysters in the northeast are, and always have been, highly susceptible to *P. marinus* (Ray 1954).

A change in the genetic structure of *P. marinus* populations that increased virulence or cold tolerance could alter the parasite's range of activity. It has always been assumed that the failure of *P. marinus* to spread into the northeastern United States was because it could not proliferate under the low temperatures typical of the region (G. Christensen 1956, unpublished observation, Andrews and Hewatt 1957, Andrews 1988). To date, there is no information on possible genetic changes in temperature tolerance of the parasite; however, mechanisms by which this might have occurred can be considered. Heightened virulence by itself (e.g., increased production of virulence factors under conditions known to favor the parasite) may be incidental to this deliberation because it would not necessarily lead to a changed geographical distribution unless the parasite could also function in the new environment. If a change in the ability of *P. marinus* to operate in a low-temperature regime has stimulated its range extension, there are at least two possible ways it could have occurred.

In the first scenario, a random mutation occurring before 1990 in a localized parasite population, say in lower Chesapeake Bay, produced a more cold-tolerant strain of the pathogen. This new strain would then have spread northward through the various means cited earlier. The potential transport mechanisms need not have accelerated to obtain the range extension, but the chances for parasite survival and proliferation in the new environment would now be much greater. In fact, it would be unlikely that both a random genetic change and more rapid means of transit would have occurred at the same time. Consequently, this scenario implies that the rate of parasite movement northward has always been regular and fast enough to produce the range extension seen since 1990.

The second scenario uses the argument that northward movement of non-cold-adapted *P. marinus* has occurred over many years. A small fraction of the introduced parasites survived but were unable to proliferate sufficiently to cause an epizootic or even to be detected by standard diagnostic methods. In response to selective pressure (temperature), these small parasite populations in a number of different locations then simultaneously became more capable of functioning at reduced temperatures. This scenario does not explain why the adaptation would have occurred during 1990–91 and not earlier.

3) THE ENVIRONMENT BECAME MORE FAVORABLE FOR THE PARASITE

Salinity and temperature are the major factors controlling the rate of growth and death of *P. marinus* in oysters (Burreson and Ragone Calvo 1993, Chu and La Peyre 1993, Chu et al. 1993, Burreson et al. 1994, Hofmann et al. 1995) and the principal correlates with the parasite's spatial and temporal distribution in nature (Andrews 1988, Soniat and Gauthier 1989, Powell et al. 1992, Burreson and Ragone Calvo 1996). A change in salinity, temperature, or both would be expected to result in a change in the parasite's distribution, abundance, or both.

Salinity

In the Chesapeake Bay, Burreson and Ragone Calvo (1996) associated the upestuary spread of the parasite between 1985 and 1988 with consecutive drought years and warm winters as well as with transplants of infected oysters from high-salinity areas in the estuary (which had been ongoing for many years). The period since 1990 has likewise been unusually dry in the mid-Atlantic. For instance, in 27 of 32 months between March 1991 and October 1993, the Delaware River, which provides about 60% of the freshwater inflow into Delaware Bay, had below average river flow (Bauersfeld et al. 1991, 1992, 1993). Resulting high salinities undoubtedly accelerated and extended upbay penetration of the parasite in Delaware Bay (see Fig. 5). Low salinity thus seems to be the primary *local* controlling influence on *P. marinus* (Burreson and Ragone Calvo 1996). Yet, many of the locations in the northeast where *P. marinus* has recently appeared are high-salinity sites (typically greater than 25 ppt) where the parasite would never be salt limited, even in periods of normal rainfall. Droughts, while a contributing factor, are probably not a major determinant in the *P. marinus* range extension.

Temperature

Temperature has a profound effect on the rate at which *P. marinus* proliferates in oysters. Parasite multiplication rates increase rapidly above 20°C and are greatest between 25 and 30°C (Andrews 1988). Associated mortalities are also highest above 25°C and slow markedly when the temperature falls below 18–20°C. In his 1988 review, Andrews (1988) pointed out that in the Virginia portion of Chesapeake Bay, water temperatures above 25°C exist for nearly 3 months each year, and for considerably longer in regions to the south. In Delaware Bay, temperatures consistently above 25°C exist, on average, for just a little more than 1 month. The difference would markedly affect the time during which the parasite could proliferate and spread. In discussing the failure of *P. marinus* to cause epizootic mortalities in Delaware Bay during the 1950s, Christensen (1956, unpublished observation), pointed out that infection levels did not begin to increase until August or September (Fig. 2A) and that water temperatures fell below 20°C in late September. Infection development was slowed before significant mortalities occurred. Further spread of the parasite was limited by the lack of infective stages dispersed by dying oysters and most parasites remaining in oysters

died over the winter (Andrews 1988, Ragone Calvo and Burreson 1994, Burreson and Ragone Calvo 1996).

Cyclic changes in sea temperature have been associated with fluctuations in the abundance of many marine species (Southward et al. 1975, Cushing and Dickson 1976, Southward et al. 1988), including range extension by some (Sauriau 1991). There is little information on changes in parasite distribution, but large-scale climate fluctuations, such as El Niño cycles that influence both temperature and rainfall, have been associated with changes in *P. marinus* activity in the Gulf of Mexico (Powell et al. 1992). Consequently, it is logical to examine possible correlation between temperature changes over the past several years and the range extension by *P. marinus*.

Long-term water temperature records, collected in a regular and consistent fashion over a wide geographic range, are rarely available. However, local air temperatures are a frequent substitute as they are collected more regularly (Jeffries and Johnson 1976, Sauriau 1991). Further, they are correlated with temperatures in nearby water bodies (Jeffries and Johnson 1976; Southward et al. 1988; Ford, unpublished). In particular, air temperatures should reflect long-term trends in adjacent nearshore water temperatures. To examine such trends, air temperature records from 1980 to 1994 were obtained from three locations along the northeastern United States coast where *P. marinus* has recently become active: 1) Millville, NJ, about 12 miles north of the Delaware Bay, NJ, leased grounds; 2) Bridgeport, CT, adjacent to major Long Island Sound oyster grounds; and 3) Hyannis, MA, representing Cape Cod (Fig. 1).

For each location, a temperature deviation for each month from 1980 through 1994 was calculated using the long-term mean of the corresponding month for the preceding 30 years (1951–80) as a baseline. To help elucidate trends, the cumulative deviation for the 1980–94 period was plotted (Fig. 6). In cumulative deviation plots, a negative slope indicates a period during which a string of months had below average temperatures; a positive slope indicates a period during which consecutive months had above average temperatures; and a line without a clear slope represents a period of near average temperatures. Plots for all three sites show a similar pattern: a warming trend from 1983 through 1986, average or somewhat below average temperatures during the rest of the decade, then a period starting in January 1990 during which the monthly temperature was well above average nearly every month for almost 2 years. The intensity of this warming is shown by the extreme steepness of the slope between January 1990 and February 1992 in plots for all three sites (Fig. 6). It is significant that the observed range extension of *P. marinus* occurred during this period.

The cumulative monthly deviation plot is an effective way of visually integrating time and temperature over a long period, but it presents difficulties in detecting potential seasonal patterns. Until recently, it was never clear whether summer or winter temperature was the more important in controlling the distribution of *P. marinus*. In their analysis of Chesapeake Bay data over the last decade, however, Burreson and Ragone Calvo (1996) associated the upbay spread of the pathogen with warm winters. To examine long-term seasonal temperature trends for the Delaware Bay region, mean air temperatures at Millville, NJ, were calculated for 3-month periods (January–March, April–June, July–September, and October–November) during each year from 1949 to 1994. The only season showing a clear and consistent association with the observed presence of *P. marinus* in the bay over the past 40 years was winter (January–March; Fig. 7A). Just after the large-scale importation of infected oysters into the bay began in the 1950s, a prolonged series of winters with below average temperatures also began. A shorter series of above average temperature winters in the early and mid-1970s culminated in the localized *P. marinus* outbreak in 1975 described earlier. The series of cold winters beginning in 1977 was associated with the disappearance of detectable *P. marinus* infections in trays of experimental oysters at the Rutgers Cape Shore Laboratory. The occasional findings in Delaware Bay in the mid-1980s were at a time when winter temperatures were either some-

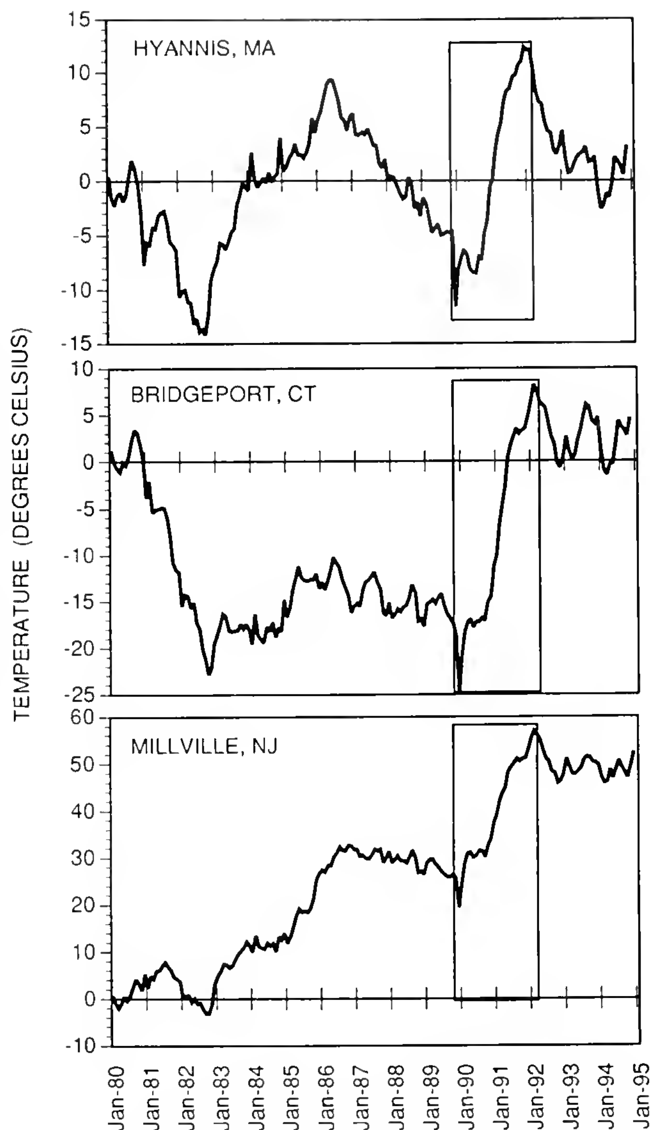

Figure 6. Cumulative monthly air temperature deviations at three stations in the northeastern United States adjacent to areas where *P. marinus* has become epizootic since 1990 (see Fig. 1). Plots were obtained by summing the difference (positive or negative) between monthly air temperatures and the long-term mean (1951–80) for that month. A negative slope indicates a period during which a string of months had below average temperatures; a positive slope indicates a period during which a string of months had above average temperatures; and a line without a clear slope represents a period of near average temperatures. The steep positive slope outlined in each plot represents a 2-year extreme warming period common to all three stations.

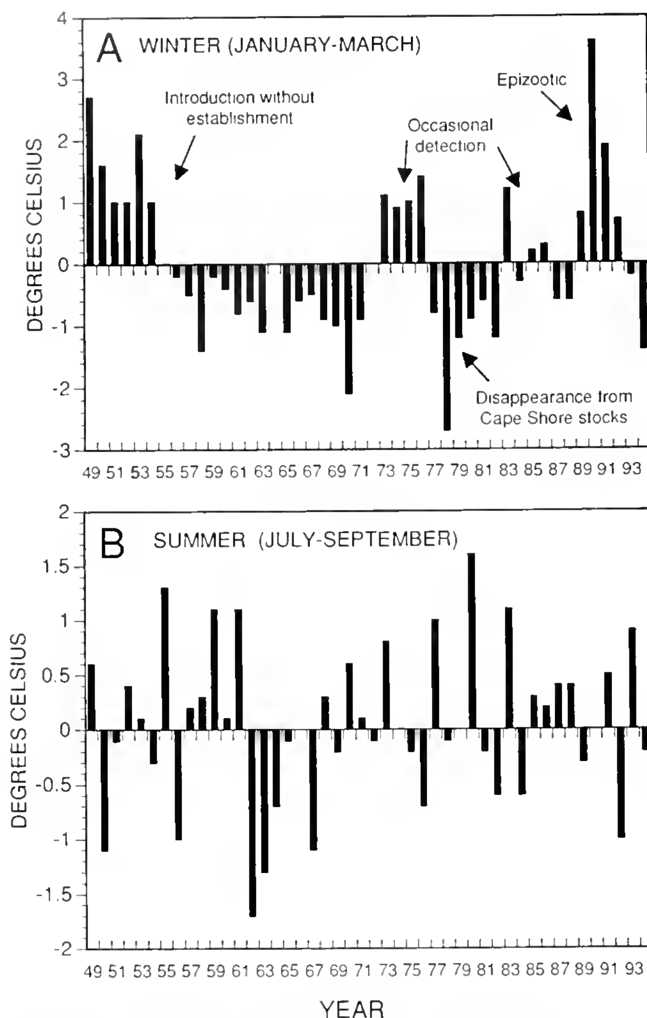

Figure 7. Seasonal air temperature deviations at Millville, NJ, from 1949 through 1994. Deviations are the difference between mean temperatures for each 3-month period for each year of record and the long-term mean for that period.

what above, or only slightly below, average and when there was also a string of above average summer temperatures (Fig. 7B). Finally, the current epizootic is correlated with four above average temperature winters beginning in 1989. No such pattern emerged from plots of any other season, including July–September when *P. marinus* is most active in Delaware Bay (Fig. 7B).

Warm winters would act by lessening overwinter parasite mortality (Bushek et al. 1994, Ragone Calvo and Burreson 1994) and accelerating the development of advanced, lethal infections in the late spring. Release of infective particles from heavily infected and dying oysters early in the season would then lead to a new round of infections resulting in multiple infection cycles in a single summer (Andrews 1988).

SUMMARY AND CONCLUSIONS

The 1990–91 observed range extension by *P. marinus* into the northeastern United States can be linked empirically to historical, rather than new, introductions of potentially infected oysters and a recent warming trend that has made the environment suitable for parasite development. The possibility that *P. marinus* has become more cold adapted has not been tested and cannot be excluded as an additional factor. Some selection for cold tolerance may have been occurring gradually, particularly during brief periods of above average temperature when heightened parasite multiplication rates would have increased the parasite population on which selection could act.

The 1950s Delaware Bay episode clearly demonstrates that the introduction of a disease agent—even in large quantity—is not, *by itself*, sufficient to cause a damaging outbreak of that disease or the establishment of a significant population of the agent. To become well established and then to trigger an epizootic in the new location, the introduced pathogen requires that certain conditions be favorable and that they last long enough for the population to attain a threshold level. Yet, even if these conditions are not met, small numbers of parasites may be able to survive in a generally unfavorable environment. Failure to detect a pathogen is not sufficient evidence that the organism is absent because population abundance may be below a "threshold of perception" (Fig. 8). The threshold is reached by the appearance of dead and dying hosts if "perception" is defined as morbidity and mortality. For example, had there not been a specific monitoring program for *P. marinus* in Delaware Bay, its presence would not have become evident until 1990, when mortalities began. If diagnostic methods that detect nonlethal infections are in routine use, the perception threshold is much lower and detected infections may not signal an imminent epizootic. Even these, however, may be too insensitive to detect very low pathogen densities. It is relevant to the following argument that the standard Ray/Mackin assay does not reliably detect very light *P. marinus* infections. A positive diagnosis typically requires total-body parasite densities greater than 10^3 g^{-1} wet weight (Choi et al. 1989, Bushek et al. 1994).

The *simplest* explanation consistent with the available data for the recent observed range extension by *P. marinus* is that the pathogen was repeatedly introduced, by many means over many years, into various northeast locations where it persisted at undetectable levels and was stimulated to proliferate into an epizootic by the recent warming trend. Increased parasite abundance in the new locations has then augmented its spread within the new range.

If this hypothesis is correct, will a return to a more typical temperature regime, especially cold winters, push the parasite from its new range? In assessing this potential, it must be recalled that *P. marinus* has not only been detected in a new geographic range, but both prevalence and infection intensity are high in many areas. Because oysters are abundant in these areas (e.g., Delaware Bay seed beds, Oyster Bay, Long Island Sound, and Cotuit Har-

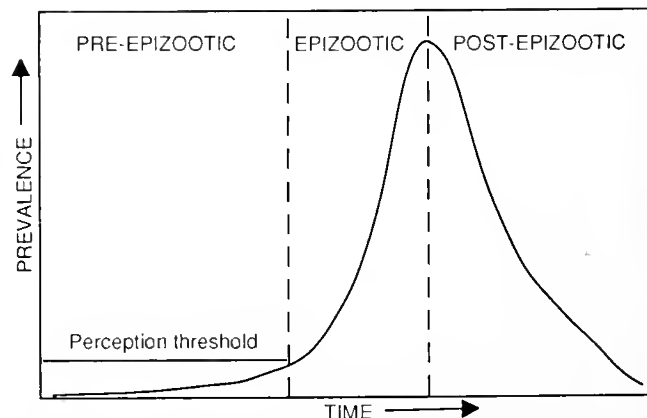

Figure 8. Schematic showing phases of an epizootic, including the pre-epizootic period when the existence of a pathogen is unknown. Adapted from Brown (1987).

bor), the parasite population is also very high. Even if adverse conditions are severe enough to destroy all but a very small fraction of the parasites, those surviving can be numerically abundant enough to rekindle an epizootic when conditions become more favorable. A recently developed mathematical population model of oyster–*P. marinus* interactions predicts that conditions, including low temperature, needed to terminate an epizootic are more extreme than those required to trigger one (Powell et al. 1996). Similarly, Burreson and Ragone Calvo (1996) concluded that a single unusually warm winter in Chesapeake Bay had a greater impact on the proliferation and spread of *P. marinus* than a cold winter did in its elimination. In fact, the cold winter of 1993–94 failed to end the epizootic in the northeast and winter temperatures in 1994–95 were again above average. Stopping or materially slowing the epizootic will require not one but a series of consecutive cold (i.e., average or below average temperatures) winters and cool springs that will delay the summer infection buildup until late in the season. A restricted period during which lethal infections can develop, kill oysters, and further disseminate infective particles appears to have helped end the 1950s epizootic in Delaware Bay. In estuaries where salinity is potentially limiting, a number of consecutive years of above average rainfall at the same time would further help, as there appears to be a synergistic effect of low salinity and low temperature that is harmful to parasites (Burreson and Ragone Calvo 1993, Burreson et al. 1994, Chu et al. 1994, Ragone Calvo and Burreson 1994). In addition, substantial freshwater inflow into any water body should diminish the abundance of infective particles by flushing (Mackin 1956).

The hypothesis that the recent warming trend in the northeastern United States, particularly the above average winter temperatures, has fostered the range extension of *P. marinus* needs to be tested further by examining trends in water temperatures and determining how closely actual seasonal temperature cycles (as opposed to long-term trends) now associated with *P. marinus* in the northeast approximate those in more southern locations that have supported the parasite for decades. In addition, continued efforts must be made to determine whether the parasite has become, or is becoming, adapted to lower temperatures. The development of lower temperature–adapted parasites might be more feasible now that large populations are established in a region where selection pressure exists for this trait. That *P. marinus* can adapt to low salinity has been demonstrated *in vitro* using cultured parasites (O'Farrell et al. 1995). Although it has not been demonstrated that a genetic change was involved, such adaptation could theoretically occur in nature and in response to low temperature as well as low salinity.

Obtaining a better understanding of the causes for the range extension has both fundamental and practical implications. Researchers have long advised against the transportation of oysters from areas where a contagious disease agent is enzootic into "disease-free" water (Rosenfield and Kern 1979, Ford 1992). The history of *P. marinus* demonstrates how risky it is to consider either areas or hosts as free of a pathogen merely because it has no obvious effect on the host or it cannot be otherwise detected. How restrictive should regulations on transportation of molluscs by commercial growers be if uncontrollable factors such as climate change are demonstrated to play a significant role in the spread of disease? This is an especially relevant question because the many other potential means of introduction occur despite such regulations. Now that *P. marinus* is found in New England, should restrictions be relaxed on introductions of southern oysters or other molluscs that might be carrying the parasite? What potential does transplantation between regions known to be enzootic for a pathogen have to exacerbate or prolong a disease problem (see Bushek and Allen 1996)? The range extension by *P. marinus* should be a stimulus for a new discussion of reasonable and rational controls on the movements of oysters in commercial culture.

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LABORATORY INVESTIGATIONS OF SUSCEPTIBILITY, INFECTIVITY, AND TRANSMISSION OF *PERKINSUS MARINUS* IN OYSTERS

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ABSTRACT The protozoan parasite, *Perkinsus marinus* (Dermo), has caused significant mortality in the eastern oyster, *Crassostrea virginica,* along the east coast of the United States and the Gulf of Mexico, since the 1950s. Because of its current expanded distribution and increased abundance, *P. marinus* is now considered more prevalent in the mid-Atlantic waters and the Chesapeake Bay in particular, than another protozoan pathogen, *Haplosporidium nelsoni* (MSX). The susceptibility, infectivity/pathogenicity, and transmission of *P. marinus* in eastern oysters were investigated in numerous laboratory studies. The influence of environmental factors such as temperature, salinity, and pollution on the interaction between the host oyster and the parasite were also examined. Three *P. marinus* life stages, the meront, prezoosporangia, and biflagellated zoospore, were found effective in transmitting the disease. The meront stage was more effective than the prezoosporangia stage in transmitting the disease in eastern oysters, suggesting that the meront is the primary transmission agent in nature. A dose of 10-10^2 freshly isolated *P. marinus* cells oyster^{-1} was required to cause infection by direct shell cavity injection. *P. marinus* susceptibility and disease progression were positively correlated with temperature, salinity, and number of infective cells the oyster encountered. Temperature appeared to be the most important factor, followed by the infective cell dose, and then salinity in determining the subsequent disease development in oysters. There was no significant interaction between temperature, salinity, and infective cell dose on the prevalence of disease in oysters. However, the interaction between either temperature and salinity or between temperature and *P. marinus* dose significantly intensified the disease. The Pacific oyster, *Crassostrea gigas,* was less susceptible, but not completely resistant, to *P. marinus* compared to the eastern oyster, *C. virginica.* However, the Pacific oyster was intolerant of high temperature (>15°C) and low salinity (<10 ppt), thus vulnerable to high mortality under high temperature and low salinity environmental conditions. Pollution has the potential to enhance *P. marinus* susceptibility and infection in oysters.

KEY WORDS: *Perkinsus marinus,* eastern oyster, *Crassostrea virginica,* disease, susceptibility, infectivity, transmission

INTRODUCTION

For the last 40 years, the protozoan parasite *Perkinsus marinus* (Dermo) has continuously caused severe mortality in the eastern oyster, *Crassostrea virginica,* from the Delaware Bay throughout the mid-Atlantic and Gulf coasts in the United States (Andrews 1988, Andrews and Ray 1988). To verify field observations and to achieve a better understanding of disease processes and transmission dynamics in nature, numerous studies have investigated the susceptibility, infectivity, and transmission of this parasite since its discovery in oysters of Louisiana coastal waters (Mackin et al. 1950). The influence of environmental factors on the host-parasite interaction has also drawn much attention. However, extensive laboratory studies on these subjects did not occur until the mid-1980s. This chapter reviews and discusses laboratory studies on susceptibility, infectivity, disease processes, and transmission of *P. marinus* in eastern oysters.

TRANSMISSION OF *P. MARINUS* IN OYSTERS

Although the life cycle of *P. marinus* is still not completely known, three life stages, meront, prezoosporangia, and biflagellated zoospores, have been identified and described (Perkins 1966, Perkins 1988). Immature meronts (merozoites) usually found in the phagosomes of hemocytes are 2–4 μm and coccoid. Meronts (10–20 μm) are mature merozoites with an eccentric vacuole that often contains a refringent vacuoplast. The mature meront, an 8 to 32 cell stage enclosed within a mother cell wall, is a sporangium (schizont, 10–40 μm). When meronts are placed in fluid thioglycollate medium (FTM) for 4–5 days, they develop into prezoosporangia (hypnospores), which are sometimes observed in moribund and dead oyster tissues and can enlarge to 150 μm. Prezoosporangia are characterized by having a large vacuole and an eccentric nucleus adjacent to the cell wall. After incubating thioglycollate-cultured prezoosporangia in sea water for 4–5 days, zoosporulation (production of biflagellated zoospores) usually occurs. However, it is unclear whether prezoosporangia released in sea water from moribund and deceased oysters would zoosporulate in nature.

Laboratory study of *P. marinus* disease and transmission processes in oysters began in the early 1950s when the thioglycollate tissue assay became available (Ray 1952). The infectivity and pathogenicity of different life stages, the required dosages for infection, and routes of transmission were of great interest.

Infectivity and Pathogenicity of Different Life Stages

The disease caused by *P. marinus* is infectious and can be transmitted from infected to uninfected oysters. Placing uninfected oysters in the same container with infected oysters, the uninfected oysters ultimately become infected (Ray and Mackin 1954). All three identified life stages, meront, prezoosporangia and biflagellate zoospore, can cause *P. marinus* infection in oysters. However, it is not known which life stage is most effective and the principal stage for transmitting disease in the field. The infectivity and pathogenicity of the two life stages, meront and prezoosporangia, were compared in our laboratory (Volety and Chu 1994). We inoculated 10^4 meronts or prezoosporangia into the shell cavity of individual oysters, at 21–25°C and 14–20 ppt, that were collected from an area outside the normal geographic range of *P. marinus* (Damariscotta River, ME) and measured infection prevalence and intensity in these oysters over a range of time intervals.

Infections developed within 40 days in oysters inoculated with either meronts or prezoosporangia. Infection prevalence (Fig. 1A) and intensity (Fig. 2A) increased with time in both groups. However, prevalence of infection was significantly higher in oysters inoculated with meronts than with prezoosporangia. At 75 days postchallenge, intensities of infections ranged from light to moderate in oysters exposed to meronts, whereas only light infections were noted in oysters exposed to prezoosporangia. A similar trend was demonstrated in oysters collected from the Ross Rock area of the Rappahannock River, VA, an area that typically has low prevalence of *P. marinus* infection (Fig. 1B, Fig. 2B), with the exception that infection first appeared in oysters 15 days after exposure to prezoosporangia. However, this may represent an infection acquired in the field since the organisms for this experiment were collected from a location affected by *P. marinus*. Infection was not detected in the Maine oysters sampled at 20 days after exposure to prezoosporangia or meronts.

It is uncertain whether the different infection rates and intensities (Figs. 1 and 2) in oysters between these two experiments were caused by batch variation in infectivity of meronts and prezoosporangia or were due to difference in response to *P. marinus* between the two oyster populations. Previous studies demonstrated that *P. marinus* infection rates varied among oyster strains or populations (Burreson 1991, Chu and La Peyre 1993a). The *P. marinus* susceptibility of six oyster strains, including two native strains from various disease enzootic areas in the southern Chesapeake Bay, one native strain from the Delaware Bay, and a *Haplosporidium nelsoni* (MSX)–resistant strain, were compared by Burreson (1991). All the native strains were found to have lower disease-caused mortality than the MSX-resistant strain. The native

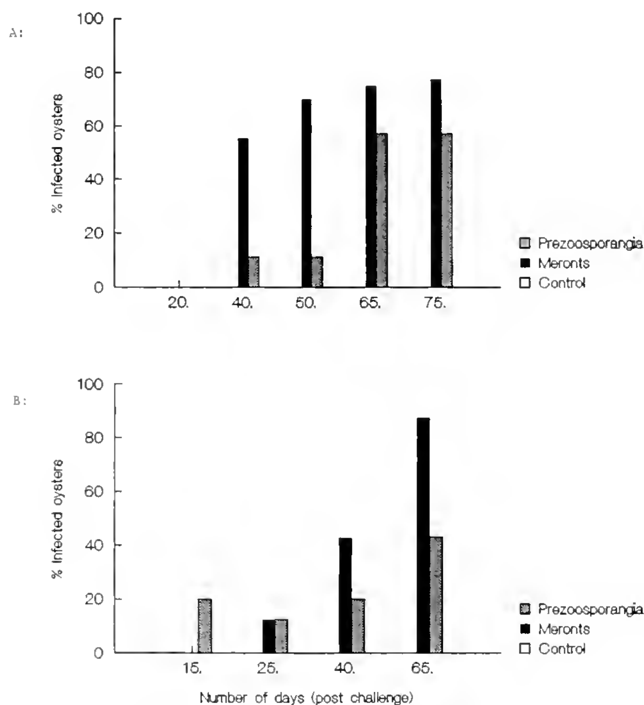

Figure 1. (A) *C. virginica. P. marinus* prevalence in oysters (Damariscotta River, ME) at 20, 40, 50, 65, and 75 days postchallenge by meronts or prezoosporangia (N = 8–9 oysters per sampling date). (B) *P. marinus* prevalence in oysters (Rappahannock River, VA) at 15, 25, 40, and 65 days postchallenge by meronts or prezoosporangia (N = 5–8 oysters per sampling date).

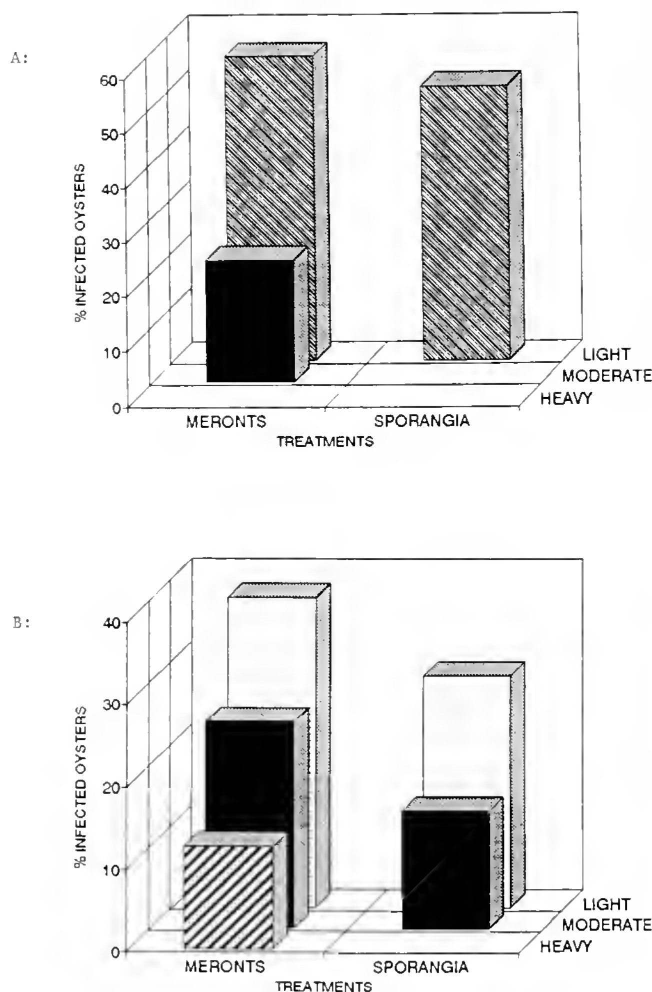

Figure 2. *C. virginica.* Intensity of *P. marinus infection in oysters inoculated with meronts or prezoosporangia after 75 days (A, oysters from Damariscotta River, ME) and after 65 days (B, oysters from Rappahannock River, VA).*

strains continued to grow, whereas the MSX-resistant strain did not, after being infected by *P. marinus*. Similarly, Chu and La Peyre (1993a) noted that the progression and development of *P. marinus* infection differed, to some extent, among three oyster populations from the Chesapeake Bay.

Results from our experiments indicate that meronts and prezoosporangia can effectively transmit *P. marinus* disease among oysters. Meronts are probably the primary transmission agent in nature. Oysters inoculated with this life stage resulted in higher infection prevalence and intensity than oysters inoculated with prezoosporangia. The proliferation rate of *P. marinus* meronts (and merozoites) cultured at 5, 12, 20, and 28°C was positively correlated with temperature (Volety 1995). The high prevalence of infection in oysters inoculated with meronts can be interpreted as a result of rapid multiplication of meronts at warm temperatures (i.e., 21–27°C). Moreover, it has been reported that 99% of *P. marinus* cells found in the water of the upper Chesapeake Bay in the warmer months (March to October) between 1992 and 1993 resembled the meront stage (Dungan and Roberson 1993). The lower infection rate of oysters inoculated with prezoosporangia compared to meronts is puzzling. The fate of inoculated prezoosporangia within the oyster is not known, although division of

prezoosporangia into meront-like structures by schizogony has been observed in *in vitro* cultures (La Peyre 1993, Perkins, personal communication). It is possible that there is a lag time for the inoculated prezoosporangia to partition to meront stage or that culture of meronts in FTM affects the infectivity of the subsequent prezoosporangia. In *in vitro* culture, prezoosporangia develop into zoosporangia in sea water, zoosporulate, and subsequently release biflagellated zoospores (Perkins 1966, Chu and Greene 1989). However, the production of zoospores by meronts or prezoosporangia in oysters or in cells isolated from oyster tissue without FTM treatment has not been documented.

The infectivity and pathogenicity of biflagellated zoospores are unknown, partly because there are still missing links in the *P. marinus* life cycle. For example, the biflagellated zoospore, which has not been seen in oyster tissue, originates from the zoosporangium that develops in sea water outside of the host. How it enters the host and how it develops into a meront stage once it penetrates into the host tissue are unknown. In the past, production of zoospores regularly occurred from prezoosporangia cultured in sea water (Perkins 1966, Chu and Greene 1989), and we routinely infected oysters with biflagellated zoospores. In experiments conducted in the early 1980s, direct injection of a dose of 10^5 zoospores per oyster into the shell cavity successfully induced infection in oysters (Chu, unpublished data; Morris Roberts, Virginia Institute of Marine Science, unpublished results). Nevertheless, recent attempts in several laboratories to culture prezoosporangia to zoosporulation have not been successful. Occasionally, some prezoosporangia zoosporulate but produce only a few biflagellate zoospores. Thus we have been unable to include the zoospore stage in infectivity and pathogenicity comparisons.

The biflagellated zoospore can swim and has an apical complex, presumably for host entry by penetration (Perkins 1988). If zoosporulation occurs in nature after prezoosporangia are released from moribund or deceased oysters, then the role of biflagellated zoospore in transmission of *P. marinus* should not be disregarded or underestimated. Unlike the meront stage, which is passive and therefore must rely on water currents of flushing rate to be transmitted between oysters, the biflagellated zoospores are motile. Moreover, the usual winter temperatures of subtropical climates would not be able to kill the prezoosporangia embedded in the oyster tissues. Prezoosporangia can withstand temperatures as low as 4°C, and zoosporulation has been observed in prezoosporangia, previously incubated at 4°C, after they were transferred to 28°C (Chu and Greene 1989).

Minimal Dose for Infection

The number of *P. marinus* cells required to transmit the disease from infected, dying, and gaping oysters to uninfected oysters has been in question for a long time. To determine whether the oyster mortality caused by *P. marinus* is correlated to the infective cell concentrations, Mackin (1962) inoculated oysters with minced tissues containing various concentrations (10–10^6) of meronts which had been incubated in FTM for 24 hr. He found that a dose of 1.0×10^2 to 5.0×10^2 cells was required to cause mortality within 41 days and mortality rate was positively correlated with the inoculated infective cell numbers. However, Mackin only monitored the oyster mortality caused by exposure to the *P. marinus* infective cells but not the infection rate or disease intensity in oysters. In a study to compare the *P. marinus* susceptibility in four different laboratory-reared stocks of *C. virginica*, Valiulus (1973) observed infection in experimental oysters at 105 days after they received a dose of 10 cells per oyster injected into their shell cavity.

Recently, Chu and Volety (unpublished observation) tested the responses of eastern oysters to different doses of meronts and prezoosporangia using oysters from Damariscotta River, ME. In two experiments, a dose-dependent response of *P. marinus* infection was found in oysters exposed to 0, 10, 10^2, 10^4, and 10^5 meronts or prezoosporangia at 25°C for 8–12 weeks (56–84 days). In both experiments, infection prevalence was found to significantly increase with increasing dose of *P. marinus* infective cells inoculated into the shell cavity of the oysters (Fig. 3A, data from one of the two experiments). A similar pattern was shown in infection intensity, expressed as weighted incidence (WI = sum of disease intensity rating/total number of oysters examined, Fig. 3B). Again, the meront stage caused much higher *P. marinus* infection prevalence and intensity in oysters than prezoosporangia. The lowest dose that initiated a *P. marinus* infection was between 10 and 10^2 meronts or prezoosporangia per oyster. No mortality occurred during these studies.

The dose response in oysters to *in vitro* cultured *P. marinus* was studied by Bushek (1994). In three separate experiments, he challenged oysters with different doses of cultured infective cells via direct injection into shell cavity or adductor muscle or by feeding. He found that oysters fed a dose as high as 10^7 cells oyster^{-1} did not succumb to infection, whereas light infections

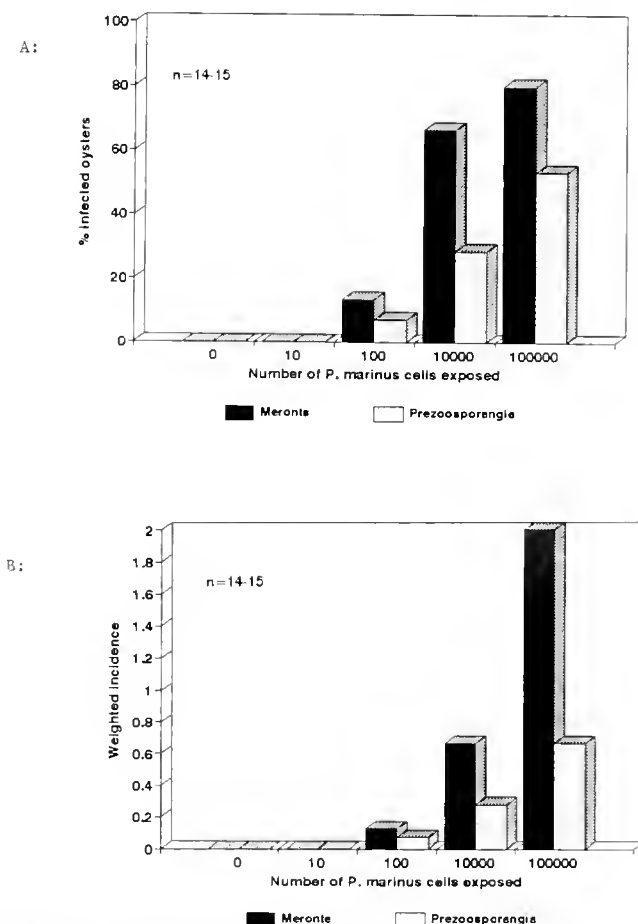

Figure 3. *C. virginica*. Prevalence (A) and intensity (B) of *P. marinus* in oysters (Damariscotta River, ME) 56 days after inoculation with 0, 10, 10^2, 10^4, or 10^5 meronts or prezoosporangia (N = 14–15).

developed within 50 days with doses above 10^4 cells oyster^{-1} when inoculated into the shell cavity or the adductor muscle. In the latter two cases, infection intensity increased as dose increased. Similarly, infection developed in oysters in 28 days after two sequential inoculations of laboratory-cultured *P. marinus* cells (1.1×10^5 and 3.3×10^5 cells oyster^{-1}) into the shell cavity, while no infection developed in oysters at 56 days after feeding three doses of laboratory-cultured *P. marinus* (e.g., 3.56×10^5, 3.36×10^7, and 1.36×10^7 cells oyster^{-1}) (Perkins 1994).

Laboratory-cultured *P. marinus* cells may be less infective/pathogenic than those freshly isolated from infected oyster tissues. Generally, oysters infected with laboratory-cultured meronts (and merozoites) did not exhibit an infection rate as high as the oysters infected with meronts freshly isolated from oyster tissues (Volety and Chu unpublished results, Perkins unpublished results). Dosing oysters with 10^6 cultured meront cells per oyster, La Peyre et al. (1993) detected only light infections in 8 weeks, and no mortality was noted. Recently, Chintala et al. (1995) compared the infectivity of laboratory-cultured *P. marinus* cells and *P. marinus* cells isolated from infected oysters. They challenged oysters with the same number of natural isolates or cultured *P. marinus* and followed mortality. At 12 weeks postchallenge, 75% of the oysters challenged with natural isolates died with heavy *P. marinus* infection while only 7.5% of the oysters challenged by laboratory-cultured *P. marinus* died. In contrast to the above findings, Gauthier and Vasta (1993) found heavy infections in oysters 4–5 weeks after two biweekly inoculations of 2×10^5 cultured meront cells and mortality occurred at 6–8 weeks. The cultured meronts used in Gauthier and Vasta's work seem more virulent than either Bushek's (1994) or La Peyre's (1993). It is not known whether the difference in infectivity in cultured meronts is due to the difference in composition of culture media or the origin of the meront isolates. The medium developed by Gauthier and Vasta (1993) is quite different from the one used by Bushek (1994), La Peyre et al. (1993), and Chintala et al. (1995). The latter three used meronts cultured in medium developed by La Peyre et al. (1993).

Bushek (1994) also tested the infectivity of isolates of *P. marinus* from different locales. Infectivity or virulence varies between isolates of *P. marinus*. Significantly heavier infections were found in oysters challenged by two Atlantic Coast isolates (Chesapeake and Delaware Bays) than in oysters challenged by the two Gulf Coast isolates (Barataria Bay, LA, and South Bay Laguna Madre, TX). The findings of Bushek's study (1994) suggest the existence of geographic *P. marinus* races.

Exactly how *P. marinus* infective cells, discharged from carriers and/or released from infected gapers, enter a new host is unclear. Entry through filtration/feeding is assumed to be the main route of *P. marinus* transmission in nature, so it is of interest that shell cavity injection is more effective than feeding in infection induction (Ray 1954, Bushek 1994, Perkins 1994). The infective cells, either meront or prezoosporangia, are particularly sticky (personal observation). They would easily adhere to the mantle and gills while passing through the host's shell cavity during the feeding and/or filtration process. If this is taken into consideration, infection generated through shell cavity inoculation of infective cells may be comparable to the natural condition.

It was demonstrated through the dose response experiments that a dose of 10–10^2 freshly isolated *P. marinus* cells oyster^{-1} is required to cause infection by direct shell cavity injection (Chu and Volety, unpublished observation). Considering the abundance of *P. marinus* (3,000–19,000 l^{-1} water) found in the water during warmer months of the year (Dungan and Roberson 1993), and the filtration rate (>8 l hr^{-1}, Galtsoff 1964) of oysters in nature, it may be obvious how *P. marinus* infection is transmitted. As Bushek (1994) speculated, chronic feeding of high levels of *P. marinus* may be required to cause infection. However, once 10–10^2 infective cells are trapped in the shell cavity, infection will result (Valiulus 1973, Chu and Volety unpublished observation).

SUSCEPTIBILITY AND INFECTIVITY; TEMPERATURE AND SALINITY EFFECTS

It has been known, since the epizootics caused by *P. marinus* in oysters in the 1950s, that the distribution and abundance of the parasite in the field are limited by temperature and salinity (see reviews by Andrews 1988, Andrews and Ray 1988). Beginning in the 1950s, studies have been carried out to determine the relationship between *P. marinus* incidence in oysters and temperature and salinity under laboratory-controlled conditions. Hewatt and Andrews (1956) studied the effect of high and low temperatures on *P. marinus* infection in oysters by holding oysters in the laboratory at 15°C and oysters in trays in the York River at 26–28°C for 6 weeks after feeding the oysters minced, heavily infected oyster tissues. They found that low temperature substantially reduced the oyster mortality caused by the parasite, thus suggesting that low temperature may inhibit the parasite's activity. In an earlier study, they (Andrews and Hewatt 1957) also found that oysters maintained in an aquarium at 15°C did not develop infection after 6 weeks, while oysters maintained at room temperature (20–21°C) were all infected by the parasite after 2 weeks. In two experiments, Ray (1954) compared the infection rate of artificially infected oysters maintained at two salinity ranges (26–28 and 10–14 ppt; 24–29 and 10–15 ppt). He found that the developmental rate of infection in the oysters at the low salinity range was delayed.

From these early studies, it was concluded that temperature and salinity affect the infection rate and development of the parasite in oysters. However, the lowest test salinities in Ray's study (1954) were 10–15 ppt and 10–14 ppt, which are much higher than the lowest salinities (3–6 ppt) that *P. marinus* can tolerate (Chu and Greene 1989), and only two temperatures or salinities were compared for *P. marinus* infection in these studies. Thus no correlation can be drawn between *P. marinus* incidence and temperature or salinity using these data. Moreover, in these investigations, oysters developed infection and died within 2 weeks, indicating that the experimental oysters may have been previously infected in the field.

The relationship, documented in field studies, between *P. marinus* infection in oysters and temperature and salinity, was reaffirmed through more detailed and comprehensive laboratory studies by Chu and her associates (Chu and La Peyre 1993b, Chu et al. 1993a). In two separate studies, they examined the effects of temperature and salinity on *P. marinus* susceptibility and infection in oysters after challenging individual oysters with 10^6 freshly isolated meronts. Their results showed that *P. marinus* susceptibility and infection in oysters were significantly correlated with experimental temperatures and salinities. Disease prevalences and infection intensity decreased as temperature decreased (Fig. 4). Likewise, *P. marinus* prevalence and intensity (WI) in meront-challenged oysters were positively related to salinity (Fig. 5). Oysters at 3 ppt exhibited lower *P. marinus* prevalence and intensities than those at 10 and 20 ppt. Heavy infections were found only in oysters at 10 and 20 ppt. However, in the salinity experiment, the

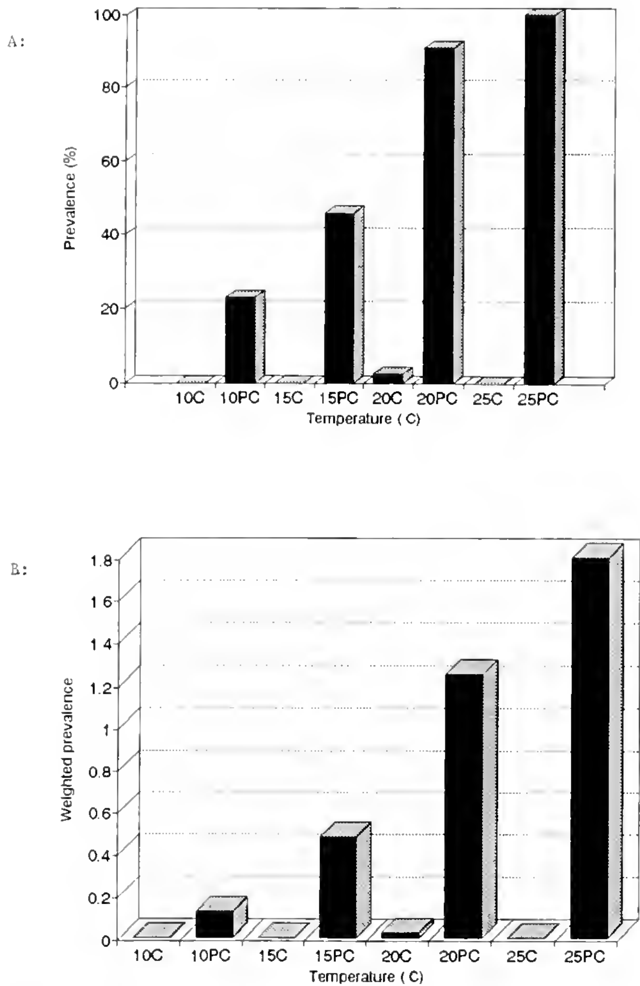

Figure 4. *C. virginica. P. marinus* **infection prevalence (A) and intensity (B) in oysters (Rappahannock River, VA) at 10, 15, 20, and 25°C (N = 40). C = Control; PC = oysters challenged by** *P. marinus.*

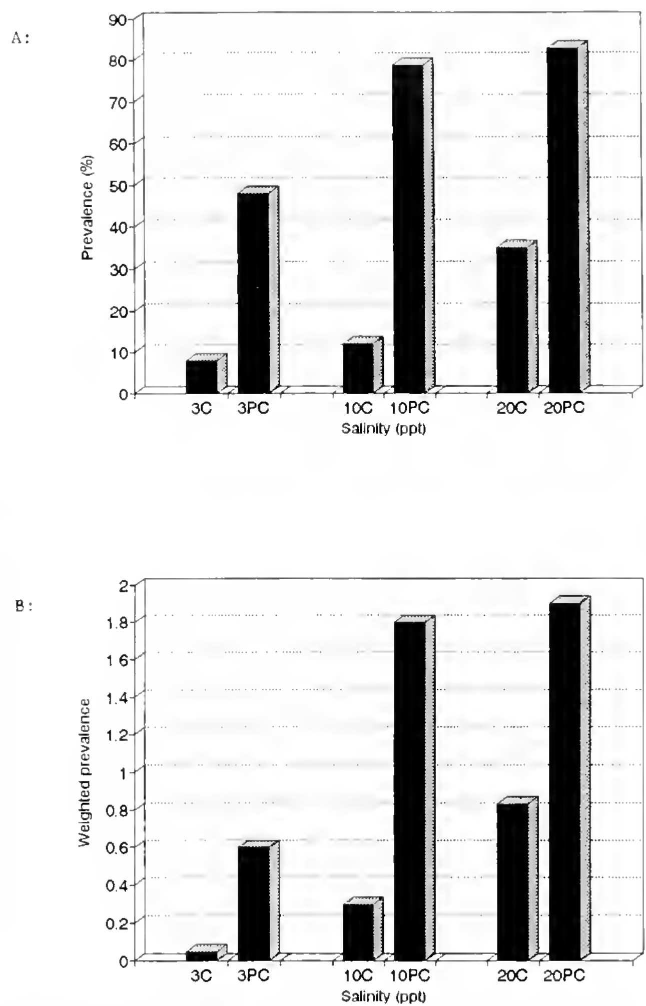

Figure 5. *C. virginica. P. marinus* **prevalence (A) and intensity (B) in oysters (James River, VA) at 3, 10, and 20 ppt (N = 19–24). C = control; PC = oysters challenged by** *P. marinus.*

unchallenged oysters showed a pattern of *P. marinus* infection similar to the challenged oysters. This is believed to be an expression of latent infection established in the field (a subsample [N = 40] taken in this experiment at the time of collection exhibited 12.5% light infection). Therefore, salinity as low as 3 ppt did not eliminate infection but did prevent intensification. Similar results were noted by holding oysters infected with *P. marinus* in water of 6 ppt (Ragone and Burreson 1993).

TRANSMISSION ECOLOGY

Field observations point to temperature and salinity as two important environmental factors regulating the activity of *P. marinus*. Certainly, the dosage of *P. marinus* infective cells is also critical. Therefore, it is important to examine the impact of the interaction among these three crucial factors on the outcome of the disease process. Recently, Chu et al. (1994) investigated the response of oysters challenged by two different doses (2.5×10^3 or 2.5×10^4 freshly isolated meronts oyster^{-1}) of *P. marinus* at nine salinity-temperature combinations: i.e., 10, 15, and 25°C at 3, 10, and 20 ppt. Results agree with their previous findings on the susceptibility and infectivity and dose response studies. Increased infection prevalence and intensity (Fig. 6) occurred at high temperatures and salinities and there was a dose-dependent response to infective particles. When the effects of temperature, salinity, and infective cell doses and their interaction on *P. marinus* susceptibility and infection intensity were analyzed using logistic regression and a log linear model, temperature was found to be the most important factor influencing the susceptibility to *P. marinus* and subsequent disease development in oysters. This was followed, respectively, by the dose of infective particles and salinity. Similarly, Fisher et al. (1992) found temperature to be more influential than salinity on *P. marinus* intensity and mortality in oysters collected from the Gulf of Mexico and maintained at different test temperatures (18–27°C) and salinities (6–36 ppt). It has also been reported that fluctuations in incidence of the parasite in oysters in the Gulf of Mexico region are more sensitive to changes in temperature than to salinity fluctuations (Mackin cited in Ray 1954). Applying these results to the field observations may explain why *P. marinus* infection in oysters is dominant in the summer months at mid-Atlantic waters and is generally confined to subtropical regions. It is also true that, in nature, river water inputs and/or fresh water runoff not only dilute the salinity, but reduce the *in situ* concentration of *P. marinus* infective cells in estuaries, thus protecting oysters to some extent from infection (Mackin 1962).

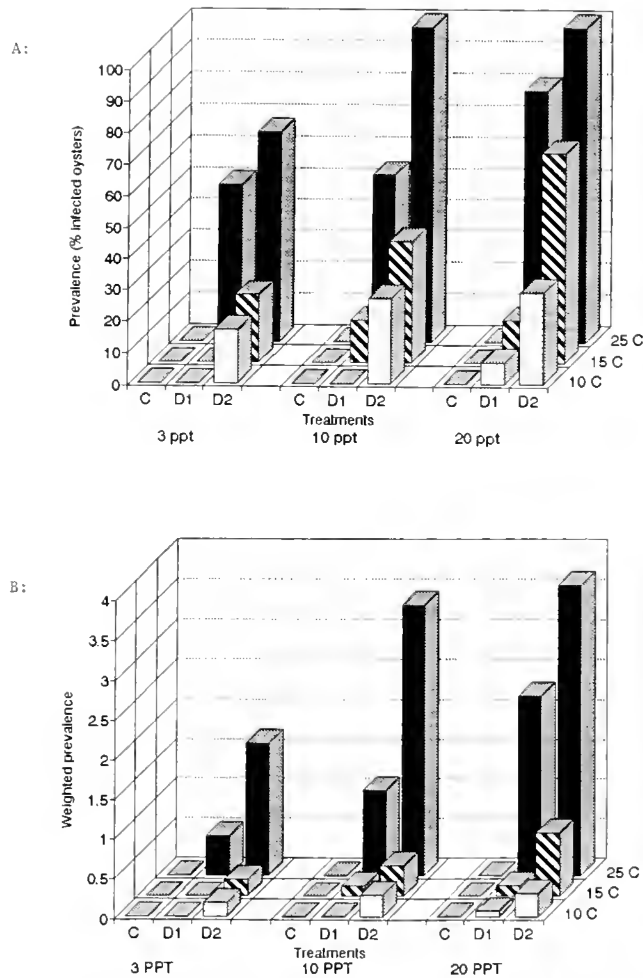

Figure 6. *C. virginica*. *P. marinus* **prevalence (A) and intensity (B) in oysters (Damariscotta River, ME) at different temperature and salinity regimes after challenge with two different doses of** *P. marinus* **infective cells. C = control; D1 = 2.5×10^3 meronts oyster^{-1}; D2 = 2.5×10^4 oyster^{-1}. N = 7–15 per group at all temperature-salinity treatments, except the groups at 25°C and 3 ppt treatment (N = 3–4 per group).**

However, along the Gulf Coast, unlike the Chesapeake Bay, water usually stays warm year-round. Therefore, the seasonal shift of *P. marinus* infection is more likely to be associated with the change of salinity caused by rainfall and river runoff rather than temperature. A program to monitor the regional distribution and yearly trends of *P. marinus* prevalence and intensity in relation to climate changes in the Gulf of Mexico between 1986 and 1989 indicated that the yearly shift of *P. marinus* prevalence was related to the rainfall/river runoff (salinity) rather than temperature (Powell et al. 1992).

The effect of three-way interactions among temperature, salinity, and meront doses on disease prevalence was found to be insignificant in our study (Chu and Volety, unpublished observation). There was a significant two-way interaction, however, between temperature and salinity, and between temperature and meront dose, on intensity of *P. marinus* infection. Based on data calculated from field samples, an effect of temperature and salinity interaction was noted for *P. marinus* infection intensity (weighted prevalence) in two different field studies conducted on the Gulf Coast (Soniat 1985, Soniat and Gauthier 1989).

A COMPARISON OF *CRASSOSTREA GIGAS* AND *C. VIRGINICA*: EFFECTS OF TEMPERATURE AND SALINITY ON SUSCEPTIBILITY TO *P. MARINUS*

The eastern oyster, *C. virginica*, has historically supported a major fishery on the east coast of the United States. Because of severe mortality, beginning from the late 1950s, in oyster populations caused by *P. marinus* and *H. nelsoni* in the mid-Atlantic region, introduction of a non-native species, the Pacific oyster (*Crassostrea gigas*), to the waters of this region was proposed to revitalize the oyster fishery. The Pacific oyster has been successfully introduced and cultured along the west coast of the United States and in Europe. This oyster species is rarely infected by the protozoan parasite, *Bonamia ostreae*, that has caused severe losses of the European oyster (*Ostrea edulis*) industry in Europe and on the west coast of the United States over the last decade (Grizel 1985, Elston et al. 1987, Grizel et al. 1988). Results of recent studies using outdoor flow-though sea water systems with quarantined effluent also indicate that the Pacific oyster is less susceptible than the eastern oyster to *P. marinus* (Meyers et al. 1991, Barber and Mann 1994).

Pacific oysters usually thrive in waters of salinity higher than 18 ppt and temperature ≤15°C, though they may be able to tolerate a temperature as high as 35°C and salinity as low as 10 ppt (see review by Mann et al. 1991). Information regarding temperature-salinity tolerance in *C. gigas* is, however, limited and the definitive temperature and salinity tolerance of this species has not been tested in the laboratory. The mid-Atlantic climate is relatively warm, between temperate and subtropical, and the ecosystem of the Chesapeake Bay is complex. The salinity range of oyster habitats in the Chesapeake Bay varies seasonally and ranges from 0 to >20 ppt. The water temperature of most tributaries of the Chesapeake Bay can reach 28–29°C and persist for more than 2 months during the summer. The oyster pathogen, *P. marinus*, can persist in salinities lower than 5 ppt and the epizootics caused by this parasite are elevated at high temperatures and salinities (Andrews 1988, Burreson and Andrews 1988). Therefore, the competence of the Pacific oysters against *P. marinus* under different salinity and temperature regimes is of particular concern.

Chu et al. (1993b) evaluated, in the laboratory, the susceptibility of diploid (2N) and triploid (3N, estimated to be 95%) *C. gigas* to *P. marinus* compared with *C. virginica* at three test temperatures (10, 15, and 25°C) at a salinity of 20–22 ppt and at three test salinities (3, 10 and 20 ppt) at a temperature of 19–22°C. Their findings (Fig. 7) show that, generally, Pacific oysters were more tolerant to *P. marinus* infection than eastern oysters at the temperature and salinity regimes tested. However, at 10°C, *P. marinus*–challenged 3N *C. gigas* had prevalences higher than *P. marinus*–challenged 2N *C. gigas* and *C. virginica*. In *C. virginica*, moderate and heavy infections developed in both control and *P. marinus*–challenged groups at 25°C and in *P. marinus*–challenged groups at 20 ppt. No heavy infection was found in *C. gigas* in any treatment. Moderate infections were detected only in diploid *C. gigas* at 10 and 25°C. Since much higher infection prevalences were found in the control (unchallenged) *C. virginica*, at any given temperature and salinity treatment, than in unchallenged 2N and 3N *C. gigas* (Fig. 7), the authors believed that part of the recorded prevalence and intensity in *C. virginica* was attributed to the expression of hidden infection carried over from the field. Unfortunately, the techniques, thioglycollate tissue (Ray 1952) and hemolymph (Gauthier and Fisher 1990) assays, currently employed

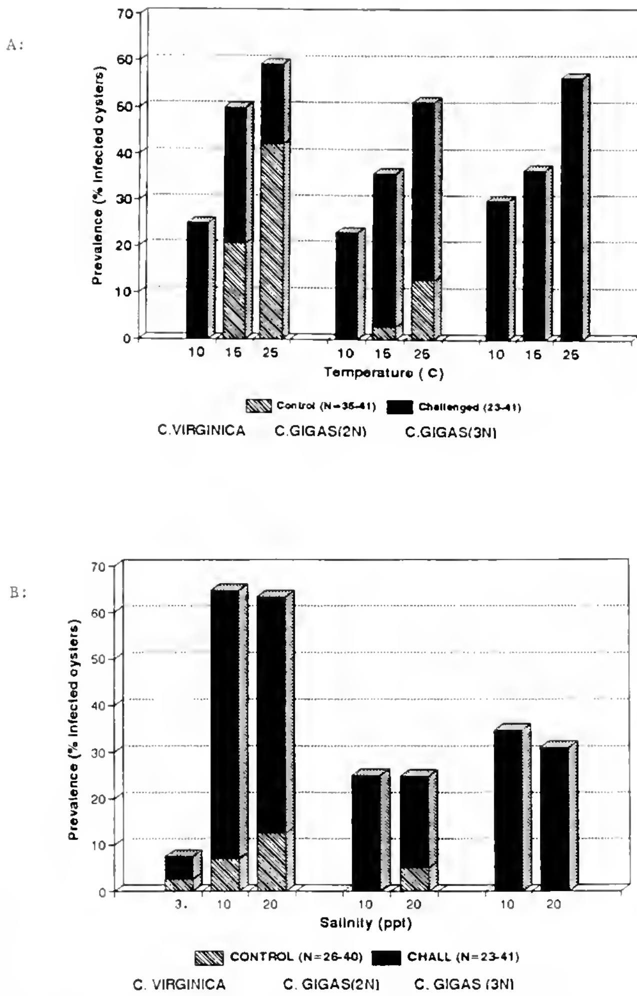

Figure 7. *C. virginica* and *C. gigas*. *P. marinus* prevalence in *C. virginica* (Rappahannock River, VA) and *C. gigas* at 10, 15, and 25°C (A, N = 35–41) and at 10 and 20 ppt (B, N = 23–43).

for routine *P. marinus* diagnosis apparently were not sensitive enough to detect cryptic infection. Therefore, in the future, eastern oysters from an area free of *P. marinus* should be employed for this kind of study.

The investigation by Chu et al. (1993b) also revealed that Pacific oysters are much less tolerant than eastern oysters to low salinity and high temperature. Heavy non-*P. marinus*–related mortality occurred during their study, in both diploid and triploid Pacific oysters at salinities 20 ppt and below and temperatures higher than 15°C (Tables 1 and 2). High non-disease-related mortality (70%) was also recorded in Pacific oysters in conjunction with low salinity (<20 ppt), in a study carried out to compare the growth and mortality of *C. gigas* and *C. virginica* challenged with *P. marinus* (Barber and Mann 1994). These results suggest that *C. gigas* may survive the *P. marinus* challenge, but may die under the environmental conditions prevailing in the mid-Atlantic, particularly the southern Chesapeake Bay. Moreover, the shells of this species held in water of lower Chesapeake Bay (i.e., York River, VA) were found to be quite susceptible to invasion by the polychaete *Polydora* sp. (Burreson and Mann 1994).

RELATIONSHIP BETWEEN POLLUTION AND *P. MARINUS* SUSCEPTIBILITY IN OYSTERS

As described earlier, susceptibility and advancement of *P. marinus* infection in oysters are associated with environmental temperature and salinity. However, it is uncertain to what extent the increased distribution and intensification of *P. marinus* infection in nature are attributable to environmental degradation. Pollution has been hypothesized to contribute to some aquatic epizootics, although this link has not been adequately examined. To evaluate this hypothesis, Chu and Hale (1994) investigated the effects of a complex mixture of sediment pollutants on the susceptibility of oysters to *P. marinus*. They exposed oysters to different dilutions (i.e., 0, 15, 30%) of water-soluble fractions (WSF) generated from sediments collected from Elizabeth River, a heavily polluted sub-estuary of the Chesapeake Bay. The Elizabeth River sediments used for the study were grossly contaminated with polycyclic aromatic hydrocarbons, characteristic of creosote. The WSFs generated from the sediments were dominated by lower molecular weight aromatic hydrocarbons and heterocyclic compounds (Table 3). The mean concentration of aromatic pollutants in the WSFs (representing more than 100 compounds) was 4.08 mg l^{-1} (S.D. = ±0.399). These oysters were then challenged with *P. marinus* meronts. Pollutant exposure in the laboratory was found to enhance preexisting *P. marinus* infections in

TABLE 1.

Mortality of *C. virginica* and *C. gigas* during temperature acclimation and after challenge with *P. marinus*

Parameter	*C. virginica*			*C. gigas* (2n)			*C. gigas* (3n)*		
Temperature (°C)	10 (N = 80)	15 (N = 80)	25 (N = 80)	10 (N = 79)	15 (N = 81)	25 (N = 85)	10 (N = 82)	15 (N = 82)	25 (N = 111)
Mortality (# of deaths) during acclimation	0	0	4	0	2	7	1	1	32
Mortality (# of deaths) after *P. marinus* exposure	1	3	10	1	7	21	1	3	1
Total mortality (%) during experiment**	1.3	3.8	17.5	1.3	11.1	32.9	2.4	4.9	29.7

* An estimated 95% of oysters were triploid.
** % = # of dead oysters/initial total number of oysters.

TABLE 2.

Mortality of *C. virginica* and *C. gigas* during salinity acclimation and after challenge with *P. marinus*

Parameter salinity (ppt)	*C. virginica* 3 (N = 79)	*C. virginica* 10 (N = 84)	*C. virginica* 20 (N = 81)	*C. gigas* (2n) 3 (N = 77)	*C. gigas* (2n) 10 (N = 86)	*C. gigas* (2n) 20 (N = 95)	*C. gigas* (3n)* 3 (N = 52)	*C. gigas* (3n)* 10 (N = 78)	*C. gigas* (3n)* 20 (N = 81)
Mortality (# of deaths) during acclimation	0	0	0	44	6	9	37	12	10
Mortality (# of deaths) after *P. marinus* exposure	4	0	0	—	20	7	—	17	17
Total mortality (%) during experiment**	5	0	0	—	30.2	16.8	—	37.1	33.3

* An estimated 95% of oysters were triploid.
** % = # of dead oysters/initial total number of oysters.

oysters from an area affected by *P. marinus* and to increase the susceptibility to experimental infection in oysters from an area outside the normal geographic range of *P. marinus* (Fig. 8). Both occurred in a dose-dependent manner.

Recently, Fisher et al. (1995) and Anderson et al. (1995) tested, independently, the effects of tributyltin (TBT) on *P. marinus* progression in eastern oysters. Both research groups observed that exposure to TBT significantly elevated mortality caused by *P. marinus* in oysters, when compared to non-TBT-exposed oysters. TBT exposure enhanced the disease prevalence (Anderson et al. 1995) and increased slightly the progression of *P. marinus* infection in oysters (Anderson et al. 1995, Fisher et al. 1995).

Pollutants may reduce disease resistance by causing physiological stress in the host or suppressing certain host immune mechanisms (Anderson 1996). Alternatively, pollution may affect disease susceptibility by elevating infection pressure through an increase in the number and activity of infectious organisms. It is currently unknown whether the increased *P. marinus* infection observed in pollutant-exposed oysters is attributable to heightened *P. marinus* virulence or decreased host resistance, although the latter is suspected. Results from the above studies do suggest that environmental degradation may increase the epizootic, although *P. marinus*–caused disease is known to be predominantly exacerbated by elevated temperature and salinity. A previous study by Winstead and Couch (1988) noted that *P. marinus* expression

TABLE 3.

Concentrations (S.D.), in mg l^{-1}, of the major organic contaminants, detected in representative water-soluble fractions (WSF), generated from Elizabeth River sediments and control water (filtered York River water) (N = 3)

Analyte	Control	WSF
Naphthalene	<0.001	1.510 (0.520)
Acenaphthene	<0.001	0.424 (0.033)
2-Methylnaphthalene	<0.001	0.224 (0.018)
Phenanthrene	<0.001	0.210 (0.026)
Fluorene	<0.001	0.201 (0.031)
Dibenzofuran	<0.001	0.195 (0.012)
1-Methylnaphthalene	<0.001	0.151 (0.022)
Carbazole	<0.001	0.148 (0.013)

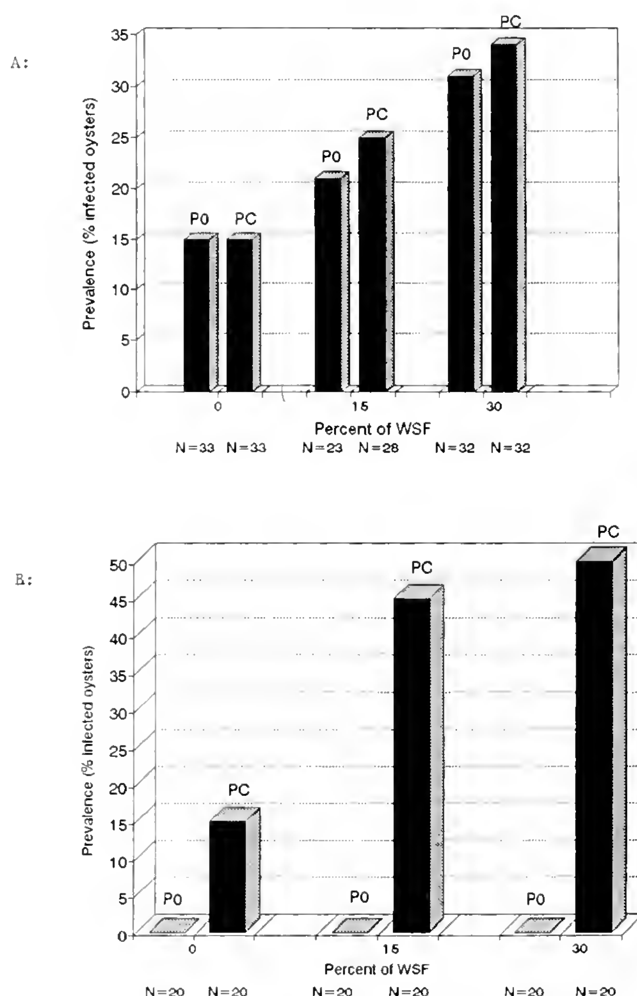

Figure 8. *C. virginica*. *P. marinus* prevalence in oysters after exposure to 0, 15, and 30% dilutions of WSF generated from contaminated estuarine sediments. PO = nonchallenged oysters; PC = *P. marinus*–challenged oysters. (A) Oysters (N = 23–33) from Rappahannock River, VA, were exposed to WSF dilutions for 35 days and then were challenged with *P. marinus*. Postchallenge WSF exposure was then continued for an additional 21 days. (B) Oysters (N = 20) from Damariscotta River, ME, were exposed to WSF dilutions for 35 days and then were challenged with *P. marinus*. Postchallenge WSF exposure was then continued for an additional 35 days.

appeared at uncharacteristically low temperatures after toxicant exposure.

CONCLUSION AND FUTURE STUDIES

All three identified *P. marinus* life stages, meront, prezoosporangia, and biflagellated zoospore, are effective in transmitting disease. The meront stage is more effective compared to prezoosporangia stage in creating *P. marinus* infection in eastern oysters and thus may be the primary transmission agent in nature (although the infectivity and pathogenicity of the biflagellated zoospore stage were not included in the comparison). Results of laboratory studies are consistent with field observations and clearly demonstrated that *P. marinus* susceptibility and disease advancement are positively correlated with temperature, salinity, and the number of infective cells to which the oyster is exposed. Among the above three environmental factors, temperature is the most important factor followed by the infective cell dose controlling the susceptibility to *P. marinus* and subsequent disease development in oysters. Salinity was a less influential factor than temperature and infective cell concentration. There was no significant three-way interaction among temperature, salinity, and infective cell dose on the prevalence of disease in oysters in the laboratory, but the two-way positive interactions between either temperature and salinity or between temperature and *P. marinus* dose significantly intensified the disease in oysters. Culture of oysters in tributaries with high river water input and/or fresh water runoff may effectively dilute *P. marinus* infective elements, thus providing some level of protection to oysters from infection. The Pacific oyster, *C. gigas*, is less susceptible to *P. marinus* than the eastern oyster, *C. virginica*. However, they may not survive if introduced into Chesapeake Bay tributaries, since they are unable to adapt well to low salinity and high temperature environmental conditions. Pollution has the potential to enhance *P. marinus* susceptibility and infection in oysters. To further understand the transmission and disease processes of *P. marinus* in eastern oysters, the life history of this parasite needs to be completely determined. It is not known why laboratory-cultured meronts seldom reach zoosporangia stage and why prezoosporangia derived from FTM-cultured meronts no longer zoosporulate. The infectivity and pathogenicity of the biflagellated zoospores life stage need further examination. Furthermore, we know little about the fate of *P. marinus* cells after they enter the host. Finally, sensitive techniques are needed to detect *P. marinus* at low infection intensities without sacrificing the oyster.

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THE STRUCTURE OF *PERKINSUS MARINUS* (MACKIN, OWEN AND COLLIER, 1950) LEVINE, 1978 WITH COMMENTS ON TAXONOMY AND PHYLOGENY OF *PERKINSUS* SPP.

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ABSTRACT A description of the structure of the *Crassostrea virginica* pathogen *Perkinsus marinus* is provided from observations at the light and transmission electron microscope levels of detail and includes cellular multiplication (palintomy) in the host and zoosporulation in estuarine water as well as observations of cells in axenic culture. The description is primarily a review of previously published information, however, new information is provided on development of walled outgrowths from hypnospores derived from fluid thioglycollate medium treatment of infected host tissue. The protoplast within the outgrowths subdivides to yield small unicells which escape into the ambient water, or the protoplast emerges from the tip of the outgrowth and then subdivides into unicells. A taxonomic and phylogenetic reevaluation of the genus *Perkinsus* is provided.

KEY WORDS: *Perkinsus marinus* structure, palintomy, trophozoites, tomonts, hypnospores, zoosporulation, zoospores, taxonomy, phylogeny

INTRODUCTION

In the last 5 years there has been a marked increase in the yearly production of scientific papers involving descriptions of the biology of the *Crassostrea virginica* pathogen, *Perkinsus marinus*. This increase is undoubtedly due to the interest in finding answers to the problem of continuing declines in oyster production in the middle Atlantic region of the United States, resulting in large part from disease caused by *P. marinus* (as well as *Haplosporidium nelsoni*). The increased funding support provided by the National Oceanographic and Atmospheric Administration for oyster disease research since 1991 has been significant in sustaining this increased level of research activity. With this interest, it seems particularly timely to revisit the subject of *P. marinus* structure. It appears that a description of all stages in the life cycle in one publication would be useful to those investigators studying the ecology, biochemistry and physiology of the pathogen as well as its interactions with its host. Thus, this paper is primarily a review of morphological descriptions which have already been published; however, new information is also provided.

Over the years since Mackin et al. (1950) first named the organism *Dermocystidium marinum*, a number of publications have appeared in which the structure of *P. marinus* has been described at the cytological (Ray 1954, Ray and Chandler 1955, Mackin and Boswell 1956, Mackin 1962, Perkins and Menzel 1966, La Peyre et al. 1993, Perkins 1993) and the ultrastructural (Perkins and Menzel 1967; Perkins 1969, 1976a, 1976b, 1987, 1991; La Peyre et al. 1993) levels of detail. Perkins (1988, 1993) has provided summary papers of both cytology and ultrastructure; however, neither paper was a complete treatment of all stages and likewise the earlier treatments were fragmentary, requiring the reader to consider numerous papers in order to acquire a reasonably complete description of the organism. The present paper is an attempt to provide a more complete source of information on the pathogen's structure in a single publication.

Since cultured cells of *P. marinus* engaged in cellular multiplication differ somewhat from those in the host, a description of the cultured forms is presented, supplementing the descriptions of Gauthier and Vasta (1993), Kleinschuster and Swink (1993) and La Peyre et al. (1993). Otherwise the descriptions are of cells as seen in the host *C. virginica* with the exceptions being cells in sea water which were either engaged in zoosporulation or involved in the production of tubular outgrowths followed by cytokinesis.

MATERIALS AND METHODS

P. marinus cells described from histological sections were present in oyster tissue which had been fixed in Davidson's fixative (Shaw and Battle 1957), paraffin embedded and stained in Harris' hematoxylin and eosin. The fine structure was described from cells fixed in glutaraldehyde and osmium tetroxide using a variety of different times and concentrations. Mostly the treatments centered around 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at room temperature for about 3 hr, followed by three buffer rinses in 0.2 M sodium cacodylate buffer over about a 60-min period, and then postfixation using 1% OsO_4 in 0.1 M sodium cacodylate buffer for 1–3 hr. The latter two solutions were held at 4°C and ranged from pH 7.2 to 8.0.

Induction of hypnospore formation was accomplished using the Ray technique (Ray 1952) in which fluid thioglycollate medium (FTM) was reconstituted in ca. 17–22 ppt estuarine water and fortified with 400–500 units/ml each of penicillin G (potassium salt) and streptomycin sulfate. Occasionally 4×10^{-3} mg/ml of nystatin was used if there were problems with molds growing in the medium. Pieces of infected oyster tissues were placed in the medium for 4–7 days at room temperature to induce formation of the hypnospores.

Hypnospores were isolated by mincing the FTM-treated tissues with a razor blade, pulling the minced material repeatedly through a 10-ml hypodermic syringe without an attached needle, and then filtering first through a 40-μm screen, followed by a 20-μm screen. The cells were washed either by gentle centrifugation or on the screens, back flushed into petri dishes with estuarine water at ca. 17–22 ppt, and then allowed to incubate at room temperature for up to 7 days in the presence of the above-mentioned concen-

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trations of penicillin G and streptomycin sulfate. The zoosporangia and zoospores shown in Figs. 30, 31, 33 and 34 were obtained as a result of using the above technique prior to 1980. The technique worked for about 20 years after it was first described by Perkins and Menzel (1966); however, in the late 1980s workers started having difficulty with inducing zoosporulation and today only a few cells in any given population of hypnospores will become zoosporangia and form motile zoospores. Those that are formed are not released from within the zoosporangial wall (Bushek, Chu, La Peyre and Gauthier, personal communications; Perkins, unpublished data). In attempts to induce zoosporulation, a wide variety of culture conditions were tried by those workers using *P. marinus* obtained from oyster populations from both the Gulf of Mexico and Atlantic coasts of the United States. This lack of normal zoosporulation is very curious and no satisfactory explanation has been provided to explain the change which has occurred.

The cells shown in Figs. 21–29 are *Perkinsus* sp. (probably *Perkinsus atlanticus*) from the clam *Macoma balthica*. They are used herein as a matter of convenience to illustrate zoosporulation in *P. marinus* because the morphogenesis readily occurs and zoospores are released as opposed to what is now observed in *P. marinus*. In addition, zoosporulation in this *Perkinsus* sp. is indistinguishable at this level of detail from *P. marinus* (Perkins and Menzel 1966; Perkins 1968, 1988; Kleinschuster et al. 1994; Perkins, unpublished data). It should be noted that *Perkinsus* spp. hypnospores from other bivalve molluscs also readily become zoosporangia and release their zoospores (Goggin et al. 1989, Azevedo et al. 1990, Perkins, unpublished data using *Perkinsus* sp. from *Macoma mitchelli* and the shipworm *Bankia gouldi*).

The walled extensions (outgrowths) of hypnospores described below form in 20–30 ppt estuarine water with the same concentrations and types of the above-mentioned antibiotics after about 4 days' incubation at 20–25°C. Isolation of the cells was accomplished using the same techniques as described above.

Since *Perkinsus* spp. now appear to be closely related to both the Apicomplexa and the Dinoflagellata (see taxonomy section of the DISCUSSION), it appeared prudent to reconsider the terms used to denote the various stages of the life cycle, particularly since the terms used most recently (Perkins 1991) have not been entirely suitable even if one considers *Perkinsus* spp. to be Apicomplexa. After reconsideration of selected protistological literature including the glossarys of Corliss and Lom (1985), Margulis et al. (1990) and Margulis et al. (1993), it continues to be obvious that there are no existing terms which are entirely suitable to denote the various stages in the life cycle of *Perkinsus* spp., due in large part to the apparent phylogenetic uniqueness of the molluscan pathogens. I am reluctant to propose new terms to further complicate the literature; therefore, I have selected preexisting words which are the most suitable in light of our current knowledge concerning the phylogenetic position of *Perkinsus* spp. The terms are generalized ones which should not stimulate an inordinate amount of controversy and have been used previously to denote life cycle stages of various species in the Alveolate clade of Protista (Apicomplexa, Dinoflagellata and Ciliata) (Patterson and Sogin 1992). They are as follows: the unicells seen in infected oysters are termed *trophozoites* (thalli of Perkins 1969 and merozoites and meronts of Perkins 1991) which undergo cellular proliferation termed *palintomy* (merogony or schizogony of Perkins 1991). *Mature trophozoites* are those which have a signet ring profile due to a large eccentric vacuole and a thickening of the cytoplasm where the nucleus is located (meronts of Perkins 1991). The dividing cells which are engaged in palintomy are termed *tomonts* (schizonts of Perkins 1991). The terms, *zoosporulation*, used to denote formation of flagellated cells (*zoospores*) from *zoosporangia*, are retained. Justification of the changes in terminology proposed herein is presented in the terminology section of the DISCUSSION.

RESULTS

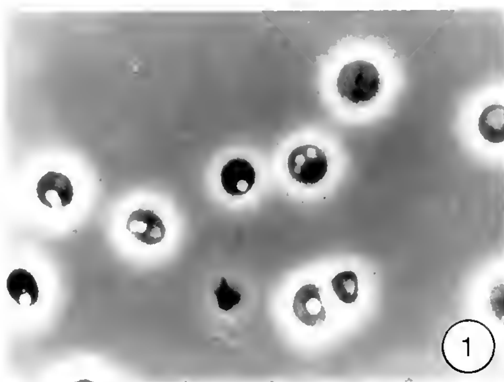
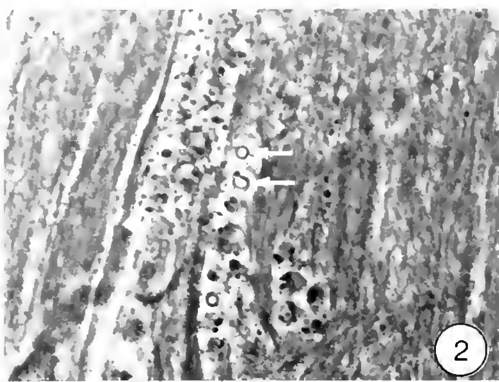
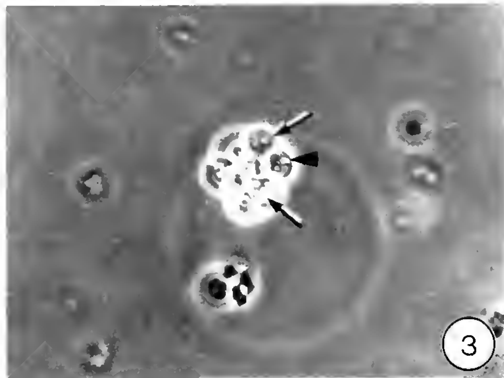
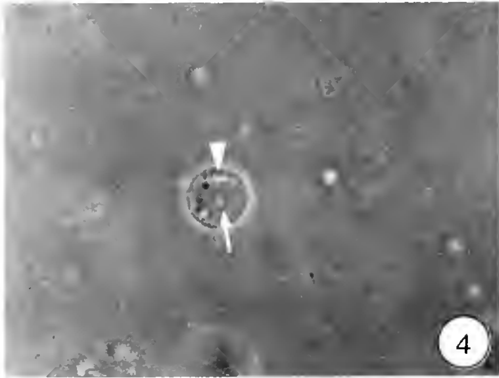
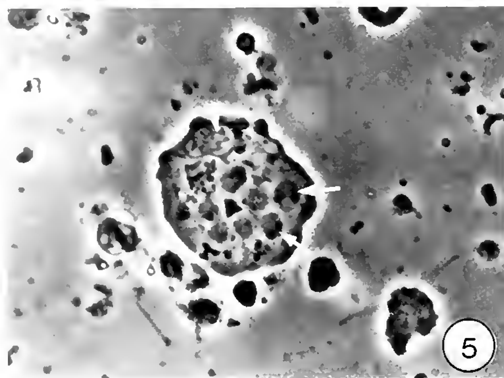
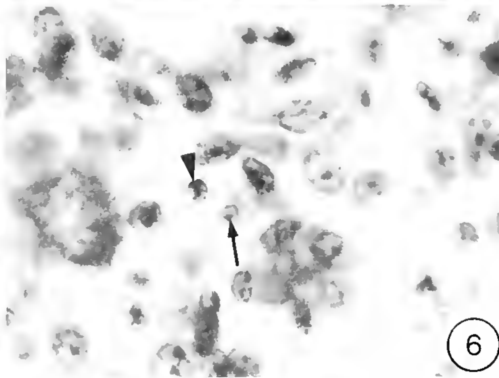
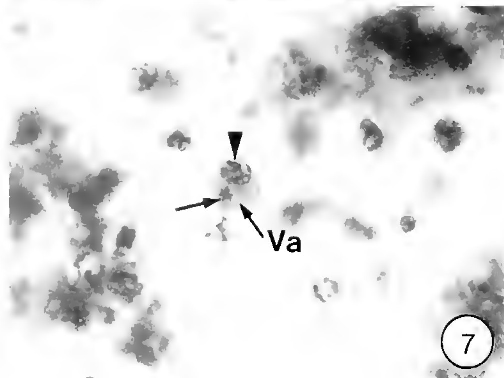
Palintomy Within the Host

Cellular stages of *P. marinus* observed in the host are found in Figs. 1–13. The smallest and least differentiated free cells of *P. marinus* are uninucleate, immature trophozoites which are the daughter cells formed by palintomy. Shortly after release by rupture of the tomont wall, they are spheroidal, ovoid or cuneiform (Figs. 1, 5, and 7).* In histological sections of Davidson's-fixed material, the spheroidal ones range from 1.9 to 2.9 μm in diameter and when unfixed they are 1.9–3.4 μm in diameter (Table 1). In phase contrast microscopy of living cells, they appear dark with one to three brightly refringent lipid droplets. In hematoxylin and eosin-stained histological sections of Davidson's-fixed oysters, the basophilic chromatin of the nuclei appears as a ring around the nuclear perimeter with a few thick regions in the ring (Figs. 6, 12, and 13). Nuclei are about 0.9–1.9 μm in diameter for delineated immature trophozoites in 8- to 16-cell tomonts (Fig. 13) and in the immature trophozoites which have been recently freed of the tomont. Most often the immature trophozoites are in the phagosomes of hemocytes but may be found free in the connective tissue, in lesions or in spaces between epithelial cells.

In the electron microscope, immature trophozoites are found to have 1) mitochondria with tubular cristae, 2) lipid droplets (0.4–0.9 μm in diameter), 3) free ribosomes, 4) cisternae of smooth endoplasmic reticulum, 5) a nucleus with a small nucleolus which is not visible in the light microscope and consists of a cluster of ribosome-sized particles, 6) virus-like particles with 5- and 6-fold rotational symmetry (icosahedrons?) of 46 to 53 nm in diameter and 7) two centrioles in parallel orientation often with a large cylindrical, electron-dense body in the lumen of each (Figs. 14 and 17). The centrioles are often found in deep depressions in the nuclear surface. The immature trophozoites are delimited by a fibrogranular cell wall (ca. 0.1 μm thick) around which unit membranes are found and in which unit membranes are sometimes found embedded. The latter are probably derived from membranes formed in the secondary lysosome of hemocytes that have phagocytized the parasitic cells. The embedded membranes are more pronounced in mature trophozoites. Also associated with the cell surface are micropores typical of the Apicomplexa (Perkins 1969). They consist of vaselike invaginations of the plasmalemma with a narrow neck around which an electron-dense ring is found.

It is not possible to morphologically define when an immature

*Figures 1–5, 18, 19, 21–29, 30 and 35–46 are from unfixed, living cells and the remaining figures are from fixed and stained cells. The types of microscopy utilized and abbreviations are: phase contrast (PC), differential interference contrast (DIC) and bright field (BF) microscopy, transmission electron microscopy (TEM) and scanning electron microscopy (SEM). All parasite cells are *P. marinus* from *C. virginica* except for those in Figs. 21–29 which are *Perkinsus* sp. from *M. balthica* and those in Figs. 18 and 19 which are hypnospores, derived from *C. virginica*, where the oysters were experimentally infected using zoospores of *Perkinsus* sp. from *M. balthica*. Parasite cells are all from infected host tissues and not from continuous cultures, unless so noted.

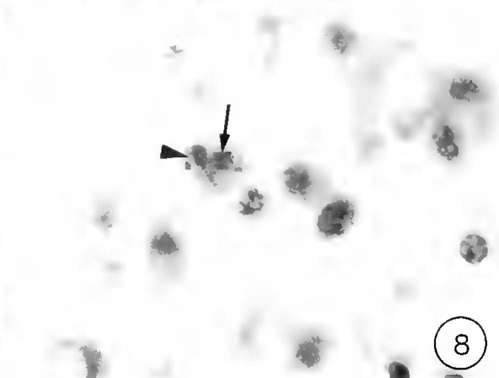


Figure 1. Immature trophozoites isolated by differential filtration and centrifugation as described in La Peyre and Chu (1994). The refringent inclusions are lipid droplets. Micrograph kindly provided by J. F. La Peyre. PC. 2,000×.

Figure 2. Fresh squash of adductor muscle with lesions containing mature trophozoites. Large vacuoplasts (arrows) are visible in some mature trophozoites but not in others (arrowheads). PC. 1,100×.

Figure 3. Trophozoites in transition from immature to mature condition, recently liberated from tomont. Vacuoplasts (arrows), lipid droplet (arrowhead). PC. 1,110×.

Figure 4. Mature trophozoite containing vacuoplast (arrow) free in eccentric vacuole. Lipid droplet (arrowhead). DIC. 1,400×.

Figure 5. Hemocyte containing seven immature trophozoites (arrows). Hemocyte nucleus (arrowhead). PC. 1,400×.

Figures 6–9. Trophozoites of varying degrees of maturity in hemocytes. The parasite cell in Fig. 6 is in transition from an immature to a mature trophozoite. The eccentric vacuole (Va) of *P. marinus* in Fig. 7 is flattened. Arrowheads in Figs. 6–8 indicate the nuclei of hemocytes containing the mature trophozoites and the arrowhead in Fig. 9 indicates the parasite cell nucleus. The arrows in Figs. 6–8 each point to a parasite nucleus and the one in Fig. 9 indicates the eccentric vacuole of the mature trophozoite. Note that enlargement of the nucleus and nucleolus occurs as maturity (enlargement) of the trophozoites progresses, with there being no nucleolus visible in the light microscope at the stage seen in Fig. 6. BF. 1,400×.

TABLE 1.

Mean sizes (standard deviations, sample sizes and ranges) in micrometers of *P. marinus* observed from Davidson's-fixed host tissue in histological sections and from unfixed host tissue smears.

P. marinus	Fixed	Unfixed
Immature trophozoites	2.1 (0.31, 25, 1.9–2.9)	2.7 (0.44, 35, 1.9–3.4)
Mature trophozoites	6.4 (1.86, 30, 2.9–9.7)	5.5 (1.94, 29, 3.9–11.6)
2-Cell tomont	4.7 (0.65, 10, 3.6–5.3)	
4-Cell tomont	5.0 (0.99, 12, 3.4–7.1)	4.9 (0.88, 10; 3.9–6.3)
8-Cell tomont	6.8 (1.29, 11; 4.5–8.6)	7.6 (0.80, 5, 6.8–8.7)
16-Cell tomont	8.0 (0.90, 12, 6.8–8.7)	—
32-Cell tomont	10.6 (1.91, 6, 7.8–12.6)	—

trophozoite becomes a mature trophozoite since there is a continuum of differentiation. At the light microscope level, the primary changes are that the immature trophozoites become spheroidal after release from within the tomont cell wall, grow in overall size and in the process acquire a prominent eccentric vacuole (Figs. 8 and 9). The nucleus enlarges and, when viewed from the side in the larger mature trophozoites of 5.8–9.6 μm, appears to be oblong and slightly convex on the centrifugal side and slightly concave on the side facing the vacuole (Fig. 9). The short axis averages 3.0 μm (range = 2.4–3.8; S.D. = 0.48; N = 11) and the long axis averages 4.1 μm (range = 2.9–4.8; S.D. = 0.59; N = 11). Viewed from the side facing the cell wall, the nuclear profile is round and averages 3.8 μm in diameter (range = 2.9–5.3; S.D. = 0.80; N = 6). The nucleolus becomes visible in the light microscope, enlarging to a mean size of 1.5 μm (range = 1.2–1.9; S.D. = 0.23; N = 17). Histological sections of Davidson's-fixed cells which have a well-defined vacuole range from 2.9 to 9.7 μm (Table 1). Immature trophozoites may contain a small primary vacuole even while still within the tomont cell wall (Fig. 17); therefore, the presence of a primary vacuole is not an indication of whether a cell is an immature or a mature trophozoite. This explains why there is a slight overlap in sizes recorded for the two types of cells (Table 1). In recording the measurements, if an eccentric vacuole could be seen, the cell was considered to be a mature trophozoite.

The larger cells have an inclusion in the vacuole termed a vacuoplast (Figs. 2–4 and 16). When fully formed (Fig. 4), the vacuoplast is free in the vacuole and moves by Brownian movement in a characteristically low-frequency oscillation due to its mass. The movement is different from inclusions in host cells which oscillate at a higher frequency; thus movement of the vacuoplast is useful in detecting mature trophozoites in fresh squashes of infected host tissue where numerous cell types are visible (Fig. 2). In addition, the vacuoplast has a characteristically light brown or golden color in unstained, living cells when viewed with bright field microscopy. A distinction of convenience between immature and mature trophozoites could be to state that mature ones have a vacuoplast and immature ones lack the inclusion. This was not used to distinguish between the two cell types in measuring cells for Table 1 because the vacuoplast is not generally preserved or visible in Davidson's-fixed and hematoxylin and eosin–stained cells.

The vacuoplast formation appears to occur by synthesis or aggregation of droplets of electron-dense material at the vacuole membrane (Figs. 15 and 16) which may, in turn, be derived from cisternae of the endoplasmic reticulum (Figs. 14 and 15). The droplets in the eccentric vacuole then coalesce and form a single inclusion body. The electron density of the droplets may either be of the same density as or may be greater than that of the vacuoplast in glutaraldehyde- and osmium tetroxide–fixed cells (Fig. 16). Whether the latter observation indicates that the droplets are in fact not precursors of the vacuoplast or there is a change in chemical composition upon coalescence is not known due to the lack of biochemical information; however, the morphological evidence indicates that the droplets do form the free inclusion. Mackin (1962) and Perkins (1969) speculated that the vacuoplast is similar to volutin (inorganic polyphosphates) found in yeast cells by virtue of its strong basophilia, high electron density and formation by vacuolar secretion of electron-opaque droplets.

Other than the differences noted above, mature trophozoites have the same ultrastructural characteristics as immature trophozoites.

When palintomy is initiated, mature trophozoites appear to subdivide the eccentric vacuole so that the cytoplasm assumes a frothy appearance (Fig. 10) as in zoosporulation (see below). Division then occurs by successive bipartitioning of the cell in which karyokinesis is followed by cytokinesis and then repeated until tomonts, containing what appear to be 4–32 (rarely 64 and most often 8 or 16) immature trophozoites, are formed (Figs. 11–13 and 17). It is not possible to accurately count the number of cells above 8 in histological sections; however, it is reasonable to assume that if successive bipartitioning continues, 16, 32 and 64 immature trophozoites could be formed. Counts of the tomonts with large numbers of immature trophozoites indicate that the 16, 32 and 64 cells are probably formed. Odd numbers of cells can be found within a tomont cell wall probably due to lack of division of a daughter cell or death of an occasional immature trophozoite. This is suspected to occur, because when cell counts are made from FTM-treated and infected oyster tissue, the number of *P. marinus* hypnospores in a cluster is often found to be an odd number. They are easy to count because of their size (often 20–80 μm in diameter), and if squashes of fibrous tissues such as adductor muscle are made, the clusters tend to remain intact and are not dispersed (Fig. 18). Single clusters as large as 32 cells which appear to have arisen from a single tomont have been observed.

Tomonts are about the same size as the larger mature trophozoites; therefore, palintomy involves subdivision of the mature trophozoite without a noticeable increase in cytoplasmic mass, resulting in smaller and smaller cells. As can be seen from the nuclear sizes, the same applies to the nucleoplasm (Figs. 12 and 13). The data presented in Table 1 were obtained by measuring only the spheroidal cells in histological sections of Davidson's-fixed oyster tissue. Only two cells which could have been in the 64-cell stage were observed. They were 9.7 and 10.7 μm in diameter. All above measurements were from infected *C. virginica* obtained from Virginia's waters of the Chesapeake Bay.

Release of the immature trophozoites occurs upon rupturing of the tomont cell wall (former mature trophozoite wall) and often occurs in a phagosome of a host hemocyte (Fig. 5). Upon lysis of the hemocyte the immature trophozoites are released and most often are phagocytized by other hemocytes, except in advanced infections where the host is overwhelmed by large numbers of trophozoites. Palintomy may occur in any tissue of the host, but is most often found in the connective tissue, between epithelial cells of the gut, gill and digestive gland and in lesions of the gut and gill epithelia. Ganglia are the least likely to be invaded.

The ultrastructure of palintomy is not well known. The tomont cell wall remains intact during cell multiplication and is ca. 0.12–

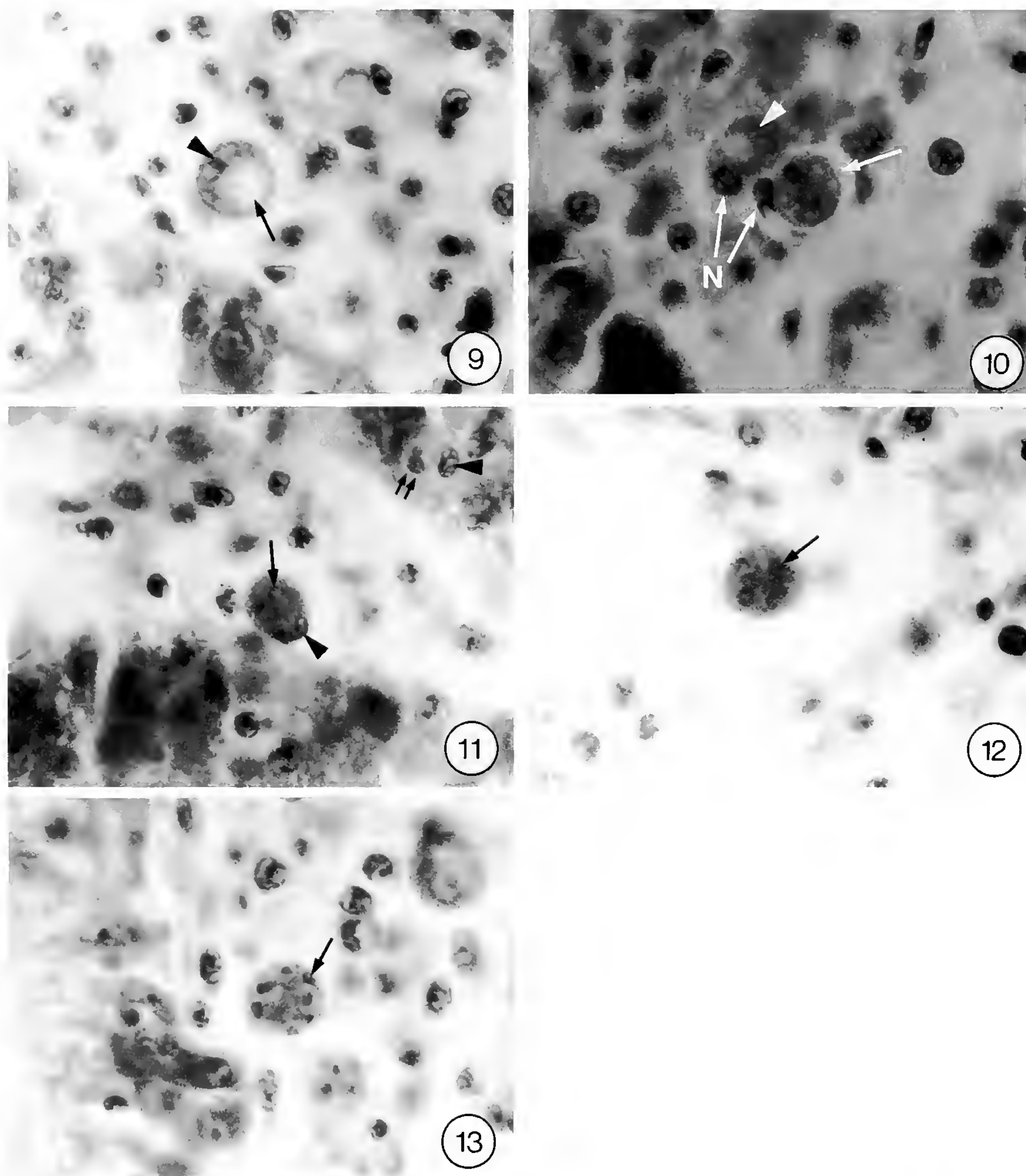


Figure 10. Mature trophozoite (arrow) in which eccentric vacuole has been subdivided prior to first cytokinesis. Mature trophozoite with eccentric vacuole (arrowhead); hemocyte nuclei (N). BF. 1,200×.
Figure 11. Two-cell tomont and young immature trophozoite (double arrow) in hemocytes. One tomont nucleus (arrow); hemocyte nuclei (arrowheads). BF. 1,400×.
Figures 12 and 13. Four- and eight-cell tomonts. Tomont nuclei (arrows). Note that the nuclei decrease in size from the two-cell (Fig. 11) to the eight-cell stage (Fig. 13) and that there are no nucleoli visible after first division of the tomont nucleus. BF. 1,300×.

0.18 μm thick. Once immature trophozoites have been delineated, a thin wall (≤0.1 μm) is formed around each. Mitosis has not been seen in the hundred or more cells that have been observed in the electron microscope, thus it must occur rapidly. Centrioles most probably serve as the spindle pole bodies during mitosis. From the limited numbers of observations of cytokinesis, it appears that the plasmalemma infolds centripetally to divide a binucleate cell resulting from a single mitotic event. The possibility exists that some cells may undergo progressive cleavage (multiple fission) by fusion of flattened vesicles lined end-to-end as was suggested by Perkins (1969). This form of cytokinesis involves several mitoses followed by cytokinesis to yield the uninucleate immature trophozoites.

Palintomic Zoosporulation

After being held in FTM, reconstituted in estuarine or sea water in the range of ca. 20–30 ppt for several days at room temperature,

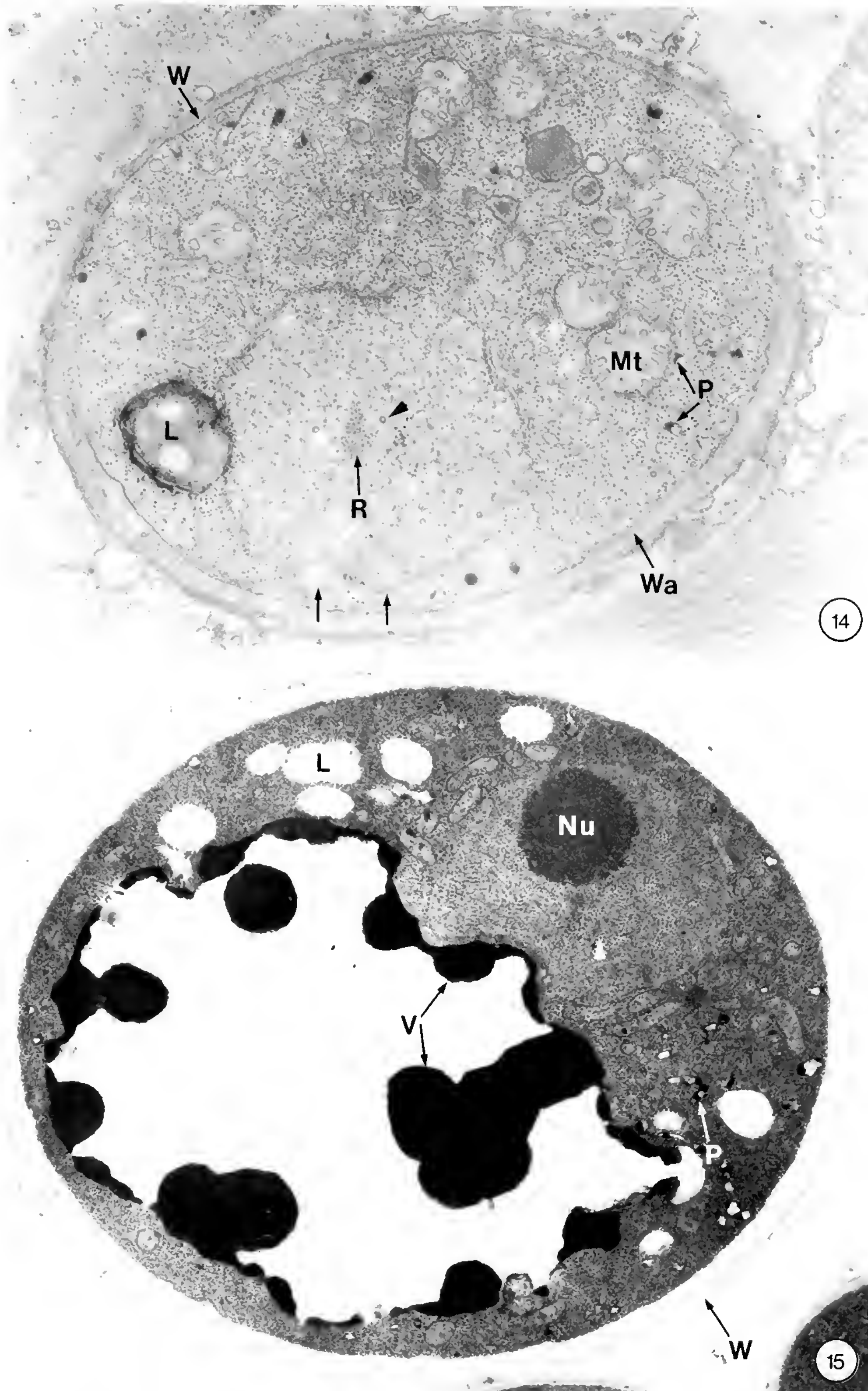
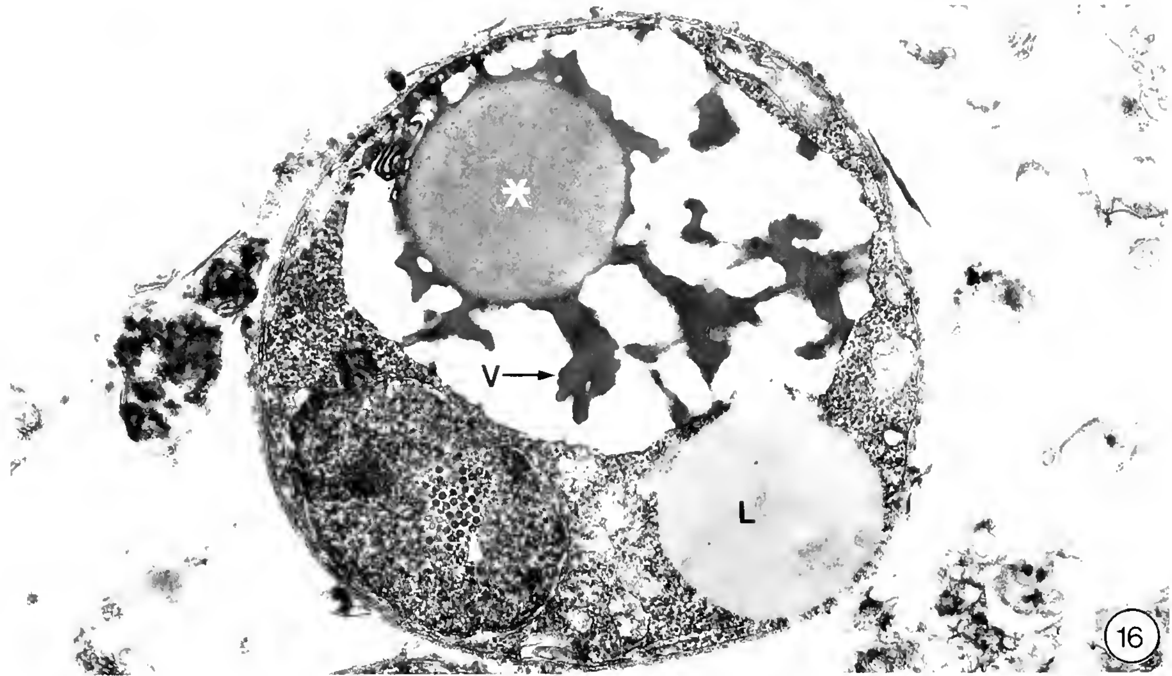


Fig. 14. Immature trophozoite. Note lack of a large eccentric vacuole. Centrioles (arrows); mitochondrion (Mt); virus-like particle in nucleoplasm (arrowhead); ribosomes of nucleolus (R); immature trophozoite cell wall (W) fused with tomont wall (Wa); lipid droplet (L); presumptive precursor material (P) of vacuoplast in elements of endoplasmic reticulum. TEM. 27,000×.

Figure 15. Mature trophozoite from axenic culture of *P. marinus*. Nucleolus (Nu); lipid droplet (L) largely dissolved during fixation and embedding; vacuoplast material (V) being secreted into eccentric vacuole; presumptive precursor material (P) of vacuoplast in cisternae of the endoplasmic reticulum; cluster of intranuclear virus-like particles (arrowhead); cell wall (W). Micrograph kindly provided by J. F. La Peyre. TEM. 13,000×.



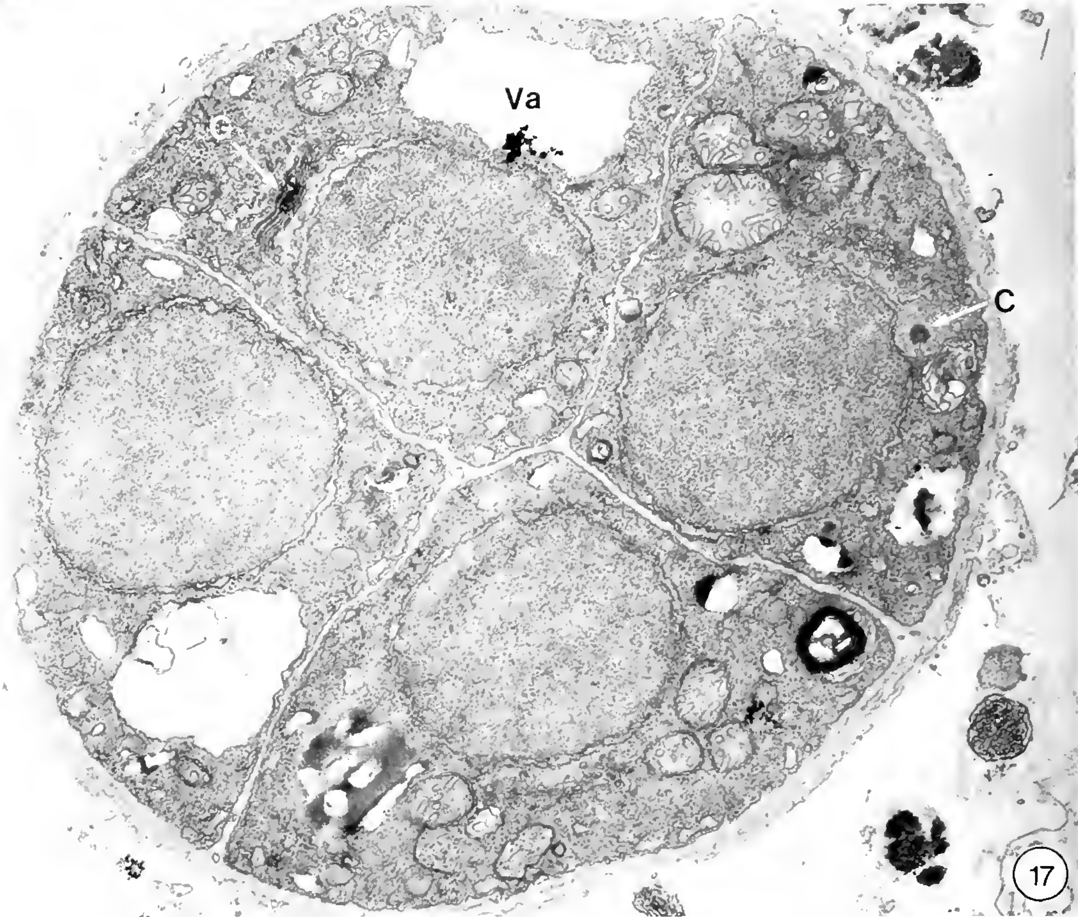
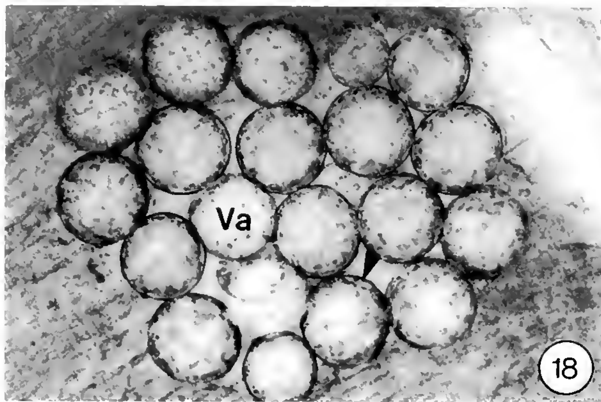
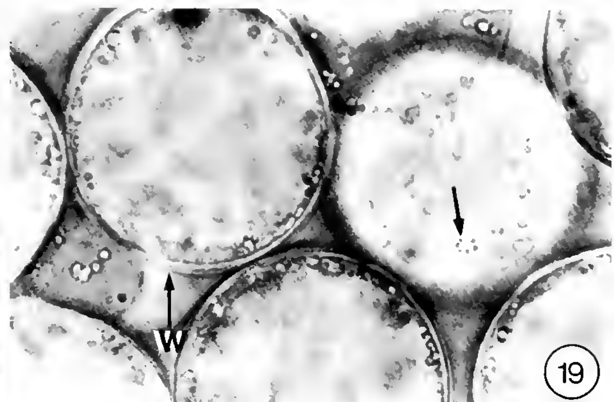


Figure 16. Mature trophozoite in which a vacuoplast (asterisk) has been formed, presumably from intravacuolar vacuoplast material (V). Unlike this cell, in most preparations the vacuoplast and vacuoplast material have the same electron density. Lipoid droplet (L); intranuclear virus-like particles (arrowhead). TEM. 26,000×.

Figure 17. Eight-cell tomont. Centriole with electron-dense inclusion (C); Golgi body (G); developing eccentric vacuole (Va); mitochondrion (Mt). Note regular pattern of cytokinesis yielding cuneiform, immature trophozoites. TEM. 28,000×.





Figures 18 and 19. Hypnospores found in adductor muscle of previously uninfected *C. virginica* which was exposed to zoospores of *Perkinsus* sp. from *M. balthica*. The hypnospores were induced to form in FTM and are indistinguishable from those of *P. marinus*. Note the large eccentric vacuole (Va) in each cell around which there is a thin layer of cytoplasm pressed against the cell wall (W). Nucleus (arrowhead); lipid droplets (arrow). BF. Fig. 18 = 155×; Fig. 19 = 500×.

Figure 20. Hypnospore of *P. marinus* equivalent to those in Figs. 18 and 19. Note the extreme size of the eccentric vacuole, the thick cell wall and the large expanses of membrane-free cytoplasm with ribosomes. Nucleus (N); lipid droplet (L). TEM. 10,600×.

most immature and mature trophozoites enlarge to various sizes, often into the 30–80 μm range. I have observed extremes of up to 480 μm in diameter. In the process, the vacuoplast disappears, the cell wall becomes thicker and the eccentric vacuole enlarges markedly resulting in a thin layer of cytoplasm pressed against the cell wall (Figs. 18–20 and 35). The mitochondria may disappear or are reduced to thin profiles with few cristae probably as a result of being held in the anaerobic or microaerophilic conditions extant in the medium. Otherwise, the ultrastructure is the same as in the mature trophozoites except that the nucleus enlarges and contains a prominent nucleolus. These cells are termed hypnospores (see terminology section in the DISCUSSION).

In his original observation of enlargement in FTM, Ray (1952) also found that a diluted (1:25) aqueous solution of Lugol's KI-I_2 stained the hypnospores blue, blue-black or blue-green. Since the solution could be applied to pieces of oysters after holding in FTM and then squashed under a coverslip, a rapid technique for detection of the pathogen was developed. Based on their studies in which they used cytochemical and enzyme extraction techniques, Stein and Mackin (1957) suggested that the thick hypnospore wall consists of hemicellulose and cellulose. They speculated that the staining reaction in Lugol's iodine solution in the absence of acid hydrolysis is due to the combination of the two polysaccharides, a combination which is lacking in the trophozoites. The latter do not stain blue, blue-green or blue-black in Lugol's solution. Perkins and Menzel (1967) demonstrated a 20–30-μm diameter, fibrillar network in hypnospore walls which had been isolated and treated with a 50% solution of 0.25N NaOH in commercial bleach (Clorox). This network most probably represents the polysaccharide complex.

If the hypnospores are washed free of the FTM and placed in estuarine or sea water of ca. 20–30 ppt, zoosporulation by palintomy ("palintomic zoosporulation" shortened herein to "zoosporulation") may occur. However, as opposed to what has been observed over the last 20 years, in recent years preparations of *P. marinus* hypnospores show a low incidence of zoosporulation without release of the zoospores (see comments in the Materials and Methods section). Therefore, rather than using earlier published micrographs, zoosporangial development is illustrated using cells of *Perkinsus* sp. from *M. balthica*, where the process is indistinguishable from that seen in *P. marinus* (see Fig. 1A–L in Perkins and Menzel 1966). The morphogenesis consists of digestion or subdivision of cytoplasmic inclusions and lipoidal droplets so that the cytoplasm becomes finely granular with active Brownian movement. A lens-shaped clear area (rarely two areas) under the cell wall can be seen to form and the nucleus enlarges markedly (Figs. 21 and 22). Over the clear area a discharge pore is formed around which the edges of the pore are ragged and outwardly flared, indicating that pore formation is an explosive event. Coupled with pore formation is the extension of a discharge tube (sometimes two) which unfolds from the lens (Fig. 23). At the base of the tube and occluding the pore is an ovoid plug. Ultrastructural observations of the pore complex reveal that the plug is an enlargement of a secondary cell wall layer which is synthesized beneath the preexisting wall. The tube is in continuity with the plug (Perkins and Menzel 1967).

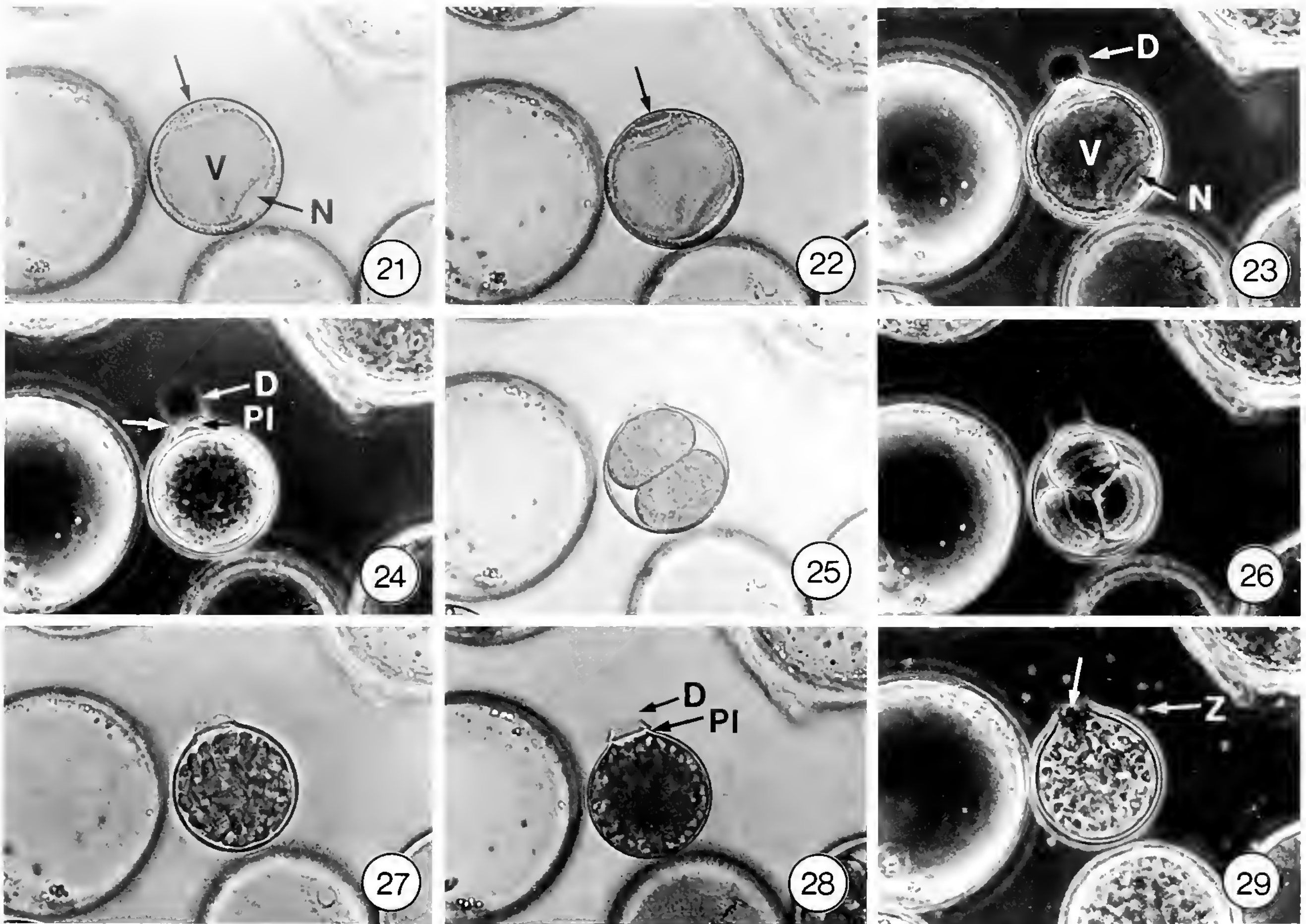
As in palintomy observed in host tissues, the eccentric vacuole is subdivided into small vacuoles distributed randomly in the cytoplasm (Fig. 24) as the cell contracts and pulls away from the cell wall. Presumably much of the fluid originally in the eccentric vacuole is squeezed into the space between the cell and its wall.

The details of mitosis have not been elucidated; however, fine structure of the few mitotic profiles which have been observed indicate that the nuclear envelope may remain intact and the centrioles serve as the spindle pole bodies. It appears that deep, channel-like invaginations of the nuclear envelope are formed with the nuclear envelope intact. Bundles of microtubules are found in the invaginations attached to the envelope at kinetochore-like structures. The details are being elucidated and will be described in a separate publication. Thus, mitosis resembles that seen in some dinoflagellates such as *Amphidinium carterae* (Dodge 1987). However, the dinokaryotic condition of coiling of the chromatin is not observed in *P. marinus*. The chromatin appears as electron-dense granular aggregates of varying density whether in interphase or during mitosis.

After the first karyokinesis, cytokinesis occurs by an equatorial pinching in half of the binucleate cell to yield two separate and distinct cells (Fig. 25). This process is repeated so that after each cycle, each nucleus and whole cell become smaller and smaller until, just before the final cytokinetic event, the cells are no longer spheroidal but have become shaped like a kernel of rice (Figs. 26 and 27). At this time synthesis of the flagella has occurred, as evidenced by movement of the cells within the zoosporangial wall and by the fact that free quadriflagellated zoospores can sometimes be observed instead of the normal biflagellated ones, presumably as a result of premature release from the zoosporangium and not isogametic copulation. A final cycle of karyokinesis, followed by cytokinesis, then occurs, and biflagellated zoospores can be seen to swim actively in the zoosporangium for periods of up to 2 days (Fig. 28) before the plug at the base of the discharge tube disintegrates and the zoospores swim out of the zoosporangium through the tube (Fig. 29). They measure 2–3 × 4–6 μm, are rounded at the anterior end and are pointed at the posterior end with a subapical indentation (groove?) on one side about one third of the cell body length from the anterior extremity. The flagella arise from the indentation (Figs. 30 and 31).

Before the final cycle of karyokinesis and cytokinesis, synthesis of the apical complex, which characterizes the Apicomplexa, is initiated. The complex consists of a conoid, polar ring, rhoptries, microneme-like structures and subpellicular microtubules (Figs. 31 and 32). The conoid is anteriorly situated and composed of microtubular units arranged in a helical coil forming a truncated cone which is open along one side (Fig. 32). Around the anterior end is a ring of electron-dense material forming the anterior polar ring to which are attached microtubules of the cytoskeleton which extend beneath the plasmalemma to the posterior end of the zoospore where there is a posterior ring. The microtubules do not appear to attach to the latter ring (Perkins 1976a). Attached to and below the anterior polar ring and surrounding the anterior third of the conoid is a truncated cone open at either end consisting of what appears to be the same material which forms the ring (Perkins 1976a). Flattened alveoli (vesicles) are found between the plasmalemma and the cytoskeleton at the anterior end of the cell but are not repeated posteriorly as in other Apicomplexa. A thin layer of granular material is present beneath the plasmalemma, instead of alveoli, starting at the posterior margin of the alveoli and extending beneath the plasmalemma around the rest of the cell.

The rhoptries are membrane-bound, flask-shaped sacs of electron-dense material which have the neck of the flask inserted into the lumen of the conoid and extend almost to the anterior end of the cell. A group of long membrane-bound organelles are also found with the anterior ends inserted into the conoid lumen and



Figures 21–29. Zoosporulation in *Perkinsus* sp. from *M. balthica* after placement of the cells in estuarine water from FTM. The morphogenesis is indistinguishable from that of *P. marinus*. After 42 hr, 45 min in estuarine water at 25°C (Fig. 21) the nucleus (N) has enlarged markedly and there is a thickening of the cytoplasm (arrow) away from the nuclear region (N). A plaque of wall material then formed beneath the original cell wall (Fig. 22; arrow), followed by formation of a pore in the cell wall and a discharge tube (D) from the plaque (Fig. 23). At the base of the pore is a plug of secondary wall material also derived from the plaque (Fig. 23: eccentric vacuole, V; nucleus, N). The cell then subdivided, the eccentric vacuole forming a frothy cytoplasm in which the first karyokinesis occurred (Fig. 24: edge of pore, arrow, beneath which is the plug, Pl). First cytokinesis occurred (Fig. 25) followed by more cycles of karyokinesis and cytokinesis (Figs. 26–28) until hundreds of motile zoospores had been formed. After the plug disappeared and the zoospores had matured, they swam through the discharge tube to the outside. Free zoospore (Z); zoospore emerging from the zoosporangium (arrow). BF and PC. 440×.

extending almost the full length of the cell. They have been called rectilinear micronemes as a term of convenience by Perkins (1976a) only because they have a diameter much the same as micronemes, are membrane bound and contain electron-dense material as in other Apicomplexa. They differ in that the length is much greater, and they are not completely filled with electron-dense material. Another type of organelle is attached to the posteriorly extended part of the conoid in a row of membrane-bound, long and wavy units of about the same diameter as the rectilinear micronemes and contains electron-dense material. These extend the length of the cell and have been termed conoid-associated micronemes, also as a term of convenience. It is not known whether either type of "micronemes" is homologous to the micronemes of the Apicomplexa (Figs. 31 and 32).

In the anterior end of the zoospore is found a U-shaped large vacuole (Fig. 31) which contains electron-dense material (not visible in Fig. 31) resembling the droplets which contribute to the vacuoplast material found in mature and immature trophozoites. The material is in contact with the vacuole membrane and does not fill the vacuole. Other organelles in the zoospore include a single Golgi body located next to the nucleus, one mitochondrion with tubular cristae located on the dorsal side of the cell (if one accepts that the side to which the flagella are attached is ventral) and a ventrally located nucleus with well-defined heterochromatin. Lipid droplets are present in the cell, generally in the posterior end, and are often visible as a single refractive inclusion when viewed using phase contrast microscopy.

The posteriorly directed flagellum is almost straight and has no mastigonemes. The anteriorly directed flagellum is about three times as long as the posterior one and is always more or less coiled whether contracting and engaging in generation of forward motion of the cell (Fig. 30) or extending in the recovery mode. The coiling has no obvious, fixed pattern or form except that at the end of the recovery mode it generally is more tightly coiled than when it has completed contraction to generate forward motion. Filamentous mastigonemes are found along one side of the anterior flagellum in groups of about five, and at the base of each group is a distally pointed, flexed and spur-like unit (Fig. 33). Units resembling the filamentous mastigonemes are found in cytoplasmic vacuoles of differentiating zoospores. Presumably, assembly of the mastigo-

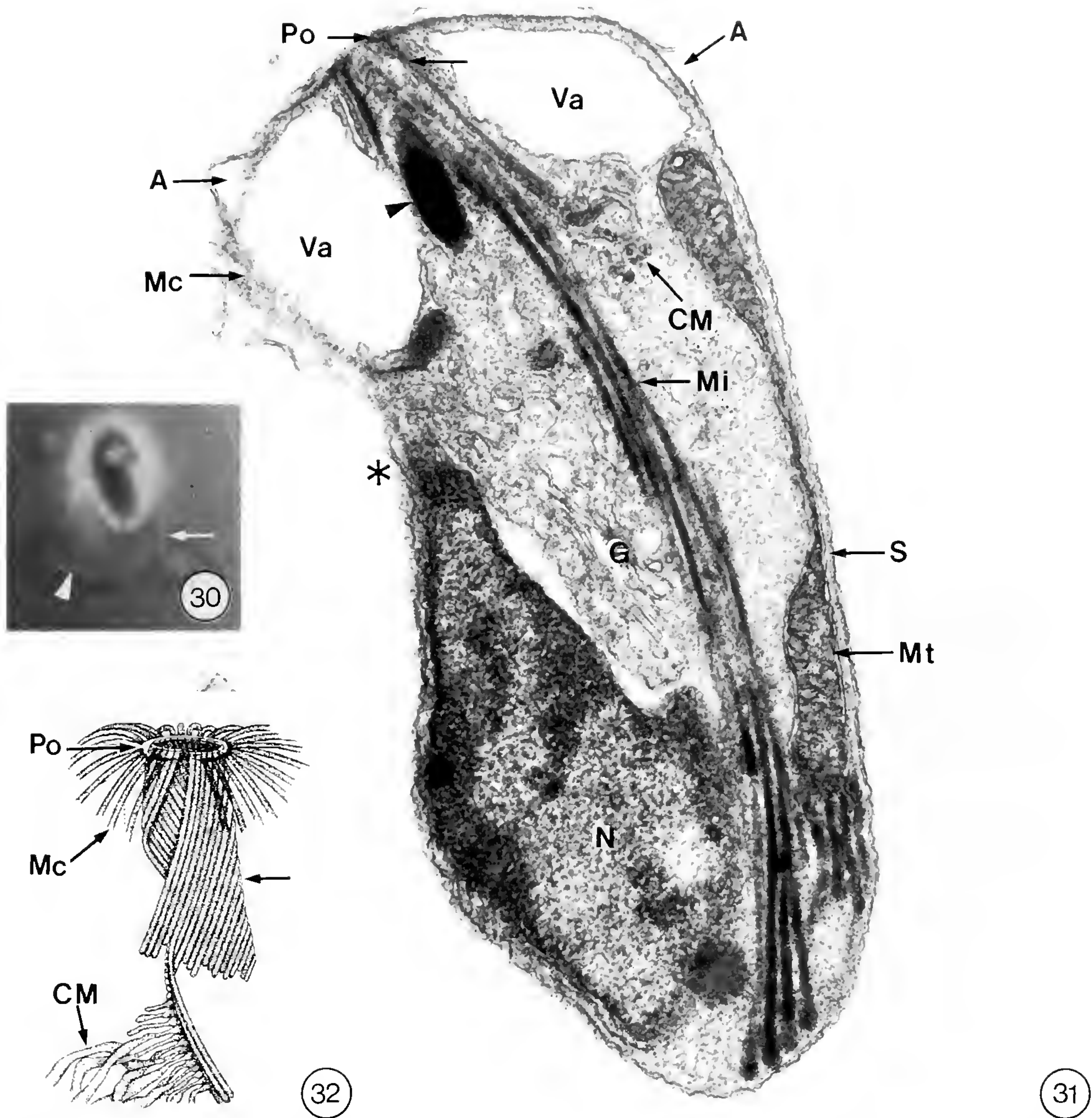


Figure 30. Actively swimming zoospore with rounded anterior end of cell facing the top of the micrograph. Note coiling of anterior flagellum (arrowhead) around the cell body in retraction phase of flagellar movement and the straight, posteriorly directed, posterior flagellum (arrow). The subapical indentation in the right side of the cell body is the site from which both flagella arise. PC with strobe flash. 2,200×.

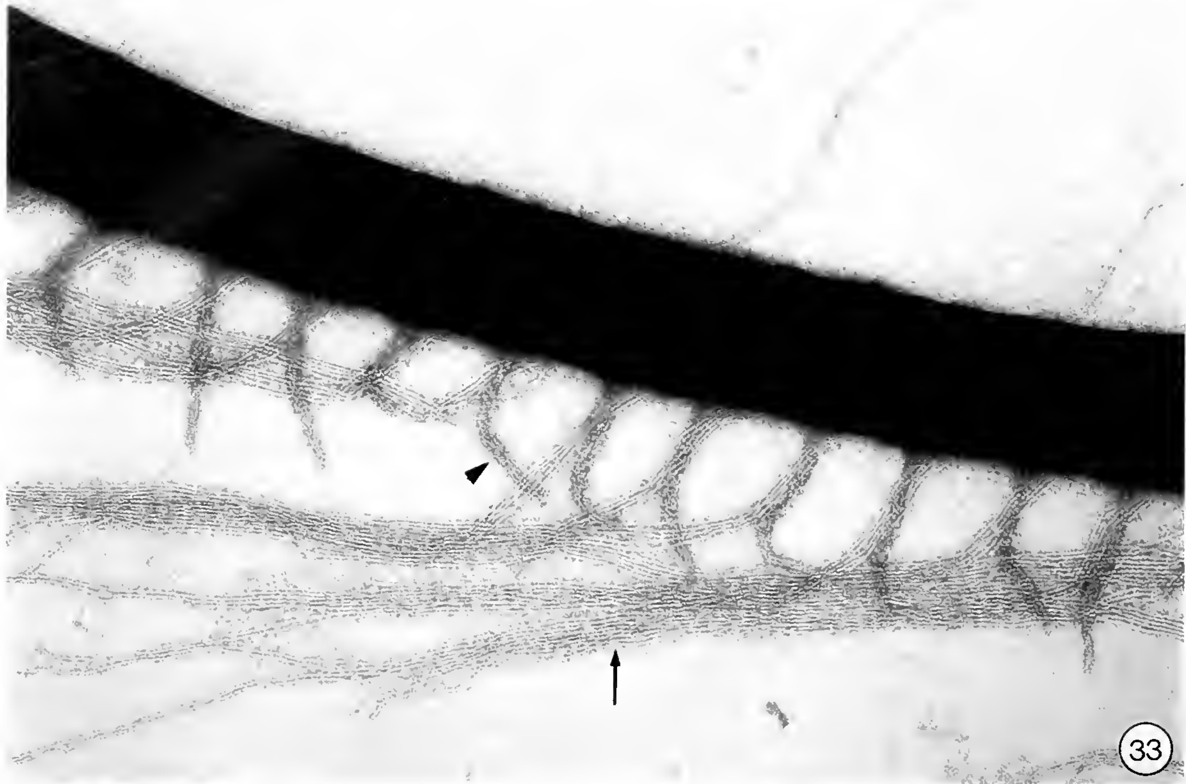
Figure 31. Longitudinal section through zoospore in which can be seen the conoid (arrow), rhoptry (arrowhead), general region of flagellar attachment (*), alveoli (A), conoid-associated micronemes (CM), Golgi body (G), microtubules of cytoskeleton (Mc), rectilinear micronemes (Mi), dorsally located mitochondrion (Mt), nucleus (N), polar ring (Po), subpellicular granular layer (S) and vacuoles (Va). TEM. 40,000×. From Perkins (1987).

Figure 32. Diagram of part of apical complex including conoid (arrow), conoid-associated micronemes (CM), microtubules of cytoskeleton (Mc) and polar ring (Po). From Perkins (1976a).

nemes occurs in the vacuoles which are derived, in turn, from the Golgi body.

The kinetosome has a prominent electron-dense, cylindrical inclusion in the mid-region of its lumen with struts connecting it to the A and B microtubules of the kinetosome triplet blades (Perkins 1988). The distal end of the kinetosome contains a cup-shaped structure with the open end of the cup facing the proximal end (Fig. 34). Several microtubular rootlets arise from a complex, electron-dense region at the proximal end of the kinetosome. The other details of the flagellar apparatus have not been elucidated.

At the base of the flagellum is a transition plate where the central pair of microtubules of the axoneme terminate. Distal to



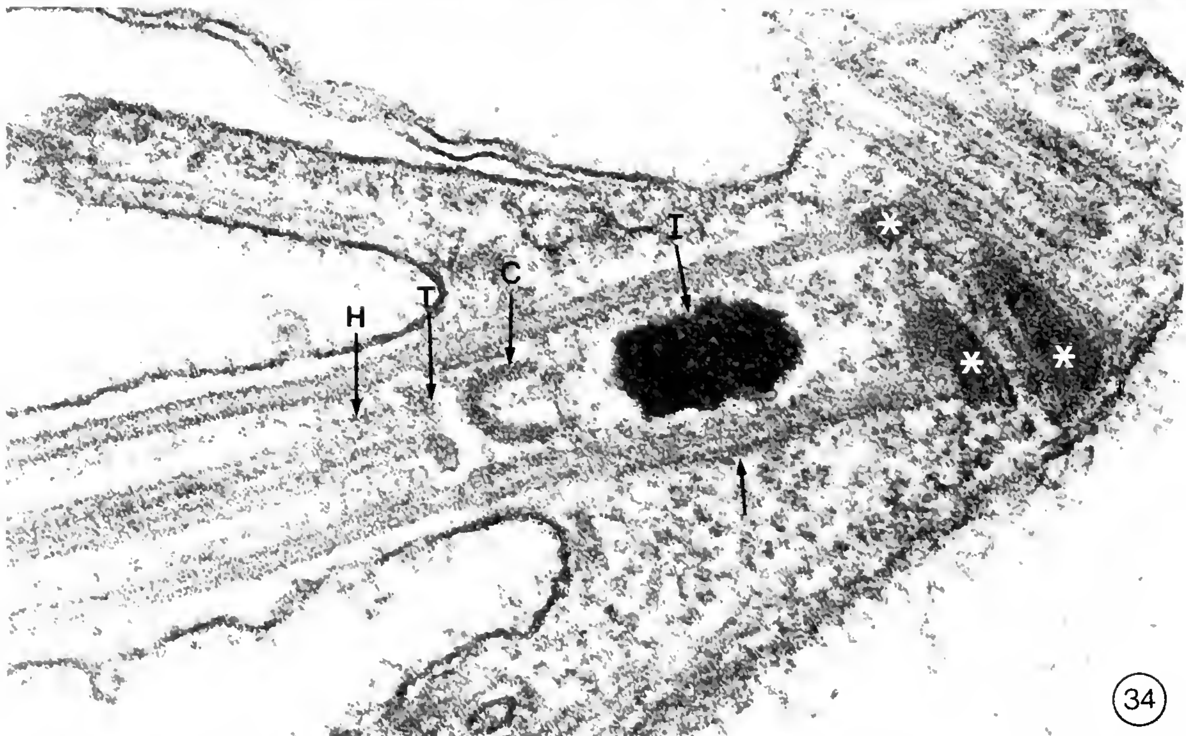
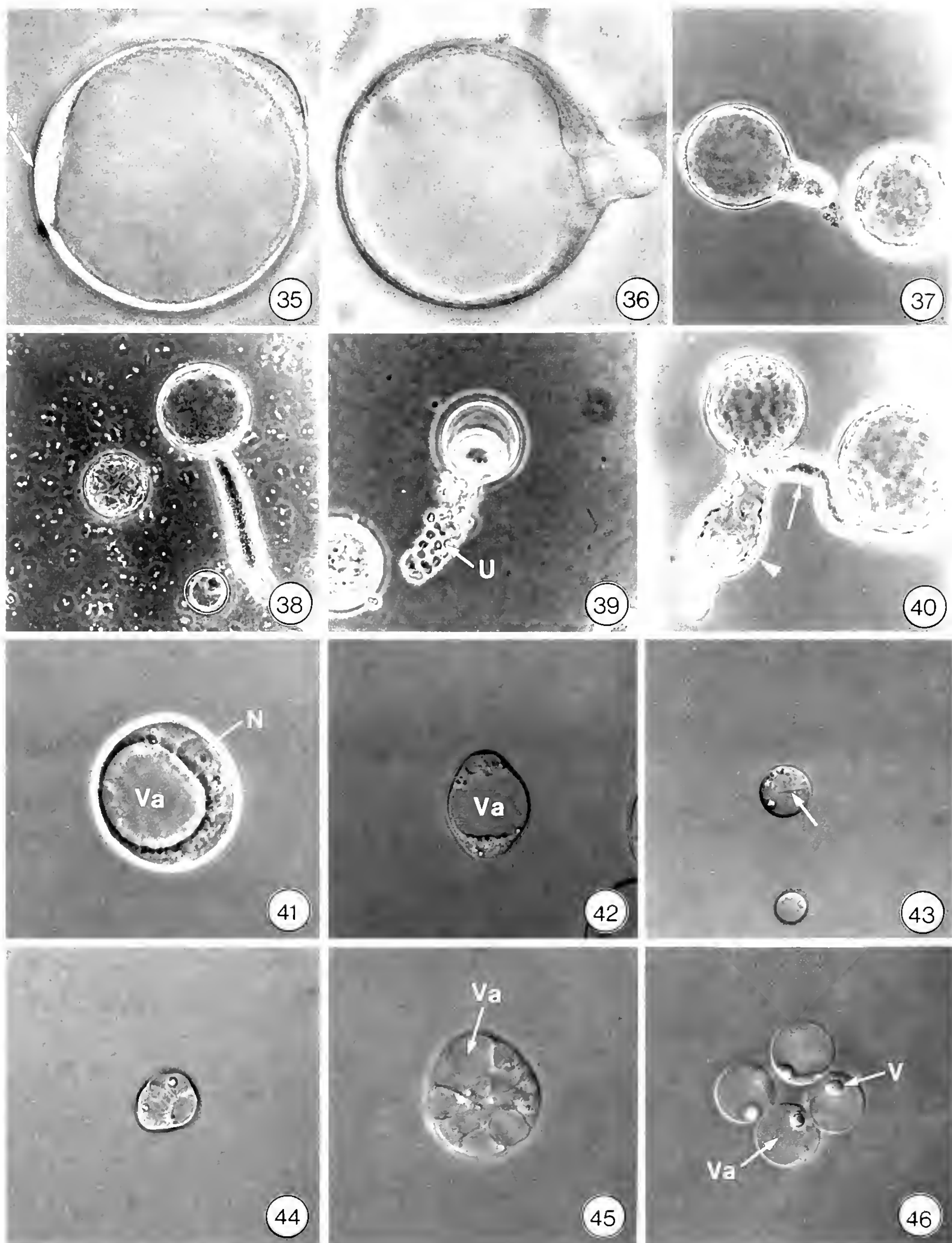


Figure 33. Uranyl acetate–stained whole mount of the mid-region of a zoospore's anterior flagellum. Filamentous mastigonemes (arrow), spur (arrowhead). There is a single row of mastigonemes and spurs attached periodically with a cluster of four to five mastigonemes accompanying each spur. TEM. 130,000×.

Figure 34. Kinetosome of zoospore (arrow) and environs in which can be seen a cylindrical inclusion (I); cup-like structure (C); terminal helix (H); terminal plate of central pair of axoneme microtubules (T); and presumptive microtubular organizing centers (*) at base of kinetosome from which microtubular rootlets arise. TEM. 156,000×.



Figures 35–39. Hypnospores in which the progression of events leading to tubular outgrowth formation, followed by subdivision into unicells, can be seen. The cell wall around the tubular outgrowth is thinner than that of the original wall of the hypnospore. Nucleus (N); cytoplasm (arrowhead); bulging of primary cell wall over the region of outgrowth formation (arrow); unicells (U). Figs. 35 and 36 = DIC; Figs. 37–39 = PC. Figs. 35 and 36 = 800×; Figs. 37 and 39 = 400×; Fig. 38 = 300×.

Figure 40. Two hypnospores connected by a tubular outgrowth. The outgrowth (arrow) of the cell on the right has fused with the outgrowth (arrowhead) of the cell on the left. Whether cytoplasmic mixing and nuclear fusion occur in cells connected in this manner has not been determined. PC. 300×.

Figures 41–46. Immature and mature trophozoites and tomonts in axenic culture. Fig. 41 = mature trophozoite; Fig. 42 = presumptive binary fission of mature trophozoite; Fig. 43 = first division of mature trophozoite engaged in palintomy. The eccentric vacuole has not subdivided as is seen in palintomy in infected oysters (see Fig. 10) (interface between two immature trophozoites = arrow). Figs. 44 and 45 = presumptive tomonts with four or five and more than eight immature trophozoites, respectively, in which the cells have large vacuoles and are irregular in shape; Fig. 46 = four mature trophozoites, in each of which is seen a single vacuoplast which appears attached to the eccentric vacuole membrane. Nucleus (N); vacuoplast (V); eccentric vacuole (Va). DIC. Figs. 41 and 46 = 1,000×; Figs. 42 and 43 = 800×; Fig. 44 = 900×; Fig. 45 = 750×.

the plate is a transitional helix wrapped around the central pair of microtubules (Fig. 34).

Although it has not been visualized, presumably the zoospore makes contact with an epithelial cell of the host and the rhoptries induce a depression in the host cell surface so that the parasite is internalized in a vacuole as occurs with other Apicomplexa (Perkins 1992). The flagella would be lost in the process as would the apical complex, and the cell would round up to become a cell resembling an immature trophozoite. Alternatively, the zoospores could be phagocytized by hemocytes with the loss of the same organelles.

Nonzoosporulating Development of Hypnospores

It has been observed by Ray (1952, 1954) that hypnospores while in FTM will form tubular outgrowths with bulbous termini, structures which he suggested were involved in microconidial development as in the Entomophthorales of the Eumycota. I have found in recent years that when hypnospores are removed from FTM and placed in sea water such outgrowths are formed, but I have not seen them while the cells are still in FTM. The outgrowths begin as a thickening in the cytoplasm away from the nucleus. This thickening bulges the cell wall outward (Fig. 35). A distinct cytoplasmic protrusion then forms encased in cell wall material thinner than that of the preexisting hypnosporal cell wall (Figs. 36 and 37). This protrusion continues to grow outward until it is as much as two to three times as long as the diameter of the hypnospore (Fig. 38). The nucleus has been observed to remain within the spheroidal part of the cell wall after the outgrowth is quite long. At some undetermined time the nucleus probably enters the outgrowth and divides because small elongate cells are cleaved from the protoplasm in the tube. Presumably the cells are nucleated but this has not been demonstrated. The cells spill out of the distal end of the outgrowth and are found clustered around the outgrowth (Fig. 39). In other hypnospores, the protoplast may migrate out of the distal end of the outgrowth, form a bulbous mass and then subdivide to form similar-sized cells as above. This bulbous mass resembles the structures described by Ray (1952, 1954).

An outgrowth may fuse with a neighboring cell (Fig. 40), but it is not clear whether mixing of cytoplasm or karyogamy occurs. In addition, an outgrowth may have irregularities in the surface which resemble limited branching of hyphae in the lower fungi. No ultrastructural investigations of the outgrowths have been conducted.

Development in Axenic Culture

When grown in the Kleinschuster and Swink (1993) medium, mature trophozoites (Figs. 41 and 46) of *P. marinus* were observed to have a wider range of sizes, from 3.8 to 43.2 μm in diameter (N = 100), than those observed in oyster tissue (range = 2.9–11.6), and the mean was 11.0 μm (S.D. = 9.4). The latter is about twice the diameter of mature trophozoites in the oyster where the means were 6.4 and 5.5 in fixed and unfixed cells, respectively. The differences could be due to the enriched nutritional environment of the culture medium. In addition, the vacuoplasts (Fig. 46) were sometimes observed to be fixed to the eccentric vacuole membrane and did not oscillate by Brownian movement as in those from oyster tissue. In the ultrastructural observations of cultured mature trophozoites (La Peyre et al. 1993 and Fig. 15), the cellular structure resembled that observed in uncultured cells from oyster tissue (Fig. 14 and Perkins 1969) including the presence of ca. 50-nm-diameter virus-like particles in the nucleoplasm.

The primary differences between cells cultured in the Kleinschuster and Swink medium and naturally occurring cells involve the forms which cytokinesis assumes as visualized at the light microscope level. No ultrastructural observations of cytokinesis were made. Both binary fission and palintomy appear to result in immature trophozoite formation in the cultured cells. In the former, the nucleus appears to divide, the cytoplasm migrates to form polar thickenings (Fig. 42), and then it is assumed that the cell pinches in half in the equatorial region without any other subdivision. Various degrees of equatorial pinching have been observed; however, completion of division has not been documented. Asymmetric binary fission (budding) of a smaller cell from the parent meront also appears to occur (see also Fig. 6 of La Peyre et al. 1993).

Palintomy in cultured cells occurs most often, as observed in infected oysters; however, there is a degree of plasticity or variability of form which is not seen in oyster tissue. The eccentric vacuole often does not markedly subdivide prior to cytokinesis as seen in Fig. 10, but rather the vacuole may be halved with each cell division or it may lie at one pole of the cell and the cytoplasm divides at the other end (La Peyre et al. 1993; their Figs. 3–5). This suggests that progressive cleavage (multiple fission) is involved. In some cases there may be only two, three or four cells formed in a crescent of cytoplasm at one pole without any change in the vacuole. In Fig. 43, cytokinesis has just occurred or is nearing completion, and the interface between two immature trophozoites is visible. After the first cytokinesis the immature trophozoites may become irregular in size and shape (Figs. 44 and 45), the irregularity being more apparent the more numerous the daughter cells. However, most often palintomy occurs as seen in uncultured cells in the host. More observations are necessary to determine whether the infrequently observed "frothy" cells similar to the one in Fig. 45 are in fact viable or are anomalous and undergo lysis without completion of cytokinesis.

DISCUSSION

An attempt has been made herein to provide a useful reference source for recognizing *P. marinus* at both the light and electron microscope levels of detail, summaries of which can be found in Figs. 47 and 48. There are a number of heterotrophic, mostly saprobic, but some pathogenic species of coccoid protist in the estuarine and marine environments which could be confused with *P. marinus* when the cells are observed free of the host, *C. virginica*. For example, this could occur in observing cultures of microorganisms or samples of sea water, sediments or tissues of organisms presumed to be parasitized. The most likely to present difficulties with identification are members of the family Thraustochytriaceae Sparrow 1943 of the phylum Labyrinthomorpha Page in Levine et al. 1980 (Corliss 1994). Members of this family superficially resemble *Perkinsus* spp. in cell size, shape and mode of cytokinesis, but generally lack the large eccentric vacuole and form biflagellated zoospores with a bilateral array of tubular mastigonemes, ectoplasmic nets from specialized organelles termed sagenogens and laminated walls consisting of circular plates (Perkins 1974, 1976c; Porter 1990). They normally do not invade tissues of *C. virginica*, but I have observed (unpublished data) small clusters of *Labyrinthuloides*-like cells in the connective

tissue beneath epithelia of the oyster. *Labyrinthuloides haliotidis* invades the host tissues and causes disease of juvenile abalone (Bower 1987); other species of Thraustochytriaceae cause diseases of other molluscs (Polglase 1980, Jones and O'Dor 1983, McLean and Porter 1987).

In addition to the differences noted above which are ultrastructural or require the observation of zoosporulation, one can differentiate between *P. marinus* and the thraustochytriaceous species by use of the Ray FTM technique. If the cells enlarge in FTM, form thick cell walls and acquire a markedly large eccentric vacuole, then they are most probably a species of *Perkinsus*. Furthermore, if the application of Lugol's iodine solution as prescribed by Ray (1952) yields a blue, blue-black, black or blue-green staining of the cell wall after incubation in FTM, then the certainty of the identification is greater. No other protistan cell walls have been shown to stain these colors in Lugol's solution unless pretreated with strong acid, with the possible exception of some ciliate cysts (Perkins and Menzel 1967). The final confirmation would be to induce zoosporulation and find biflagellated zoospores with a unilateral row of filamentous mastigonemes and spurs on the anterior flagellum and an apical complex. However, as noted above, induction of zoosporulation in *P. marinus* has been shown to be difficult in recent years, as opposed to other species of *Perkinsus*. Short of ultrastructural information, the best characters which can be used to differentiate between the thraustochytriaceous forms and *P. marinus*, in the light microscope and without treatment in FTM, are the presence, in mature trophozoites of *P. marinus*, of a large eccentric vacuole often with a vacuoplast and hyaline cytoplasm with a few refringent inclusions. The cytoplasm of the thraustochytriaceous species is most often finely granular and a large eccentric vacuole is infrequently seen, never with a prominent vacuoplast.

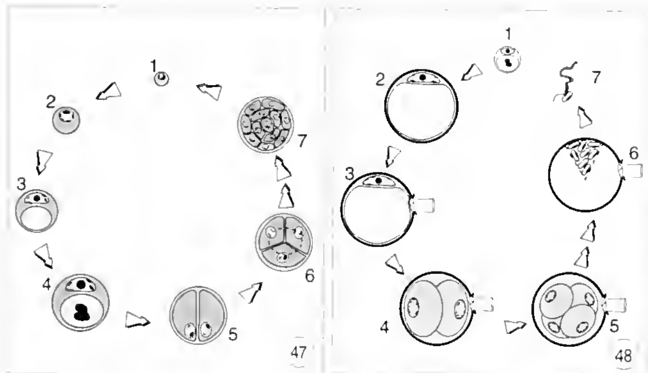


Figure 47. Developmental cycle of *P. marinus* in *C. virginica*. Immature trophozoite (1) becomes a mature trophozoite (4), in the process acquiring a large eccentric vacuole with a vacuoplast, free in the vacuole, and a nucleus with a centrally located nucleolus. Palintomy (5–7) occurs during which the nucleus becomes progressively smaller through the first three karyokineses and the nucleolus becomes invisible in the light microscope. Most often 8–16 immature trophozoites (range = ca. 4–64) are liberated from the tomont (5–7) through a tear in the wall (7 to 1).

Figure 48. Zoosporulation of *P. marinus* in sea water, free of the host. Mature trophozoite enlarges markedly, losing the vacuoplast (1 to 2); a discharge tube and pore, occluded by a plug of secondary wall material, develop in the wall (3); palintomy results in the formation of numerous biflagellated zoospores (4 to 6) during which the nucleus and individual cells are reduced in size and the nucleolus becomes invisible in the light microscope; dissolution of the wall plug and liberation of the swimming zoospores then occur through the tube (6 to 7). Zoospores swim to or are pulled into an oyster's mantle cavity, ultimately resulting in new infections being established in or beneath the gill, mantle or gut epithelia. Presumably the zoospores lose their flagella and apical complex, become rounded and result in cells which can then be termed immature trophozoites.

Dungan and Roberson (1993) significantly advanced the ability to microscopically detect cells of *Perkinsus* spp. through the use of antibody labelling. As they noted, this technique will undoubtedly prove to be very useful in detecting the presence of the pathogens in environmental samples and determining whether there are cells of *Perkinsus* sp. which are not detected in host tissues when histological or FTM techniques are used. Despite the high level of specificity involved in the use of antibody labelling, there will nevertheless be the necessity to compare the results with observations of cytological and fine structure to eliminate any doubts. There still may be cellular stages in the life cycle yet to be detected and those techniques may prove to be critical in finding such stages, particularly in samples from the host-free environment (Li et al. 1994). Of particular interest may be the daughter cells formed from outgrowths of hypnospores (Figs. 35 to 39). The question arises as to whether the formation of these daughter cells represents the beginning of a saprobic phase in the life cycle or is a terminal phase which results in death of the cells if they do not enter *C. virginica* or another host organism.

In addition to the immunolabeling approach to detecting cells of *Perkinsus* spp., the recent characterization of the small rRNA gene of *P. marinus* (Fong et al. 1993) has provided the ability to synthesize DNA probes which should be species specific. Such a capability should permit detection of *P. marinus* as opposed to any other microbe, including distinction of one species of *Perkinsus* from another. A wide diversity of bivalve molluscs are known to harbor cells of *Perkinsus* sp. or spp. as evidenced by the formation of zoosporangia in FTM (Ray 1954, Andrews 1955, Goggin and Lester 1987).

The sizes of cells in Table 1 are about the same as recorded earlier (Mackin et al. 1950; Ray and Chandler 1955; Mackin 1962; Perkins and Menzel 1967; Perkins 1969, 1976b, 1988), with the exception of the upper limits for mature trophozoites where sizes of 10 μm (Perkins 1976b), 20 μm (Perkins 1969, 1988) and 20 or, rarely, 30 μm (Ray and Chandler 1955) have been recorded. The largest mature trophozoite measured for this paper was 11.6 μm (Table 1). An explanation for the larger sizes that I recorded in earlier publications (and probably also those of Ray and Chandler 1955) is the observation that in moribund oysters mature trophozoites may become much larger than usual and are probably cells equivalent to those formed in FTM (i.e., hypnospores) (Fig. 13; Perkins 1988). However, this is speculation because zoosporulation has never been demonstrated in any *P. marinus* cells removed from infected oyster tissue and placed directly in estuarine or sea water without FTM treatment. Zoosporulation can be induced in the larger cells of *Perkinsus* sp. from *M. balthica* by removing them from the host and placing them in estuarine water (Perkins 1968, Valiulis and Mackin 1969). If it is accepted that cells of *P. marinus* larger than about 12 μm in diameter in oyster tissue are actually hypnospores, then there is a natural equivalent to zoosporulation observed in the large cells (hypnospores) derived from FTM.

Terminology

As noted in the MATERIALS AND METHODS section, terminology used to denote stages in the life cycle of *Perkinsus* spp.

over the years has varied and has not been entirely satisfactory due in part to uncertainties concerning the phylogenetic affinities of the molluscan parasites and the unique cellular stages in the life cycle. The following justifications are presented for the use of terminology which is new to descriptions of *Perkinsus* spp. and for retention of some previously used terms. Changes are being proposed due to the recent evidence for *Perkinsus* spp. having close affinities to both the Dinoflagellata and the Apicomplexa and due to the recognition that stricter use of the terms merogony, merozoites and meronts is justified.

Palintomy is used herein because it denotes most accurately what occurs in *Perkinsus* spp., i.e., a "rapid sequence of binary fissions, typically within a cyst and with little or no intervening growth, resulting in production of numerous, small offspring cells" (Margulis et al. 1993). This is in keeping with Chatton (1937), who coined the term and considered palintomy to be division where at each successive stage the cells became smaller and smaller: ". . . il se scinde coup sur coup en deux puis en quatre, et les scissions se poursuivent sans intervalle de croissance compensatrice, de sort que les produits sont beaucoup plus petits que l'élément initial." Thus the term is the same as successive bipartitioning (Perkins 1988). It has been used to denote asexual cellular proliferation in some parasitic dinoflagelletes (Cachon and Cachon 1987) and ciliates (Lynn and Small 1990, Margulis et al. 1993).

Thus, for *Perkinsus* spp. palintomy is proposed as a replacement for the term merogony, which is used primarily to denote "multiple fission of apicomplexans" (Margulis et al. 1993). Multiple fission is synonymous with progressive cleavage where repeated karyokineses are followed by a multiple cytokinetic event yielding several daughter cells. "Successive multiple fissions" as used in Margulis et al. (1993) is considered to be a misleading term and should be avoided. The term merogony was used previously for *Perkinsus* spp. because it is used to denote asexual cellular replication in the Apicomplexa to which *Perkinsus* spp. are related. However, I now believe that I did not adequately recognize the importance of *multiple fission*. Merogony is used to denote one type of multiple fission in the Apicomplexa (the others being sporogony and gametogony). Use of the term merogony was not previously considered to be a problem, because, even though cellular proliferation of *Perkinsus* spp. within the host most often occurs by successive bipartition, multiple fission is believed to occur infrequently (Perkins and Menzel 1967) and is known to occur in axenic cultures of *P. marinus* (La Peyre et al. 1993). In addition, merogony was used previously for *Perkinsus* spp. because the term is used in the microsporidian literature to denote cellular proliferation involving both successive bipartitioning and progressive cleavage (multiple fission) (Perkins 1991). The reasons I am not now using the term merogony are as follows: 1) whereas merogony is associated with both the Apicomplexa and the Microspora, *Perkinsus* spp. are closely related to the Apicomplexa, not to the Microspora; therefore, the apicomplexan sense of the term (multiple fission) should dominate; 2) merozoites of the Apicomplexa (the daughter cells resulting from merogony) are motile and have an apical complex whereas the daughter cells of *Perkinsus* spp. are not motile and do not have apical complexes; and 3) the dominant form of cellular proliferation in *Perkinsus* spp. is successive bipartition not multiple fission.

The cells engaged in palintomy are called tomonts which is a generalized term for a dividing cell. The term has been used in descriptions of ciliates to denote a prefission or dividing stage (Lynn and Small 1990). Despite its association with ciliates the term is being adopted due to lack of a better preexisting term. The term schizont is not used herein for tomont because the former is defined as a "multinucleate organism that will undergo schizogony" (Margulis et al. 1993), and schizogony is reserved for multiple fission.

The cellular products of palintomy have been termed herein as trophozoites, because the term is a generalized one used for parasitic protists, meaning the growing, feeding or trophic stage which is also an interfissional form. It is not used herein to denote necessarily the adult stage in the life cycle (Corliss and Lom 1985) nor is it used to denote a motile stage as defined by Margulis et al. (1993). As part of the definition, Corliss and Lom (1985) did not consider it necessary for the cell to be motile. The term trophont which has the same definition is not being used here because it is more often used in place of trophozoite for nonparasitic species of protists (Corliss and Lom 1985).

The term tomite has been used in descriptions of ciliates to indicate one or more products of palintomy and generally has been reserved for a stage in the life cycle which is small, free swimming and nonfeeding (Margulis et al. 1993). The term could be applied to *Perkinsus* spp., because the term is a general one indicating a product of division; however, since it is associated with the ciliates, I have chosen to simply refer to the cellular products of palintomy as being immature trophozoites (the smaller cells lacking a large eccentric vacuole). The larger cells which are differentiated from the immature trophozoites and which have a large eccentric vacuole are the mature trophozoites. Thus, immature trophozoites are the same cells which were termed merozoites and mature trophozoites are the same as meronts (Perkins 1991). The mature trophozoites of *Perkinsus* spp. have also been termed spores (Mackin et al. 1950), thalli or prehypnospores (Mackin 1962), mature thalli (Perkins 1969), and aplanospores (Perkins 1976b). In an earlier publication, I (Perkins 1988) used the term trophozoite as employed herein. I also continued to use the term sporangium to denote the tomont stage and the resulting complement of immature trophozoites contained in a mother cell wall prior to rupture of that wall. I now recognize that the term sporangium is inappropriate because *Perkinsus* spp. are either apicomplexans or dinoflagellates, and the term is not used for either taxonomic group despite the fact that it is otherwise suitable in that it is a "hollow unicellular or multicellular structure in which propagules (cysts or spores) are produced and from which they are released" (Margulis et al. 1993).

The term spore, instead of trophozoite, is not used herein because it is too general in its meaning (Corliss and Lom 1985), and a more specific term is appropriate.

"Zoospore" is retained from my previous publications to denote the biflagellated cells or swarmers which are formed outside of the host in estuarine or sea water. This is appropriate because the term is used sometimes in the dinoflagellate literature to denote asexually produced, flagellated cells instead of the more commonly used "dinospores" (Freudenthal 1962, von Stosch 1973). In addition, the term is generally used in protistological literature. Since *Perkinsus* spp. are closely related to the dinoflagellates, use of "zoospore" is justified. It is not a term appropriate for the more highly evolved Apicomplexa. However, since the species of *Perkinsus* comprise a phylogenetically intermediate group between the dinoflagellates and Apicomplexa, there is justification for use of the term even if those molluscan pathogens ultimately are accepted as members of the Apicomplexa, the reasoning being that

it would be best to use a dinoflagellate term for the most primitive apicomplexan species. There is no comparable cellular stage in the more highly evolved Apicomplexa. The nearest cell type is the biflagellated microgamete formed by a few species of gregarines and the Coccidea (Perkins 1991). There is no evidence that the zoospores of *Perkinsus* spp. are microgametes. The flagella of the microgametes have no mastigonemes, and there is no perforatorium, like that found in microgametes, in the zoospores (Perkins 1991).

The term dinospore is avoided in denoting the swarmers, because it has not yet been demonstrated that *Perkinsus* spp., although related to dinoflagellates, are in fact dinoflagellates. "Swarmers" is avoided because it is a term which is too general.

The process by which zoospores are differentiated is best termed palintomic zoosporulation (herein shortened to zoosporulation) instead of palintomic sporogenesis, as is used for a number of parasitic dinoflagellates (Cachon and Cachon 1987). The adjective palintomic is used because palintomy (successive bipartitioning) is involved, as discussed above, for the cell stages of *Perkinsus* spp. found in the host. The term sporogenesis is not used, because the term spore is being avoided for reasons already noted above. In addition, in the dinoflagellate literature, the term sporocyst is used in association with palintomic sporogenesis where the sporocysts each differentiate into dinospores (Cachon and Cachon 1987). It is best to avoid the term sporocyst since it has a very specific connotation in apicomplexan literature where it denotes a cyst containing sporozoites and formed within an oocyst, or in the gregarines it is the oocyst itself, there being no sporocyst. Oocysts develop from a zygote, and there is no evidence that zoospores of *Perkinsus* spp. result after karyogamy. The cell which forms zoospores is termed a zoosporangium, thus adopting another term which is used in the dinoflagellate literature to denote the cell which forms swarmers (von Stosch 1973, Spero and Morée 1981). It should be noted that I have avoided use of the term sporangium (see discussion on trophozoites above), because it is not used in the apicomplexan and dinoflagellate literature, but I am using the term zoosporangium for the reasons given previously.

Those mature trophozoites which have enlarged markedly, most notably in FTM, to form cells that are at least twice the size of the largest mature trophozoites are herein termed hypnospores, a term originally proposed by Mackin and Boswell (1956) and used by a number of investigators. In previous papers, I have used the term prezoosporangium in recognition of the fact that the cells may engage in zoosporulation, thus forming zoosporangia. I have resisted use of the term hypnospore because 1) it has been defined as a resting cyst (Taylor 1987) and has been used for a resistant stage in the life cycle where the cell can become viable after long periods of dormancy, and 2) it is a term associated with the dinoflagellates and not the Apicomplexa. I have changed my position on the matter because Margulis et al. (1993) defined it as simply a thick-walled aplanospore with no mention of it being a resting stage. This definition, therefore, includes the hypnospores which have thick walls but which are not resistant or dormant cells in that they do not appear to survive beyond a week or two in sea water. Most die in sea water within 10 days. The judgment as to when death occurs is based on the disorganized appearance of the cells' cytological structure (Perkins, unpublished data). In addition, since it is now recognized that *Perkinsus* spp. are closely related to the dinoflagellates, the use of a dinoflagellate term is appropriate. Furthermore, the hypnospores do not necessarily form zoospores, but may form hyphal-like outgrowths from which nonflagellated unicells emerge. Thus, the term prezoosporangium is less suitable.

Morphologically defining when a mature trophozoite becomes a hypnospore is not possible because the transition is a gradual one whereby the vacuoplast disappears and the eccentric vacuole becomes proportionately larger and is accompanied by a thickening of the cell wall. In *P. marinus*, the mature trophozoites average about 6 μm in diameter; however, zoosporulating cells have been observed which are as small as 15 μm. Thus, when approximately a doubling of the mature trophozoite's diameter has occurred, the transition to hypnospore is assumed to have been completed

In the case of *Perkinsus* sp. (probably *P. atlanticus*) from *M balthica*, zoosporulation occurs in the larger trophozoites (up to 48 μm in diameter; Perkins 1988) which are isolated directly from the host tissues and which have not been treated by FTM (Perkins 1968, Valiulis and Mackin 1969) as well as in those cells induced to enlarge in FTM (Perkins 1968). In *Perkinsus* sp. from *M. balthica*, the distinction between mature trophozoites and those cells which zoosporulate is even less well defined than in *P. marinus* in that the eccentric vacuole in the former is often not as pronounced. The use of the term hypnospore in the former species may not be appropriate. Apparently mature trophozoites are able to differentiate directly into zoosporangia.

In *P. atlanticus* from *Ruditapes decussatus*, zoosporulation was induced after treatment with FTM (Azevedo et al. 1990). It is not known whether this occurs without such treatment. It is known that a wide diversity of *Perkinsus* spp. from bivalve molluscs, other than *C. virginica*, can form hypnospores as a result of treatment in FTM and this is followed by zoosporulation (Goggin et al. 1989, Azevedo et al. 1990, Perkins, unpublished data).

Taxonomy

The taxonomic position of *P. marinus* has undergone a number of changes since first being described by Mackin et al. (1950) as *D. marinum*. They chose the name primarily because of the signet appearance of the mature trophozoites and the large eccentric vacuole containing a vacuoplast. Mackin and Ray (1966) changed the name to *Labyrinthomyxa marina* as a result of observations of "amoeboid stages in the host oyster" and observations of presumed cultures of *P. marinus* initiated by using hemolymph of infected oysters as an inoculum. They found amoeba-like plasmodia with rhizoid-like mucoid processes. The plasmodia were observed to "segment into small spherical cells (3 to 5 μm) which produce mucoid tracks on which they travel in the gliding motion characteristic of spindles" (Mackin and Ray 1966). Subsequent work by myself (Perkins 1976b) and others (reviewed in Olive 1975 and Porter 1990) has shown that the Labyrinthomorpha (Page 1980) Pokorny, 1985 (syns., Labyrinthomycota according to Porter 1990; Labyrinthulina according to Olive 1975; Labyrinthulomycetes according to Moss 1991), of which the genus *Labyrinthomyxa* was considered to be a member, are not closely related to *Perkinsus* spp. (Perkins 1976b). It is likely that Mackin and Ray (1966) observed cells of a labyrinthomorphid, probably *Labyrinthuloides* sp. (Perkins, 1973), which were contaminants in their cultures. The labyrinthomorphids are members of the monophyletic assemblage known as the stramenopiles and probably evolved early in the evolution of the group (Leipe et al. 1994). As the information accumulated concerning the characteristics of the labyrinthomorphids, I discontinued use of the generic name *Laby-*

rinthomyxa and reverted to use of the generic name *Dermocystidium* (Perkins 1976b) until 1978.

Following the demonstration of an apical complex in zoospores of the oyster pathogen (Perkins 1976a), Levine (1978) renamed the pathogen *P. marinus* and established a new class Perkinsea, order Perkinsida and family Perkinsidae in the phylum Apicomplexa in recognition of the significance of the apical complex and the uniqueness of the pathogen. He later modified the endings of the class and order names so that they became class Perkinsasida and order Perkinsorida (Levine 1988). These modifications in name endings are not accepted by some workers. *Perkinsus* is now the name used for the genus of molluscan pathogens described herein.

Four species have been described, all from marine molluscs: *P. marinus* (Mackin, Owen and Collier, 1950) Levine, 1978 from all tissues of *C. virginica; Perkinsus olseni* Lester and Davis, 1981 from hemolymph, adductor muscle and mantle of the Australian blacklip abalone, *Haliotis ruber; P. atlanticus* Azevedo, 1989 from the gills of the Portuguese clam, *R. decussatus;* and *Perkinsus karlssoni* McGladdery, Cawthorn and Bradford, 1991 from tissues of the bay scallop, *Argopecten irradians*. The latter species is of questionable validity, because *Perkinsus* spp. zoosporulation, typical of *Perkinsus* spp., was not observed. It was not determined whether the biflagellated cells, believed by McGladdery et al. (1991) to be zoospores, have filamentous mastigonemes and an apical complex nor was the response to FTM typical of *Perkinsus* spp. (McGladdery et al. 1991). In the case of *P. olseni* no attempts were made to determine if filamentous mastigonemes or an apical complex were present; however, typical zoosporulation was observed with formation of a discharge pore and tube, and the response to FTM was typical in terms of enlargement of trophozoites and staining with Lugol's iodine solution. Therefore, it is likely that the pathogen described by Lester and Davis (1981) is a species of *Perkinsus. P. atlanticus* has been shown to possess all characteristics of the genus including the formation of biflagellated zoospores with filamentous mastigonemes and an apical complex (Azevedo et al. 1990).

Whether there are numerous species of *Perkinsus* parasitizing marine and estuarine molluscs worldwide or only a few species is not yet clear. Using the Ray FTM technique (Ray 1952), 67 species of molluscs, mostly bivalve species, have been found to contain species of *Perkinsus*, with the distribution being from coastal temperate, subtropical and tropical waters (the possible exception being *P. karlssoni*) (Perkins 1993). It is not unreasonable to suggest that all species of bivalve molluscs from those coastal waters can serve as hosts of *Perkinsus* spp. It is uncertain how many species of *Perkinsus* are involved in the 67 mollusc species identified thus far, particularly since Goggin et al. (1989) were able to demonstrate that there is a low level of host specificity for the pathogens. Using zoospores they were able to readily cross-infect from host to host. In unpublished host specificity studies conducted at the Virginia Institute of Marine Science using species of Chesapeake Bay molluscs, I have obtained results which confirm in large part the observations of Goggin et al. (1989). It is also noteworthy that the structural differences among *P. marinus, P. olseni* and *P. atlanticus* are not striking, with *P. karlssoni* again being the exception. The question that arises is whether the differences can be attributed to the host environment presented to the parasite. The number of species of *Perkinsus* parasitizing molluscs worldwide could be very small. Further transmission studies and DNA-specific probes will be useful in answering these questions concerning species identity. In using their polyclonal antibodies coupled to a fluorescent stain, Dungan and Roberson (1993) have already provided a very useful technique for detecting the presence of *Perkinsus* cells, but the stain can not distinguish between species of *Perkinsus*. They have provided evidence that *P. karlssoni* is not a member of the genus nor are *Perkinsus*-like cells found in three species of Australian marine mollusc members of the genus.

Phylogeny

Recent molecular data and interpretations coupled with some of the newer morphological information described herein warrant a reevaluation of the phylogeny of *Perkinsus* spp. Goggin and Barker (1993) determined the nucleotide sequence (1,792 bp) for the small subunit rRNA gene of *Perkinsus* sp. from *Anadara trapezia* and compared it to nucleotide sequences of other organisms. From their analyses they concluded that ". . . *Perkinsus* is phylogenetically closer to dinoflagellates and to coccidean and piroplasm apicomplexans than to fungi or flagellates." Comparisons were made to a chytrid and a species of yeast to represent the fungi and to three species of zooflagellates. Of the possibilities considered, they concluded that *Perkinsus* sp. is most closely related to the dinoflagellate *Prorocentrum micans* and the coccidean apicomplexan *Sarcocystis muris*. In another molecular study, Fong et al. (1993) determined the base sequence (1,793 bp) of the small subunit rRNA gene of *P. marinus*. Their analyses led them to conclude that "Rather than being derived from some apicomplexan lineage, dinoflagellates are descendants of an ancestor that shares common properties with *Perkinsus marinus*."

In a third study, Marsh et al. (1995) cloned and sequenced a 3,200-bp mtDNA fragment from *P. marinus* that contains the 5S ribosomal RNA gene. Their phylogenetic analyses resulted in their conclusion that the oyster pathogen is more closely related to the dinoflagellates than to the Apicomplexa.

The morphological information contained herein leads me to conclude that *Perkinsus* spp. have affinities with both the Dinoflagellata and the Apicomplexa. The apical complex and micropores indicate affinities with the Apicomplexa. The flagellar spurs indicate affinities with the dinoflagellates in that the structures have been reported only on the transverse flagellum of three species of dinoflagellates (Dodge 1967, Leadbeater and Dodge 1967, Lee 1977) and on the shorter flagellum of *Cyathomonas truncata*, a flagellate which has been classified with the cyptomonads; however, the latter may not be a cryptomonad (Kugrens et al. 1987). Mitosis in *Perkinsus* has not been adequately elucidated, but preliminary observations indicate that it is dinoflagellate-like (Dodge 1987) in that 1) the nuclear envelope appears to remain intact during nuclear division; 2) deep channels, in continuity with the cytoplasm, lined by the nuclear envelope and containing bundles of microtubules, are formed in the nucleus; and 3) kinetochore-like structures are formed on the nuclear envelope as attachment loci for the microtubules. I am developing a more complete description of the spurs and mitosis in *Perkinsus* spp. for publication at a later date.

Obviously, more molecular and morphological information is needed from studies of the parasitic dinoflagellates and primitive Apicomplexa, such as the archigregarines, before a revision of the classification of *Perkinsus* spp. is attempted. It will be important to see whether the spurs are found in the parasitic dinoflagellates.

This will require negative staining or shadow casting of whole mounts of the transverse flagella from a number of species for transmission electron microscopic studies, not just scanning electron microscopic observations, which do not yield enough resolution and which have been performed on most of the flagella of species of dinoflagellates characterized thus far. Parasitic dinoflagellates such as *Coccidinium duboscqui* will be of particular interest in such studies. The dinospores of *C. duboscqui* (Chatton and Biecheler 1936) bear an interesting resemblance to zoospores of *Perkinsus* spp., with the transverse flagellum of the former resembling the anterior flagellum of the latter in the manner in which it loosely coils around the anterior third of the cell body. In addition, the dinospore cell body resembles that of *Perkinsus* spp. A major difference is that dinospore formation in *C. duboscqui* occurs by multiple fission, not by palintomy.

Another component of these considerations is the determination of the taxonomic position of predatory flagellates such as *Colpodella perforans* (Hollande, 1938) Patterson and Zolffel, 1991, syns. *Bodo perforans* Hollande, 1938 and *Spiromonas perforans* Brugerolle and Mignot, 1979, as well as *Spiromonas gonderi* Foissner and Foissner, 1984, which should be placed in the genus *Colpodella*. The flagellated cells of *C. perforans* have a three-membrane pellicle (alveolate structure), micronemes, subpellicular microtubules, micropores and trichocysts (Brugerolle and Mignot 1979). *S. gonderi* lacks trichocysts but has a conoid (Foissner and Foissner 1984). Thus, it has been suggested that the ancestor to the Apicomplexa was similar to *Colpodella* (*Spiromonas*) (Myl'nikov 1991). Krylov and Myl'nikov (1986) and Myl'nikov (1991) noted that there are strong similarities between *P. marinus* and *Colpodella* (*Spiromonas*), including a transitional cylinder (or helix?) at the base of each flagellum and filamentous mastigonemes on the anterior flagellum. They indicated that "it is reasonable to combine these groups." It is possible that the species of *Colpodella* (*Spiromonas*) are predatory dinoflagellates. It would be helpful for the molecular phylogenists to evaluate these predatory flagellates and thus contribute to elucidation of their affinities.

At this stage in the development of our knowledge concerning *Perkinsus* spp., it is interesting to note that Levine (1985) suggested that dinoflagellates may have given rise to the Apicomplexa, with *Perkinsus* spp. possibly being the first apicomplexan to diverge from the evolutionary line which gave rise to the rest of the Apicomplexa. Future findings will probably support his suggestion. Based on the discovery of a 35-kb circular genome in all apicomplexans which resembles a chloroplast genome as well as the recognition that both the Apicomplexa and the Dinoflagellata are alveolates, Farmer (1995) has already stated that "the likely ancestor for the apicomplexans, a group of obligate parasites, is therefore a photosynthetic dinoflagellate."

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PROPAGATION AND *IN VITRO* STUDIES OF *PERKINSUS MARINUS*

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ABSTRACT The development of continuous cultures of *Perkinsus marinus* (Apicomplexa) is a major breakthrough that will lead to a better understanding of this deadly oyster pathogen. More than 10 *P. marinus* isolates are currently in continuous cultures. Culture media used to propagate *P. marinus* range from media designed for the culture of mammalian cells to protein-free chemically defined media. Continuous cultures of *P. marinus* can be initiated from a variety of infected oyster tissues or from isolated hypnospores (i.e., the enlarged stage of *P. marinus* from oyster tissue incubated in Ray's fluid thioglycollate medium). *P. marinus* cells adapt well to culture conditions and optimal temperature, osmolality, pH and seeding density for its propagation are reported. The availability of several *P. marinus* isolates in cultures has prompted investigations that address the parasite's genetic makeup, virulence and environmental tolerance. These studies, once completed, will provide valuable insights into disease pathogenesis and host-parasite interactions. Several important findings have already been made in the short time since the original culture. For example, it was found that *P. marinus* secretes serine proteases that digest oyster tissues and plasma. Acid phosphatases and heat shock ("stress") proteins are also produced by *P. marinus*. Moreover, it was found that *P. marinus* extracellular products suppress some oyster host defenses. Studies on the mechanisms of adaptation of *P. marinus* to environmental conditions as well as on parasite biochemistry and nutritional requirements have also begun. Finally, cultured cells are being used in screening chemotherapeutic agents for their potential use in treating infected oysters. This review is a collation of the available literature on the methods used to propagate *P. marinus in vitro* and on current investigations conducted with cultured cells. Although it is important to realize some of the limitations of *in vitro* studies, research using cultured *P. marinus* cells is indispensable and may lead to novel ways of controlling the parasite.

KEY WORDS: *Perkinsus marinus*, oysters, protozoa, culture procedures, culture conditions, *in vitro* propagation

INTRODUCTION

The importance of developing techniques for the *in vitro* propagation of parasitic protozoa has long been recognized. The ability to mass culture parasites *in vitro* enables essential biological, metabolic and morphological research that would otherwise be difficult or impossible to accomplish. Moreover, diverse studies including *in vitro* drug screening, physiological, biochemical and nutritional studies can be performed without interference from the host. Hence, development of *in vitro* cultivation techniques is often heralded as a major breakthrough.

Many techniques have been developed for the cultivation of protozoan parasites of medical and veterinary importance (reviewed in Jensen 1983). In contrast, cultivation of protozoan parasites that affect marine organisms, especially invertebrates, has not received as much attention. This is unfortunate since according to Sparks (1985) "protozoan parasites are the most common cause of disease in invertebrates." Some of these protozoans (e.g., *Perkinsus marinus*, *Haplosporidium nelsoni*, *Bonamia ostrea*) have been responsible for devastating mortalities in species of economic importance, including oysters.

Several pioneering scientists realized the importance of propagating the oyster pathogen *P. marinus in vitro* and attempted to adapt the protozoan to culture conditions more than 40 years ago (Prokop 1950, Ray 1952a, Ray 1954a, Mackin 1962, Perkins 1966). There are several factors that may have contributed to the inability to establish continuous cultures of *P. marinus* at the time. First, it was almost impossible to control microbial contamination since antibiotics were just becoming available by the late 1940s. Second, none of the media that were used to culture bacteria, fungi or protozoa could support the proliferation of *P. marinus*. Finally, uncertainties about the morphology of the parasite and its life stages may have contributed to improper identification of organisms grown in cultures from infected oysters.

It was not until the early 1990s that the development of continuous cultures of *P. marinus* was achieved. La Peyre et al. (1993) first showed that *P. marinus* could be propagated *in vitro*. They discovered that continuous cultures of the parasite could be established from infected oyster heart fragments in a medium designed to resemble bivalve plasma composition. Following this finding and using modified commercial media, Kleinschuster and Swink (1993) isolated and propagated *P. marinus* from primary cultures of tissue explants of visceral ganglia, and Gauthier and Vasta (1993) initiated continuous cultures of the parasite from infected oyster hemocytes. Other researchers have since propagated several *P. marinus* isolates *in vitro* (Bushek 1994, Dungan and Hamilton 1995).

This ability to propagate *P. marinus in vitro* has provided a versatile system to study the biology of the parasite. It allows, for example, investigations of *P. marinus* physiology, biochemistry, nutrition, genetics and pharmacology. Information derived through experimentation using cultured *P. marinus* is helping to improve our understanding of the host-parasite interaction at the organismic, cellular and biochemical level. Consequently, rational methods to control this parasite may be developed. This review will summarize the methodologies used to initiate and propagate the oyster pathogen *P. marinus in vitro*. An overview of current investigations using *in vitro* culture to understand *P. marinus* disease will then be presented. Also, the potential for *in vitro* culture to resolve issues related to the pathobiology of *P. marinus* disease will be discussed.

REVIEW OF CULTURE METHODS

Methodologies for the propagation of *P. marinus* have recently been described in several reports (Gauthier and Vasta 1993, Kleinschuster and Swink 1993, La Peyre et al. 1993, Dungan and Hamilton 1995, Gauthier and Vasta 1995, Gauthier et al. 1995, La

Peyre and Faisal 1995b). Procedures varied greatly among researchers in the type of medium used, in the culture conditions, in the method used for the establishment of *P. marinus* primary cultures and in the evaluation of growth rates of protozoal cells.

A. *Media and Initial Culture Conditions*

A variety of culture media have been used to propagate *P. marinus* (Table 1). Differences in methodology also extend to the original culture conditions such as temperature, osmolality, pH and gas phase (Table 2).

1. Serum Free Culture Media

The original medium used to propagate *P. marinus*, designated JL-ODRP-1, was formulated to resemble the known composition of oyster plasma (La Peyre et al. 1993). This partly defined medium contains more than 80 defined constituents and is supplemented with solutions of cod liver oil, bovine serum albumin (BSA) and yeastolate ultrafiltrate. Cod liver oil provides a rich source of lipids including long-chain ω-3 polyunsaturated fatty acids such as eicosapentaenic (20:5ω3) and docosahexanoic (22:6ω3) acids that are typically found in marine organisms (Langdon and Waldock 1981, Chu and Webb 1984). Yeastolate ultrafiltrate (10 kDa) provides vitamin B_{12} as well as nutritional peptides. BSA was initially added because of the reported beneficial properties of albumin (e.g., binding of lipids, metals and hormones with enhanced delivery of these to the cell, detoxification and provision of additional metabolic source of amino acids) (Barnes and Sato 1980, Maurer 1992).

The serum free medium JL-ODRP-1 presents several advantages over commercial media in that 1) no animal serum is present thus decreasing considerable problems associated with the use of this complex mixture in experiments (Barnes and Sato 1980, Maurer 1992); 2) the salt composition and osmolality of the medium can be easily adjusted to any desired value, which is essential for studying the osmolality tolerance of *P. marinus* (O'Farrell et al. 1995); 3) the concentration of individual components or groups of components (e.g., amino acids, vitamins, carbohydrates) in the media are similar to their concentrations in oyster plasma and can be independently manipulated for biochemical and physiological studies. Elimination of BSA from the culture medium JL-ODRP-1 also enables the study of parasite-derived proteins (>10 kDa) without interference from extraneous proteins (La Peyre and Faisal 1995a, La Peyre et al. 1995a). Moreover, purification of serine proteases from conditioned medium is vastly simplified in the absence of BSA (Faisal et al. 1995a).

2. Commercial Media Supplemented with Serum

Commercially available media used to propagate *P. marinus* include Dulbecco modified Eagle's medium (DME), DME:Ham's nutrient mixture F-12 (DME:HAM's F-12), Leibovitz's L-15 medium, NCTC-135 and RPMI-1640, supplemented with 5–20% fetal bovine serum (FBS) and/or 5–20% oyster plasma (Gauthier and Vasta 1993, Kleinschuster and Swink 1993, Dungan and Hamilton 1995). Additional ingredients, such as taurine and trehalose, which are abundant in oyster plasma, are added for growth enhancement of *P. marinus* to a number of these commercial media originally designed for vertebrate cell cultures (Kleinschuster and Swink 1993, Dungan and Hamilton 1995).

Proliferation of *P. marinus* in several of these commercial media was recently compared (Dungan and Hamilton 1995, Gauthier and Vasta 1995). Dungan and Hamilton (1995) evaluated the proliferation of *P. marinus* in three commercial media (i.e., NCTC-135, RPMI-1640 and 1:1 DME:Ham's F-12) as well as in the culture medium JLP, their modification of the medium JL-ODRP-1. Each medium was supplemented with 10% FBS as well as yeast extract ultrafiltrate (0.4 mg/ml), L-glutamine (2 mM) and a lipid mixture (1%). *P. marinus* growth was measured by the MTS/PMS cell proliferation assay (Cell Titer 96 AQ™; Promega, Madison, WI). They found that proliferation of the parasite was greatest in 1:1 DME:Ham's F-12 medium. A concentration of 5% FBS in 1:1 DME:Ham's F-12 medium was then reported to be optimal following a comparison of various serum concentrations (Dungan and

TABLE 1.

Selected media and supplements used to propagate *P. marinus in vitro*.

Medium	Reference*	Supplement	Buffer	Antibiotic
JL-ODRP-1	1	—	HEPES 25 mM,** $NaHCO_3$ 43 mM	Chloramphenicol 5 μg/ml
1:1 DME:Ham's F-12	2	FBS 10%, oyster plasma 5%	HEPES 100 mM, $NaHCO_3$ 7 mM	Penicillin G 100 U/ml, streptomycin 100 U/ml
1:2 DME:Ham's F-12	3	Fetuin 1.7 mg/ml	HEPES 100 mM, $NaHCO_3$ 7 mM	Penicillin G 100 U/ml, streptomycin 100 U/ml
L-15 (Leibovitz)	4	FBS 10%, oyster plasma 20%, taurine 0.5 mg/L, glucose 5 mg/L, galactose 5 mg/L, trehalose 5 mg/L, yeast extract 1 g/L, lactalbumin hydrolysate 3 g/L, (100×) MEM vitamin solution 10 ml/L, (100×) lipid mixture 1 ml/L	—	Penicillin G 100 U/ml, streptomycin 100 μg/ml
NCTC-135 RPMI-1640 1:1 DME/Ham's F-12 JLP	5	FBS 10%, yeast extract 0.4 mg/ml, L-glutamine 2 mM, lipid mixture 1% (v/v), JL-ODRP-1 carbohydrates 1% (v/v)	HEPES 25 mM, $NaHCO_3$ 4 mM	Penicillin G 100 U/ml, streptomycin 100 μg/ml

* References: 1, La Peyre et al. (1993); 2, Gauthier and Vasta (1993); 3, Gauthier and Vasta (1995); 4, Kleinschuster and Swink (1993); 5, Dungan and Hamilton (1995).

** 5% CO_2 tension.

TABLE 2.

Selected culture conditions for the propagation of *P. marinus in vitro*.

Basal Medium	Reference*	pH	Osmolality (mOsm/kg)	Temperature (°C)	Gas Phase
JL-ODRP-1	1, 2	7.5	650	21–28°C	5% CO_2
1:1 DME:Ham's F-12	3	7.4	~900	27°C	Ambient
1:2 DME:Ham's F12	4	6.6	~900	28°C	Ambient
L-15	5	7.6	750	28°C	Ambient
NTCT-135	6	7.5	665	28°C	Ambient
RPMI-1640	6				
1:1 DME/Ham's F-12	6				
JLP	6				

* References: 1, La Peyre et al. (1993); 2, La Peyre and Faisal (1995a); 3, Gauthier and Vasta (1993); 4, Gauthier and Vasta (1995); 5, Kleinschuster and Swink (1993); 6, Dungan and Hamilton (1995).

Hamilton 1995). Gauthier and Vasta (1995) assessed the effects of various FBS concentrations in combination with oyster plasma on the growth of *P. marinus* in the basal medium DME, 1:1 DME: Ham's F-12 or 1:2 DME:Ham's F-12. The optimal medium was found to be 1:2 DME:Ham's F-12 supplemented with 5% FBS (Gauthier and Vasta 1995).

3. Chemically Defined Media

Recently, two chemically defined media have been used to propagate *P. marinus* (Gauthier et al. 1995, La Peyre and Faisal submitted a). *P. marinus* was cultured in DME:Ham's F-12 (1:1) supplemented with 1.7 mg/ml of fetuin (Gauthier et al. 1995). Fetuin is a widely used component of culture medium (Barnes and Sato 1980). It enhances cell attachment and presumably mediates transport of nutrients and growth factors (Puck et al. 1967, Abdullah et al. 1986). The major advantage of this medium is its ease of preparation, since each component is commercially available. However, the protein fetuin could interfere with studies of parasite-derived cellular and extracellular proteins. To confirm that *P. marinus* growth in the fully defined medium was comparable to that in serum-supplemented media, Gauthier et al. (1995) followed growth over 17 days in two slightly different media–DME:Ham's 1:1 and 1:2 supplemented with either 5% FBS or 1.7 mg/ml fetuin. They found that the stationary growth phase was reached by day 8 in all media, but that the cell populations in DME:Ham's 1:2 exhibited 30–40% higher optical densities than did those in DME: Ham's 1.1.

P. marinus has been cultured in a protein-free, chemically defined medium, in part to facilitate production of polyclonal and monoclonal antibodies against parasite extracellular and cellular proteins (La Peyre and Faisal submitted a). This protein-free defined medium was modified from JL-ODRP-1 medium by elimination of BSA, yeastolate ultrafiltrate and cod liver oil, increasing the amino acid and vitamin concentrations and adding a defined lipid solution, as well as vitamin B_{12}. This new medium, designated JL-ODRP-3, supported a reasonable rate of growth, with a doubling time of 18 h, and the propagation of at least six isolates of *P. marinus*. Moreover, two of these isolates were subcultured for at least 10 passes. Subculturing was required to permit full acclimation of the cells and to show that the defined medium supported the continuous growth of *P. marinus*.

B. Establishment of Primory Cultures

Several tissues from infected oysters were used to initiate *P. marinus* cultures. These included heart (La Peyre et al. 1993), visceral ganglia (Kleinschuster and Swink 1993) and hemocytes (Gauthier and Vasta 1993, Dungan and Hamilton 1995). The oyster heart was chosen initially by La Peyre et al. (1993) since this organ is relatively free of microbial contaminants compared to other oyster tissues (Hetrick et al. 1981). Excised hearts were decontaminated with a solution of several antibiotics including chloramphenicol (50 µg/ml), gentamicin (500 µg/ml), kanamycin (1 mg/ml), penicillin G (1,000 U/ml), polymyxin B (500 µg/ml), streptomycin (1 mg/ml) and rifampicin (50 µg/ml). Oyster-associated bacteria were found to be more sensitive to chloramphenicol than to other antibiotics, including broad spectrum antibiotics such as gentamicin or kanamycin. Therefore, chloramphenicol (5 µg/ml) was routinely added to JL-ODRP-1 medium. Gauthier and Vasta (1993) washed hemocytes in 4,000 U/ml each of penicillin G and streptomycin in sea water, and reported a minimum effective concentration of 100 U/ml each of penicillin and streptomycin for their medium. Likewise, Kleinschuster and Swink (1993) and Dungan and Hamilton (1995) added 100 U/ml of penicillin G and 100 µg/ml of streptomycin to their media. It is worth noting that *P. marinus* has a high tolerance to four tested antibacterial agents—penicillin G (1,000 U/ml), streptomycin (1,000 µg/ml), gentamicin (5,000 µg/ml) choramphenicol (50 µg/ml)—so that they may be used at high concentrations to eliminate bacterial contaminants during primary isolation or from existing cultures (Dungan and Hamilton 1995).

A simple method for initiating continuous cultures of *P. marinus* directly from the parasite, without having to establish a primary culture from infected oyster tissue, was described by La Peyre (1993) and La Peyre and Faisal (1995b). This procedure consists of incubating the visceral mass of an infected oyster in Ray's fluid thioglycollate medium (RFTM) to produce large hypnospores (also called prezoosporangia) from the smaller histozoic stages of the protozoan. Hypnospores are then purified, decontaminated and transferred into JL-ODRP-1 culture medium, where they divide rapidly, producing large numbers of merozoites that further proliferate. This procedure allows large numbers of cultured *P. marinus* cells to be obtained relatively rapidly from individual infected oysters.

C. Characterizotion of Cultured Cells

To confirm cultured organisms were indeed *P. marinus*, isolates were characterized by ascertaining: 1) their morphology at the light and transmission electron microscope levels, 2) their ability to form hypnospores that stained blue-black with Lugol's

solution after incubation in RFTM, 3) their reactivity to antibodies raised against *P. marinus* isolated from oyster tissue (i.e., hypnospores, merozoites) and 4) their infectivity to *P. marinus*–free eastern oysters.

1. Morphology

Cells cultured in JL-ODRP-1 with either a low concentration of BSA (<4 mg/ml) or no BSA, as well as in JL-ODRP-3 or DME: Ham's F-12 with fetuin, are morphologically similar to histozoic *P. marinus* (Gauthier and Vasta 1995, La Peyre and Faisal, submitted a). Moreover, the diameter of the largest dividing cells seldom exceeds about 25 μm, which is the approximate size of *P. marinus* schizonts reported *in vivo* (Perkins 1966, 1969). The size of the smaller cultured cells (3–6 μm) is identical to the size of merozoites isolated from infected oysters by La Peyre and Chu (1994). Small cultured cells have prominent refractile bodies, presumably lipid droplets, like those observed in isolated merozoites.

In contrast, cells cultured in JL-ODRP-1 with high (>4 mg/ml) BSA concentration, or in commercial media supplemented with FBS and/or oyster plasma, are much larger than cells found *in vivo* (Gauthier and Vasta 1993, Kleinschuster and Swink 1993, La Peyre et al. 1993). The presence of a high protein concentration from BSA (originally 12 mg/ml) or from FBS appears to cause enlargement of cultured cells to diameters as great as 45 μm, which is considerably larger than cells in protein-deficient media or those observed *in vivo*. These larger cells acquire prominent vacuoles that occupy more than 75% of cell volume, and resemble hypnospores (Kleinschuster and Swink 1993).

The ultrastructure of cultured cells is typical of *P. marinus* as described by Perkins (1969) (Gauthier and Vasta 1993, 1995, La Peyre et al. 1993). All of the ultrastructural details and cellular organelles observed in cultured cells, such as granular cell walls, lomosomes, vacuoles containing electron-dense volutin-like material, lipid droplets, and nuclei with prominent nucleoli containing torus-shaped ribosome aggregates, are identical to those described for *P. marinus*.

Two types of division of cultured cells have been observed: division by schizogony (Gauthier and Vasta 1993, Kleinschuster and Swink 1993, La Peyre et al. 1993) and division by binary fission or budding (Gauthier and Vasta 1993, La Peyre et al. 1993). Division by schizogony involves enlargement of cells to 20–40 μm in diameter, depending on the type of medium, cleavage of the cytoplasm, internal formation of daughter cells and rupture of the mother cell wall. *P. marinus* at various stages of schizogony have been observed *in vivo* (Perkins 1966, 1969), and this process is considered as the method of division of *P. marinus in vivo* (Perkins 1993). Divisions by binary fission or budding have also been observed *in vitro* by La Peyre et al. (1993) and Gauthier and Vasta (1993). This type of division, however, is not generally described for *P. marinus* (Perkins 1976, 1993) although there are some reports that it occurs *in vivo* (Mackin et al. 1950, Mackin and Boswell 1956). The importance of binary fission or budding *in vivo* is not clear. Division by budding or fission is prominent *in vitro* in cell populations that exhibit a high growth rate (La Peyre, personal observation).

2. Ray's Fluid Thioglycollate Test

P. marinus–cultured cells enlarge in RFTM and stain blue-black with Lugol's solution (Gauthier and Vasta 1993, Kleinschuster and Swink 1993, La Peyre et al. 1993). This capacity for enlargement is characteristic of *Perkinsus* spp. and is the basis of a common diagnostic test for infection (Ray 1952b, reviewed by Bushek et al. 1994a).

3. Immunoassay

Cultured cells are strongly positive in immunoassays with anti-*Perkinsus* antibody using a polyclonal rabbit anti-*P. marinus* serum raised against hypnospores, produced by Dungan and Roberson (1993) (La Peyre et al. 1993). Likewise, Gauthier and Vasta (1993, 1995) reported cross-reactivity of protein extracts from both cultured cells and freshly isolated merozoites (trophozoites) on Western blots probed with the same antiserum, as well as with their own rabbit anti-cultured merozoite serum.

4. Infectivity

Finally, the ability of cultured cells to infect eastern oysters was demonstrated by La Peyre et al. (1993), Vasta and Gauthier (1993) and Bushek et al. (1994b). La Peyre et al. (1993) found that *P. marinus*–free Maine oysters challenged with 10^6 cultured cells/oyster by injection into the mantle cavity developed 100% prevalences of light-intensity *P. marinus* infections, 8 weeks postexposure.In contrast, Gauthier and Vasta (1993, 1995) reported heavy *P. marinus* infections in oysters 4 to 5 weeks following two biweekly systemic or mantle cavity injections of washed cultured cells (2×10^5). Bushek et al. (1994b) investigated the dose response of oysters to cultured *P. marinus* and the fate of those cells, following exposure of oysters by three different methods (feeding, mantle cavity injection and adductor muscle injection). Only mantle cavity or adductor muscle injections were effective for development of infections. It was suggested that most of the cultured cells were either destroyed or eliminated by the oysters since low percentages of cells were recovered 4 days postexposure.

D. Measurement of Growth Rate of P. marinus In Vitro

Despite all of the information on culture procedures for *P. marinus* in the early publications, there were few data on growth rates or optimal culture conditions. However, two recent reports on optimization of culture conditions, for the propagation of *P. marinus*, in commercial media supplemented with FBS, are now published (Dungan and Hamilton 1995, Gauthier and Vasta 1995). In addition, the effects of several variables such as osmolality, temperature, pH, gas phase and seeding density on the propagation of *P. marinus in vitro* in BSA-free JL-ODRP-1 medium were measured (La Peyre and Faisal, submitted a).

In each of these studies a different method was used to determine the growth rate of parasite cells. Growth rates were measured either 1) by microscopically counting cells with a hemacytometer (La Peyre and Faisal, submitted a), 2) by using a tetrazolium-based cell proliferation assay (Dungan and Hamilton 1995), or 3) by measuring the optical density of cell suspensions spectrophotometrically at 600 nm (Gauthier and Vasta 1995). Each of these methods has its advantages and disadvantages.

The advantage of counting with a hemacytometer is that it provides a direct measurement of the actual number of cells. However, one of the difficulties in measuring cell number is that *P. marinus* enlarge and can divide by schizogony causing clumps of daughter cells. Cells were disaggregated by three passages through a 25-gauge needle. Microscopic cell count was used most successfully when the parasite exhibited high proliferation rates and division was predominantly by binary fission. Using this technique, population doubling time was defined as the time required for a

cell population to double its actual number, regardless of cell volume.

Spectrophotometric estimation of cell proliferation by tetrazolium-based assay is based on reduction of sulfonated internal tetrazolium salts to colored formazan products by mitochondrial dehydrogenase activity in viable cells. Dungan and Hamilton (1995) used a commercial cell proliferation assay (CellTiter 96 AQ™, Promega) which produces a soluble formazan product and thus permits direct reading of proliferation assays in microplate cultures using a microplate reader at 490 nm. This assay is rapid and convenient and inherently incorporates viability measurements. However, this assay measures mitochondrial activity which varies as a combined function of not only cell number, but also cell size and metabolic activity. Therefore, only the relative proliferation of a specific cell population propagated under different conditions can be compared. Doubling time was defined by Dungan and Hamilton (1995) as the time required for a cell population to double its biovolume, regardless of number.

Growth of *P. marinus* was also determined by measuring the optical density of the parasite cell suspension spectrophotometrically at 600 nm (Gauthier and Vasta 1995). This technique is simple and rapid but depends not only on the number of cells but also on their sizes and degree of aggregation. All of these cell population characteristics may vary widely among cultures, rendering standard curve estimates of population density inaccurate. Hence, only the relative proliferation of a specific cell population propagated under different conditions can be compared. Doubling time would have to be defined as the time required for a cell population to double its biovolume, regardless of number.

It is, therefore, important to keep in mind the parameter used in assessing cell growth when interpreting experimental data. It is conceivable that growth measurements obtained from either cell number, cell mitochondrial activity or optical density may differ. Ideally, a combination of a quantitative cell number technique and one that measures biovolume should be used in characterizing growth of *P. marinus*.

Nonetheless, doubling times of *P. marinus* have been reported (Gauthier and Vasta 1993, Dungan and Hamilton 1995, La Peyre and Faisal, submitted b). Gauthier and Vasta (1993) reported an estimated log phase doubling time of 24 h. Dungan and Hamilton (1995) calculated a 13-h log phase doubling time for *P. marinus* isolate ATCC 50439, under optimized conditions. The minimum log phase doubling time for *P. marinus* isolate *Perkinsus*-1 was 17 h (La Peyre and Faisal, submitted b). Strict comparison of *P. marinus* growth rates is, however, not possible because of many differences between studies, such as in media, isolates, seeding densities, proliferation assays and period of cell proliferation selected to calculate doubling time.

E. Optimization of Culture Conditions

Culture conditions for *P. marinus* proliferation in FBS-supplemented commercial media, as well as in a protein-deficient medium (i.e., BSA-free JL-ODRP-1), have recently been optimized (Dungan and Hamilton 1995, Gauthier and Vasta 1995, La Peyre and Faisal, submitted a) (Table 3). The apparent variation in the obtained results emphasizes the limitations imposed by the technique used to measure *P. marinus* cell growth.

The culture system used for optimization varied between researchers. Dungan and Hamilton (1995) selected 1:1 DME:Ham's F-12 with supplements to determine the optimal temperature, osmolality and pH conditions for *P. marinus*. *P. marinus* growth was measured by the MTS/PMS cell proliferation assay. Their experiments were conducted in 96-well titer plates with a seeding density of 1×10^6 to 5×10^6 cells/ml, depending on the parameter measured. Variable effects were usually measured 48–72 h postinoculation. The optimization experiments of Gauthier and Vasta (1995) were conducted in 24-well plates at 28°C and usually at a seeding density of 1×10^6 cells/ml. They used their optimal medium, 1:2 DME:Ham's F-12 supplemented with 5% FBS. Cell growth was determined spectrophotometrically by measuring the optical density at 600 nm following an experimental incubation period, generally 10 days. La Peyre and Faisal (submitted b) optimized culture conditions in BSA-free JL-ODRP-1. Cell propagation was determined by measuring cell density with a hemacytometer. Generally, flasks (75 cm^2) were seeded with 1×10^6 cells/ml and cell densities were determined on days 1, 3, 7, 9, 12 and 15 postinoculation.

Temperature has the greatest effect on the growth of *P. marinus in vitro*, with an optimum at 28°C in all of the above exper-

TABLE 3.

Optimization of culture conditions.

Culture Condition	Reference*	Conditions Tested	Recommended Conditions
Temperature (°C)	1	4, 10, 15, 20, 25, 28, 35, 40	28
	2	4, 20, 28, 32, 38	28–32
	3	4, 17, 22, 28, 36	28
Osmolality (mOsm/kg)	1	320, 480, 640, 800, 960, 1,120, 1,920	800
	3	372, 661, 963, 1273	661
Salinity (ppt)	2	12, 18, 24, 30, 36, 42	24–36
pH	1	6.0–8.5 at increment of pH 0.5	7.0
	2	5.6–7.8 at increment of pH 0.2	6.6–6.8
	3	7.0, 7.5, 8.0	7.5
Seeding density			
Optical density	2	0.02, 0.05, 0.1, 0.15, 0.3, 0.4	0.4
Cell counts (10^6 cells/ml)	3	0.1, 0.2, 0.4, 0.8, 1.6	0.1
FBS concentration (%)	1	0, 1, 2, 3, 4, 5, 7.5, 10, 15	10
	2	0, 0.1, 1, 5, 10, 20	5

* References: 1, Dungan and Hamilton (1995); 2, Gauthier and Vasta (1995); 3, La Peyre and Faisal (submitted a).

iments (Dungan and Hamilton 1995, Gauthier and Vasta 1995, La Peyre and Faisal, submitted b). Differing results, however, were obtained at suboptimal temperatures and were most likely due to limitations of the proliferation assays used. Dungan and Hamilton (1995) reported increasing cell proliferation with increasing temperature between 10 and 35°C. Gauthier and Vasta (1995) found maximum growth at 32°C; however, they reported that their cells looked less healthy than at 28°C. La Peyre and Faisal (submitted b) found that cells enlarged but did not divide at 36°C, and eventually died. Using lower temperatures, Dungan and Hamilton (1995) reported cell proliferation at 10°C, while Gauthier and Vasta (1995) did not report significant growth between 4 and 20°C. La Peyre and Faisal (submitted a) reported that cells at 17°C were slow to enlarge but enlarged to a greater extent before dividing, than at higher temperatures. The tetrazolium cell proliferation assay was the most sensitive to changes in cell activities. These changes in cell activities, however, may or may not indicate cell propagation under suboptimal conditions (i.e., 10 and 35°C). The optical density assay appears to be least sensitive, since no proliferation was detected for cells incubated at 20°C for 10 days.

P. marinus can be propagated in media with a wide range of osmolality (i.e., 340–1,920 mOsm/kg). Salinities of media or sea water solutions with osmolalities between 340 and 1,920 mOsm/kg, would have salinities between 12.7 and 68 ppt. From these studies it would appear that *P. marinus* growth is greatest in salinities in the range of 24–30 ppt. La Peyre and Faisal (submitted b) found that the propagation of the parasite was greater at 661 mOsm/kg (~24 ppt) than at either 372 mOsm/kg (~14 ppt) or 963 mOsm/kg (~34 ppt). In comparison, Dungan and Hamilton (1995) reported near-optimal proliferation of *P. marinus* between 475 mOsm/kg (~17 ppt) and 959 mOsm/kg (~34 ppt) with a maximum at 794 mOsm/kg (~28 ppt). Moreover, Gauthier and Vasta (1995) found that growth of *P. marinus* was significantly lower at 18 ppt than at 24 or 30 ppt but not at 36 ppt. It is important to note, however, that the results obtained represent growth rates of cells that were acclimated to 724 mOsm/kg (La Peyre and Faisal, submitted b), 650 mOsm/kg (Dungan and Hamilton 1995) and 960 mOsm/kg (Gauthier and Vasta 1995) and then transferred to the designated osmolalities without acclimation. It is possible that the growth rate of *P. marinus* might increase at lower or higher osmolalities after a period of acclimation. Indeed we have found that cells cultured in a medium with an osmolality as low as 341 mOsm/kg (12.7 ppt) for more than a year had a growth rate similar to that of cells cultured at 724 mOsm/kg in BSA-free JL-ODRP-1 medium (La Peyre unpublished data, O'Farrell 1995).

P. marinus can be propagated in a wide pH range. The optimal pH for the propagation of *P. marinus in vitro* depended on the group of researchers but encompassed a pH range of 6.6–7.5. These pH values correspond to the reported pH range of oyster plasma (Cousserans 1975).

Seeding density dramatically influences *P. marinus* growth rate. In our study we found that the growth rate of cultured cells was significantly increased by decreasing the seeding density from 16×10^5 to 1×10^5 cells/ml. The optimal seeding density of 10^5 cells/ml for *P. marinus* is within the range (i.e., 10^4–10^5 cells/ml) of seeding density generally used for cell cultures (Freshney 1994). Presumably, decreasing cell density increases the amount of nutrients available per cell. In contrast, Gauthier and Vasta (1995) reported that growth rate of their cultured cells increased with inoculum size. Their original data indicate that wells receiving the highest inocula had the greatest optical density after 8 days. Growth rate of the cultured cells, however, decreased with increasing seeding density if doubling times are calculated between initial and final optical density data.

F. Additional Techniques: Mass Culture, Cloning and Cryopreservation

The scaling-up of *P. marinus* culture allows production of a large number of cells, as well as conditioned medium containing secreted extracellular products. *P. marinus* adapts well to mass culture conditions. We routinely propagate cells in large culture flasks (75–150 cm^2, 50–100 ml) and use multitray units (1-10 × 600 cm^2, 1-10 × 200 ml; Nunc cell factories) to produce conditioned medium (La Peyre et al. 1995a and unpublished data). Other researchers have used roller bottles to scale up cultures (Kleinschuster et al. 1994, Gauthier and Vasta 1995). Gauthier and Vasta (1995) showed that higher densities of *P. marinus* were accomplished in 500 ml or 1 liter of culture medium in roller bottles than in 75-cm^2 flasks containing 50 ml of culture medium. In addition to the methods mentioned, a variety of culture equipment for scaling up cell culture is reviewed by Griffiths (1992) and might be used for *P. marinus* mass culture.

The ability to clone *P. marinus* is extremely helpful since it provides a source of genetically identical cells. Consequently, potential problems associated with the use of a mixed cell population are eliminated, and interpretation of experimental results from a wide range of studies (e.g., genetic, virulence factors, physiological, biochemical) is greatly facilitated. *P. marinus* has been cloned by standard limiting dilution method (La Peyre et al. 1993, Gauthier and Vasta 1995). Gauthier and Vasta (1995) reported that clonal cultures were successful only when a substantial volume (1:1) of conditioned medium was added to fresh medium, suggesting that certain excreted/secreted parasite products stimulated proliferation. Cloning methods described for other protozoal and animal cells, such as cloning on semi-solid medium, might be useful for *P. marinus* cloning (Iovannisci and Ullman 1983, Freshney 1994) but have not been reported for use with *P. marinus*.

Genotypic alterations, as well as alterations in phenotypic expression, may occur during long-term culture of any cell population. Most continuous cell strains, even after cloning, contain a range of genotypes that are constantly changing (Freshney 1994). It is thus important to be able to preserve cells early after the continuous establishment of cultures and periodically thereafter. Cryopreservation of *P. marinus* is done by freezing in the presence of dimethylsulfoxide (Bushek 1994, Dungan and Hamilton 1995, Gauthier and Vasta 1995). The ability of *P. marinus* to survive freezing was reported as early as the 1950s. Andrews and Hewatt (1957) froze *P. marinus* in oysters for several days, but failed to kill the parasite, which subsequently enlarged in RFTM upon thawing. Cultured *P. marinus* can also be maintained for extended periods of time (>1 year) without change of medium, or in sea water (Bushek 1994, Dungan, personal communication, La Peyre unpublished data). During this time, division of cultured cells occurs without enlargement and produces small cells of about 2 μm. Once placed in fresh medium, these cells enlarge and propagate.

A number of cryopreserved *P. marinus* isolates have been deposited at the American Type Culture Collection (Rockville, MD) and are now available to researchers (Dungan and Hamilton 1995, Bushek personal communication).

RECENT FINDINGS AND FUTURE STUDIES

For the first time in over four decades of *P. marinus* research we have access to large quantities of uncontaminated parasite cells. Moreover, culture of the parasite provides a much simplified system to study the biology of *P. marinus*, independent of host influences. The success in propagating *P. marinus in vitro* has already permitted several important studies.

A. Systematics, Genetics, Diagnostics and Detection

1. Systematics and Genetics

The combination of culture methodology and molecular biology techniques is a powerful tool to investigate many aspects of *P. marinus* biology, including its taxonomy and genetics. *P. marinus* taxonomy has always been ambiguous and is still controversial (Vivier 1982, Wolters 1991, Perkins 1996). Moreover, the distinction between different *P. marinus* races, or between *P. marinus* and other *Perkinsus* spp., is not evident since there are no criteria to differentiate them morphologically (Perkins 1993).

Recently, genetic molecular techniques (i.e., polymerase chain reaction and molecular cloning) have been used to evaluate the phylogenetic position of *Perkinsus* spp., including *P. marinus* (Fong et al. 1993, Goggin and Barker 1993, Lester et al. 1993, Goggin 1994, Goggin and Cawthorn 1994, Marsh et al. 1995a). The availability of uncontaminated cultured cells has been very helpful to confirm a small subunit rRNA gene sequence of *P. marinus* which was originally characterized from cells of infected oyster hemolymph (Fong et al. 1993). In another study, Marsh et al. (1995a), using cultured cells, reported to have cloned and sequenced a 3.2-kbp mtDNA fragment that contains the 5S ribosomal RNA gene. Results from these studies indicate that *P. marinus* is closely related to the dinoflagellates.

The existence of different races of *P. marinus* could have important implications for disease management but has received little attention until recently (Andrews 1955, Ray and Chandler 1955, Bushek 1992, 1994). Bushek and Allen (1994) established continuous cultures of several isolates of *P. marinus* from the Atlantic and Gulf coasts and compared their virulence. Isolates from the Atlantic Coast (New Jersey, Virginia) appear to be more virulent than isolates from the Gulf Coast (Texas, Louisiana). More extensive studies are needed to identify races of *P. marinus* and to compare their virulence and environmental tolerance (Bushek 1994).

2. Diagnostics and Detection

The ability to propagate *P. marinus in vitro* facilitates the development of DNA probes for its diagnosis in oysters and its detection outside of oysters (Fong et al. 1993, Marsh et al. 1995b). Marsh et al. (1995b) developed a semiquantitative assay to detect *P. marinus* in oyster hemolymph using polymerase chain reaction amplification of an intergenic mtDNA domain of cultured *P. marinus*. Development of DNA probes may also be useful to differentiate between races of the parasite in oyster populations. Other specific probes such as monoclonal and polyclonal antibodies against *P. marinus* have been used to monitor parasite abundance in the water column (Dungan and Roberson 1993, Roberson et al. 1993). These molecular probes for *P. marinus* may help reveal the ecology of this parasite, as well as explain transmission dynamics.

Experiments on cultured cells can also help improve existing methods that use RFTM for *P. marinus* diagnosis. Enlargement of cultured cells in RFTM is significant, about threefold, but is lower than expected (La Peyre et al. 1993). *In vitro* studies with cultured cells indicate, however, that enlargement and viability of *P. marinus* can be greatly enhanced by the addition of lipids and vitamins to RFTM (La Peyre, unpublished data). RFTM thus appears to contain necessary, but not sufficient, nutrients for the optimal enlargement of *P. marinus*. Enlargement of *P. marinus* generally occurs when infected oyster tissue is placed in RFTM. It is thus likely that the degrading oyster tissue provides needed nutrients for the optimal enlargement of the parasite in RFTM. As a result of these studies, it was found that abundance of *P. marinus* cells could be increased by the addition of lipid to heavily infected oyster tissue in RFTM since the previously undetected smallest cells (<10 μm) enlarged sufficiently to be detected and counted (La Peyre unpublished). Methods that use RFTM for *P. marinus* diagnosis might thus be improved in the future by the addition of certain supplements such as lipids.

B. Life Cycle and Growth Characteristics

P. marinus exhibits the typical growth curve of cultured cells. Upon seeding, *P. marinus*–cultured cells enter a lag period, generally lasting a day, during which little proliferation occurs. Cells then start dividing at an exponential rate (log phase), generally during the first week of culture. Finally, the growth rate of cells is drastically reduced as cells enter the stationary phase. Measurements, such as cell densities and length of each growth phase, vary depending on isolates and culture conditions.

Cells in medium or in culture conditions that support the highest growth rate are relatively small in size and divide predominantly by fission or budding. In contrast, the slowed growth of cultured cells in suboptimal conditions is associated with increase in cell size and division that is primarily by schizogony. Cells in late stationary phase are able to undergo cell division in the absence of enlargement. These cells can be maintained for an extended period of time (>1 year) in what appears to be a "dormant" state.

Division by schizogony is considered to be the method of division of *P. marinus* in oyster tissue (Perkins 1993). It is conceivable, however, that division of *P. marinus* by binary fission or budding may occur early in infection when the parasite is actively dividing and propagation is not nutrient limited. Division by binary fission or budding may thus have been missed in histological sections since the chance of encountering the parasite in sections of oysters with light *P. marinus* infection is low.

In addition to the production of merozoites, *P. marinus* can undergo zoosporogenesis and produce zoospores. The availability of cultured cells may help elucidate some of the determining factors involved in zoosporogenesis. Zoosporogenesis of hypnospores, obtained from cultured cells, has been observed with *Perkinsus* sp. originally isolated from the hard clam, *Macoma balthica*, but not with *P. marinus* (Kleinschuster et al. 1994). It is possible that additional nutrients or specific physicochemical factors are needed for inducing hypnospore zoosporogenesis from cultured cells. *P. marinus* zoosporogenesis occurs to a limited extent when hypnospores are isolated from infected oyster tissue incubated in RFTM and placed in culture medium (La Peyre 1993, La Peyre and Faisal 1995a). In this case, transformation of zoospores to merozoites has been observed.

C. Physiology

It is becoming clear from *in vitro* studies that *P. marinus* can be propagated in a wide range of environmental conditions and can survive extreme culture conditions. Among these environmental conditions, temperature and salinity are the most important in controlling the abundance of *P. marinus* in oysters. Until recently, interpretation of results from field and laboratory studies examining the effects of temperature and salinity on *P. marinus* infection was tentative since the parasite could not be studied independent of the host. In this context, information on the growth rate of cultured *P. marinus* cells at different temperatures and salinities helps explain the epizootiology of the disease and may provide new ideas for management practices for disease control. Overall the results obtained *in vitro* are consistent with recent epizootiological and *in vivo* studies.

Investigations of the mechanism of adaptation of *P. marinus* to various environmental conditions may suggest novel ways to disrupt parasite biology in the host. Several studies have recently reported the response of *P. marinus* to acute osmolality and temperature exposure (Burreson et al. 1994, O'Farrell 1995, O'Farrell et al. 1995, Tirard et al. 1995).

The acute osmotic tolerance of *P. marinus* was investigated by Burreson et al. (1994). They showed that most cultured cells are killed by acute hypo-osmotic shock although some cells are able to survive at very low osmolalities. For example 10% of cells are viable 24 h following transfer from 630 to 136 mOsm/kg. Following this initial study, cultured cells were successfully acclimated and propagated at low osmolalities (168, 341, 433 mOsm/kg; La Peyre unpublished data). Moreover, a much greater percentage (60%) of cultured cells propagated at 168 mOsm/kg survive extremely low osmolality shock (56 mOsm/kg, 2.5 ppt) than cells acclimated to higher osmolalities (O'Farrell et al. 1995, O'Farrell 1995). The ability of cultured cells to withstand such low osmolality explains recent reports on the persistence of *P. marinus* infection in oysters exposed to salinity lower than 3 ppt (Burreson and Ragone Calvo 1994, Ragone Calvo and Burreson 1994).

The ability of *P. marinus* to volume regulate was recently demonstrated by O'Farrell (1995). Cultured cells swelled within the first minute following a moderate hypo-osmotic shock and then returned toward original size within the next 4 minutes without lysing. Mechanisms of osmoregulation are, however, poorly understood. The amino acid concentration of cells at higher osmolalities was not greater than that of cells at lower osmolalities (O'Farrell 1995). O'Farrell (1995) suggested that amino acids are not the primary osmolytes used by *P. marinus* in osmoregulation and that other osmolytes such as polyols may be involved.

Exposure of *P. marinus* to acute hyperthermic shock induced the production of heat shock proteins (Tirard et al. 1995). These heat shock proteins of *P. marinus*–cultured cells differ in size and immunochemical specificity from oyster heat shock proteins. Thermal threshold for heat shock protein induction was higher in *P. marinus* than in oyster hemocytes.

D. Virulence Factors, Infectivity and Pathogenicity

1. Virulence Factors

Multiple proteases are present in *P. marinus* culture supernatants (La Peyre et al. 1995a, 1995b). These proteases digest a variety of proteins including gelatin, casein, fibronectin, laminin and plasma proteins. They belong to the serine class of proteases and are chymotrypsin-like enzymes. Protease production by *P. marinus* increases with increasing growth rate of the parasite at high temperature (28°C) and salinity (24 ppt) (Garreis et al. unpublished data). Although the exact role(s) of *P. marinus* proteases is still unknown, several preliminary findings suggest that *P. marinus* proteases may play a role in invasion by the parasite of host tissues and in counteracting both cellular and humoral defenses of the oyster.

The finding that *P. marinus* proteases degrade extracellular matrix proteins (i.e., fibronectin and laminin) may explain the extensive tissue lysis observed in heavily infected oysters and provides a possible mechanism by which *P. marinus* can gain access to the connective tissue (Mackin 1951, Ray et al. 1953, Ray 1954a, La Peyre et al. 1995a). The parasite body burden in oysters fed liposomes containing extracellular products (ECP) in conditioned medium and then challenged with *P. marinus* was significantly higher than that in oysters fed liposomes containing fresh medium (La Peyre et al., submitted a).

There is also an indication that *P. marinus* proteases suppressed cellular and humoral parameters of oyster host defenses (Garreis et al. 1995). Oyster hemocyte motility was reduced by purified proteases in a dose-dependent manner. Observations of hemocyte monolayers also indicate that *P. marinus* ECP enhanced degranulation of granulocytes and inhibited granulocyte motility and spreading of large hyalinocytes (La Peyre et al. unpublished data). It is possible that ECP, including proteases, may be partly responsible for the reported failure of hemocytes to successfully encapsulate groups of dividing *P. marinus* cells (Perkins 1976) and for the inability of hemocytes to kill and degrade or expel *P. marinus* at high temperature. Lysozyme activity and hemagglutinin titer also decrease following incubation of oyster plasma with *P. marinus* ECP or its purified proteases (Garreis et al. 1995). The decline of lysozyme activity and hemagglutinin titer, *in vivo*, have been reported in heavily infected oysters (La Peyre 1993, Chu and La Peyre 1993a).

These initial studies suggest that *P. marinus* proteases play a significant role in the pathogenesis of the disease. Further investigations on the role of proteases are needed. Moreover, the location, concentration and activity of *P. marinus* proteases *in vivo*, in infected oysters, need to be determined in order to better interpret *in vitro* results.

Acid phosphatase is another potential virulence factor that has been measured in cell-free culture supernatants (Volety and Chu 1994a, Volety 1995). Acid phosphatase activity has been located in the nucleus and the cell membrane of *P. marinus* as determined ultrastructurally by lead phosphate precipitation (Volety 1995). Extracellular acid phosphatase concentration in the culture medium is positively correlated with *P. marinus* cell number and temperature. Volety and Chu (1995) suggest that acid phosphatase may be responsible for the observed suppression of hemocyte chemiluminescence by *P. marinus* in response to zymosan, since other anti-oxidant enzymes, such as superoxide dismutase, catalase and glutathione peroxidase, were not detected in *P. marinus* merozoites. The significance of this finding to *in vivo* infections is unknown, since it has also been reported that the chemiluminescent response to zymosan was enhanced in hemocytes collected from heavily infected oysters (Anderson et al. 1992, 1995, La Peyre 1993). The increase in hemocyte chemiluminescent response to zymosan has been attributed to hemocyte activation in diseased oysters (La Peyre 1993, Anderson et al. 1995).

P. marinus produces specific proteins as a response to heat

shock (Tirard et al. 1995). Synthesis of heat shock proteins is generally induced as a response to stressful conditions. These "stress" proteins may be important for the survival of *P. marinus* in oysters, especially in phagolysosomes of hemocytes (Lathigra et al. 1991). In this respect, the ability to differentiate heat shock proteins of *P. marinus* from those of oyster hemocytes as reported by Tirard et al. (1995) will be very useful.

Further identification of *P. marinus* extracellular proteins and determination of their role in pathogenicity are needed. Cell surface proteins also need to be investigated as virulence factors. For example, cell proteases and hemagglutinins are present on the surface of *P. marinus* and need to be characterized (La Peyre et al. unpublished data). The way in which *P. marinus* merozoites interact with the surface of oyster cells, including hemocytes, has not yet been investigated. Cell surface hydrophobicity and lectins of microbes have been implicated in a wide variety of microbial adhesion phenomena and are considered major determinants of virulence in microbial infections (Mirelman 1986, Doyle and Rosemberg 1990).

2. Infectivity and Pathogenecity

The ability to mass culture *P. marinus in vitro* and to quantify total parasite burden provides obvious advantages for studying *P. marinus* pathogenesis and infection kinetics. The availability of an axenic and quantifiable inoculum for infection experiments is certainly very useful for investigating the mechanisms of infection and pathogenicity of *P. marinus*. Caution must be exercised, however, when interpreting results with cultured cells since *in vitro* expression of virulence factors may not entirely correspond to expression *in vivo*. Of major concern are possible changes in the parasite resulting from prolonged *in vitro* growth.

P. marinus is still infective and pathogenic after several years in culture; however, some studies suggest a loss of virulence in cultured cells (La Peyre et al. 1993, Bushek et al. 1994b, Chintala et al. 1995). In the most comprehensive study so far, the mortality rate of oysters challenged with natural parasites was found to be much greater than that of oysters challenged with cultured parasites (75 vs 7.5%) 12 weeks postchallenge (Chintala et al. 1995). The number of cultured cells expelled (i.e., recovered from feces and pseudofeces) was nearly 20 times greater with cultured cells than with natural cells. Chintala et al. (1995) speculated that the surface of wild cells may have receptors or other characteristics that are reduced or lacking in cultured cells and that favor retention by the oyster.

Results from recent infectivity studies suggest that greater prevalences and infection intensities are produced when oysters are exposed to natural *P. marinus* cells than similar dosage of cultured cells in analogous studies (Volety and Chu 1994b, Volety 1995). Interestingly, virulence of natural cells seems much greater in early infection studies of the 1950s (Ray 1954b, Ray and Mackin 1954, Andrews and Hewatt 1957) than it is today. The level of parasite virulence has not been duplicated in recent years except for the studies of Gauthier and Vasta (1993, 1995). The extreme virulence of Gauthier and Vasta's isolate compared to other cultured cells, as well as natural cells, if confirmed, will be useful in relating parasite characteristics (i.e., surface and extracellular proteins) to virulence. Apparent differences in virulence between natural and cultured cells, as well as between cultured isolates, provide a powerful tool for analyzing virulence determinants. Differences in either entry, interaction with host defenses and/or the ability to propagate in the host, between natural and cultured *P. marinus* cells, need to be investigated.

E. Interactions With Oyster Host Defenses

One requirement for a pathogen to become established in a host is that the pathogen must overcome host defenses. The pathogen may passively avoid being recognized or actively inhibit or interfere with host defense mechanisms. In cases where the host is able to recognize and kill pathogen cells, rapid proliferation of the pathogen in host tissues may overwhelm host defenses. This may result from: 1) infection (entry) by an overwhelming number of pathogen cells, 2) high rate of pathogen proliferation in host tissues or 3) depressed defense response. In the case of *P. marinus*, it is likely that a number of these scenarios are extant under conditions that favor the parasite. For example at high temperatures, propagation of cultured cells is greatly increased, secretion of factors by cultured cells with the potential to suppress cellular and humoral host defenses is increased, oyster hemocyte activities are depressed (Fisher 1988, Fisher et al. 1989, Chu and La Peyre 1993b) and natural exposure to potentially infective *P. marinus* is increased (Roberson et al. 1993).

While *P. marinus* rapidly overwhelms oysters at high temperatures (>25°C), there is increasing evidence that *P. marinus* can be degraded or expelled by the oyster defenses at low temperatures (<15°C). The availability of *P. marinus* cultures is important in this analysis since it reveals that *P. marinus* can still proliferate at 6 ppt and 17°C, albeit slowly, and can survive extremely low temperatures and salinities. The decrease in parasite abundance in oyster tissue in winter (Ray 1954a, Andrews and Hewatt 1957, Ragone Calvo and Burreson 1994) must therefore be due to an active elimination of the parasite by oyster host defenses. Additional studies have shown that hemocytes can kill and degrade natural or cultured *P. marinus* cells, *in vitro* (La Peyre 1993, La Peyre et al. 1995c) and *in vivo* (Bushek et al. 1994b), respectively. Expulsion of *P. marinus* cells by hemocytes may also be important since hemocytes have been shown to cross epithelia and excrete undigestible biotic and abiotic materials (Stauber 1950, Tripp 1960, Alvarez et al. 1992). Further studies are needed to identify the mechanisms of killing as these may provide markers for disease resistance.

In addition to hemocytes, humoral factors may also play a role in oyster defenses against *P. marinus* infection. Unfortunately, the role of humoral factors in host defenses against *P. marinus* has received little attention. These humoral factors may include lysozyme, protease inhibitors and iron-binding proteins.

Plasma lysozyme was proposed to be a potential defense factor against *P. marinus* (Chu et al. 1993, Chu and La Peyre 1993b) since its activity is much greater in oysters maintained at low temperatures and low salinities, conditions that are favorable for the elimination of *P. marinus*. In addition, preliminary studies indicate that *P. marinus* cells are lysed by plasma with high lysozyme activity from oysters maintained at low salinity as well as by commercial hen egg white lysozyme suspended in a low-salt solution (6 ppt) (La Peyre and Chu, unpublished data). Low concentrations of sea salts were used because lysozyme activity was found to be drastically reduced at high salt concentrations. It is possible that lysozyme may accelerate *P. marinus* elimination at low salinity and temperature. Prevalence of infection in oysters maintained at low salinity (5 ppt) showed a more pronounced decline than the infection prevalence in oysters maintained in high

salinity (~20 ppt), during winter (Ragone Calvo and Burreson 1994). This decrease is probably due to the action of the host defenses since *P. marinus in vivo* or *in vitro* can survive such salinity (Ragone 1991, Ragone and Burreson 1993, La Peyre unpublished data, O'Farrell 1995).

Additional preliminary studies also suggest that plasma protease inhibitors may be involved in oyster resistance to *P. marinus* infection (Faisal et al. 1995b). Plasma protease inhibitors are present in oyster plasma from susceptible eastern oysters as well as resistant Pacific oysters (Faisal et al. 1995b). However, their activity against *P. marinus* proteases is greatly increased in challenged Pacific oysters but not in eastern oysters (Faisal et al. 1995b). Moreover, protease inhibitors such as human α_2-macroglobulin have also been found to inhibit the proliferation of *P. marinus in vitro* (La Peyre et al. submitted b). Purification and identification of oyster plasma protease inhibitors active against *P. marinus* proteases may facilitate development of resistant oysters through either physiological regulation or genetic manipulation.

Other plasma factors that inhibit the growth of *P. marinus* may include iron-binding proteins. Gauthier and Vasta (1994) demonstrated that iron chelators such as transferrin inhibit the growth of cultured cells, by sequestering free iron needed by the parasite. They speculated that *in vivo* proliferation of *P. marinus* may be due in part to excess free iron, which they suggested would be available in oyster plasma during summer.

Antimicrobial peptides are host defense factors that have recently been discovered in vertebrates and invertebrates. A number of such antimicrobial peptides (e.g., tachyplesin 1, magainins, cecropins and defensin HNP1) have been tested against cultured *P. marinus* cells *in vitro* and can be lethal to this parasite (Morvan et al. 1995). Transfer of genes coding for antimicrobial peptides, or other resistance factors, may eventually produce resistant oysters. However, the development of techniques for transgenic technology in oysters is still limited.

In conclusion, like other host-parasite systems, there is a dynamic interaction between *P. marinus* and the oyster which varies with environmental conditions to favor either the host or the parasite. It is hypothesized that the apparent ability of oysters to eliminate *P. marinus* at low temperatures is counteracted at high temperature by the high growth rate of the parasite and its increased production of virulence factors that suppress oyster defenses. It is evident that knowledge about the interactions of *P. marinus* with oyster host defenses is still very limited and further investigation is needed to test this hypothesis.

F. Biochemistry, Nutrition and Chemotherapy

One of the major reasons for investigating the biochemistry of *P. marinus* is that the results may suggest ways to control the parasite. A rational approach to chemotherapy is dependent on a working knowledge of the biochemistry of both parasite and host. Little is known, however, about the nutrition, composition and metabolism of *P. marinus*. The availability of large numbers of axenic parasite cells through *in vitro* culture has made biochemical studies on *P. marinus* feasible.

1. Biochemistry and Nutrition

The biochemical characterization of *P. marinus* has just begun. The lipid and fatty acid composition of merozoites and hypnospores was recently characterized (Volety 1995, Volety et al. 1995). It was found that merozoites had a high percentage of phospholipids (61%) and a low percentage of triacyglycerols (18%) whereas hypnospores had a low percentage of phospholipid (8.3%) and a higher percentage of triacylglycerols (67%). The higher percentage of phospholipids, primary membrane constituents, may be explained by the smaller size and high proliferation rate of the merozoites compared to the much larger hypnospores which contained a high percentage of triacyglycerol, a storage lipid. Surprisingly, merozoites and hypnospores had relatively high levels of arachidonic acid (20:4n-6) compared to oysters and culture media. It is important to keep in mind that the merozoites were propagated in two different culture media supplemented with FBS, which contains animal lipids, whereas the hypnospores were isolated from infected oyster tissue following incubation in RFTM, which also contains lipids in addition to lipids from the oyster tissue. Comparison of the lipid and fatty acid compositions of merozoites isolated from oysters and merozoites propagated in defined media should provide more useful data and confirm the lipid composition of *P. marinus*. Interestingly, the chemically defined medium used by Gauthier et al. (1995) contained linoleic acid as the sole source of fatty acid for the propagation of *P. marinus*.

Comparative analysis of input and spent defined media will be useful in determining which nutrients are utilized by *P. marinus*. For example, some amino acids may be selectively depleted from the medium. This simple approach could give preliminary insight into the nutritional requirements of the parasite. Moreover, it may explain some of the pathogenicity associated with *P. marinus*, such as its reported interference with oyster osmoregulation (Paynter et al. 1995, Paynter 1996). As would be expected, some medium components, such as soluble iron, are essential for the parasite (Gauthier and Vasta 1994). Gauthier and Vasta (1994) speculated that the increase of iron in oyster tissues in summer, possibly reflecting pollution, promotes parasite proliferation. In addition, they proposed that in hemocytes, parasites may avoid oxidative damage by depleting hemocyte iron that is required for superoxide and hydroxyl radical production. Information gathered from nutritional and biochemical studies of *P. marinus* is important since it may lead to novel ways of controlling this parasite.

2. Chemotherapy

The availability of cultured cells has permitted screening of an array of chemotherapeutic agents for their ability to kill or inhibit the proliferation of *P. marinus in vitro* (Calvo 1994, Calvo and Burreson 1994, Krantz 1994, Dungan and Hamilton 1995). Prior to the development of culturing procedures, the only *in vitro* technique available was screening the effect of drugs on *P. marinus* enlargement in RFTM (Ray 1966a). The relevance of enlargement of *P. marinus* in RFTM in relation to proliferation of *P. marinus* in oysters is ambiguous. Nonetheless, this technique was useful in selecting cycloheximide for *in vivo* experiments. Cycloheximide is still the only drug that has been found to reduce infection intensities and oyster mortality (Ray 1966b, Calvo 1994).

CONCLUSION

The resurgence of *P. marinus* as the most important oyster pathogen in the Chesapeake Bay in the mid-1980s stimulated research to better understand this deadly parasite. Although numerous reviews discussed *P. marinus* morphology, life history and epizootiology prior to 1990, the lack of continuous culture of *P. marinus* had hindered important investigations on parasite biology and on host-parasite interaction. Fortunately, it was recently discovered that *P. marinus* could be propagated *in vitro*. This con-

tinuous culture generates large quantities of uncontaminated *P. marinus* for research material and has provided a much simplified system to study the biology of *P. marinus*. This new tool has opened new doors to many investigations that were either impractical, difficult or previously impossible.

Although the breakthrough in propagating *P. marinus in vitro* is recent, several important studies have already been accomplished that provide important insights into disease pathogenesis, host-parasite interactions and parasite physiology. There is a need to further pursue these studies and expand on them. Particular emphasis should be placed on studying genetic and phenotypic variations between isolates and how these variations influence virulence of the protozoan. It is also important to realize some of the limitations of cultured cells and to follow up *in vitro* findings with *in vivo* studies.

In conclusion, research should be intensified since the development of techniques for the propagation of *P. marinus* is bound to yield many rewards. Investigations using cultured cells have the potential to generate new knowledge that scientists and managers may use to develop rational approaches to prophylaxis and control of this deadly oyster disease.

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RACES OF *PERKINSUS MARINUS*

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ABSTRACT The existence of parasite races is an integral component of host-parasite interactions with significant implications for host-parasite coevolution, ecology, and management. Despite nearly 50 years of research, few studies have considered the existence or implications of races of *Perkinsus marinus*. Nonetheless, several field and laboratory observations indicate races exist that vary in virulence or environmental tolerance. One of the keys to understanding and managing *P. marinus* lies in the identification and characterization of races. A related and equally important key is elucidation of its genetic population structure. This paper discusses our current knowledge concerning *P. marinus* races and population structures.

KEY WORDS: Dermo, disease, host-parasite interaction, resistance, virulence, *Crassostrea virginica*, population genetics

INTRODUCTION

Perkinsus marinus has long been recognized as a serious oyster pathogen that is often blamed for widespread mortality of the eastern oyster *Crassostrea virginica* (see Ray 1996, Andrews 1996). The economic and ecological value of the eastern oyster has motivated numerous studies on this parasite, but few have considered the existence of races. Races are phenotypically and genetically distinct groups of conspecific individuals (King and Stansfield 1990). Their existence is an important component in the genetics and evolution of host-parasite interactions. Identification and characterization of pathogen races have played an important role in protecting agricultural crops from disease (Day 1974) and might also be important in protecting populations of the eastern oyster from *P. marinus*.

Any trait can be used to distinguish races, but we are primarily interested in environmental tolerance and virulence because these traits have economic and ecological significance—they determine the impact of a particular race on host populations. Mackin (1962) conceded that there may be races of *P. marinus*, but in the absence of clear evidence, he assumed that all variation in virulence was a result of environmental variation. This same assumption on the part of other researchers may be one reason that few studies have considered the implications of *P. marinus* races. Another reason for the lack of studies on pathogen races may be technological, specifically, the logistics of acquiring and sustaining races of the parasites for disease transmission. Recently, *in vitro* culture methods for *P. marinus* have been developed (La Peyre et al. 1993, Kleinschuster and Swink 1993, Gauthier and Vasta 1993). In this paper, we review information on the population structure of *P. marinus* and evidence for the existence of *P. marinus* races and discuss the implications of races for managing oyster populations.

POPULATION STRUCTURE AND THE FORMATION OF RACES

The term "population" is often used in many different contexts. It always refers to a group with something in common. To an ecologist, a population is simply a group of conspecific individuals that share a common geography. It is defined as those individual living in a particular space at a particular time (Krebs 1985). To a geneticist, a population is a group of conspecific individuals that share a common gene pool (King and Stansfield 1990). Thus, genetic populations are defined by the extent of gene flow (i.e., the exchange of genetic information) between groups of individuals. Restrictions in gene flow between populations, i.e., genetic isolation between populations, may lead to the formation of races via random genetic drift or differential selection. Gene flow normally occurs when individuals migrate from one population to another. Hence, population structure plays an important role in racial development.

Several levels of population structure can be defined for *P. marinus* based upon its ecological distribution. Originally discovered off the coast of Louisiana (Mackin et al. 1950) *P. marinus* has been found from Massachusetts to Florida along the Atlantic Coast and from Florida to Texas along the Gulf Coast (Andrews 1988, Ford 1992). Its extension south of the United States is poorly documented, but Burreson et al. (1994) have described a *P. marinus*–like parasite from oysters along the Yucatan coast of Mexico. Infected oysters can harbor more than 10 million *P. marinus* g^{-1} wet tissue weight and virtually 100% of the adult oysters on a bed may be infected year-round (Bushek et al. 1994). Infective stages are transmitted between oysters through the water column (Ray 1954), where concentrations as high as 1.9×10^4 cells L^{-1} have been reported (Dungan and Roberson 1993). Other vectors of transmission have been described (White et al. 1987), but direct water-borne transmission is probably the predominant mechanism. Because they are filter feeders, oysters subsample the planktonic infective stages during feeding and respiration. Hence, each oyster is probably infected with multiple clones of the parasite—sexual reproduction of *P. marinus* has not been observed (Levine 1988)—and the parasites within an infected oyster probably represent clones from a single population. Parasites in adjacent oysters, on different oyster beds, in separate tidal creeks, in distinct estuaries, or on different coasts (i.e., Atlantic versus Gulf) represent various levels of *P. marinus* population structure. From a genetic standpoint, there may be no difference among populations at any of these levels.

Gene flow within, and possibly among, populations of *P. marinus* occurs during transmission of the parasite. Natural dispersal distances during transmission are unknown. Epizootiological data and transmission experiments indicate that distances as short as 15

m can significantly reduce rates of transmission (Andrews and Hewatt 1957). This may approximate a dispersal distance limit, but the reduction is more likely due to dilution of the propagules as they move away from their source (Mackin 1962). Nonetheless, the probability that an infective cell will be transmitted from one oyster to another decreases as distance between the oysters increases. Distance represents a restriction to gene flow as fewer and fewer clones are transmitted between oysters. It follows that the probability that two oysters are infected with the same array of *P. marinus* clones (i.e., the same population) also decreases as distance increases. The physical separation of estuaries represents a second potential restriction in gene flow. We call these "potential restrictions" because at the present time we have no idea of their effectiveness. Considering both possibilities and the extensive range of *P. marinus*, genetic isolation is likely among many populations. The stronger the isolation, the greater the chance races will form.

Populations lacking gene flow may differentiate due to genetic drift or natural selection. It is unlikely that genetic drift will lead to races due to the enormous population sizes of *P. marinus*. A more probable cause is differential selection, which may result from environmental variation, variation in host resistance, or intraspecific competition. For environmental causes, temperature and salinity appear to be the two most important factors governing the distribution and abundance of *P. marinus*. Below about 15°C, epizootics go into remission (Andrews and Hewatt 1957). In the field, prevalence tends to decline with decreasing salinity (Mackin 1956, 1962, Soniat 1985, Soniat and Gauthier 1989) and salinity below 12 ppt retards the development of infections in the laboratory (Ragone and Burreson 1993). Both factors vary latitudinally and have resulted in physiological races of *C. virginica* (Stauber 1950) that appear to be genetically distinct (Barber et al. 1991). Variation in host resistance may cause variation in the parasite by selecting various phenotypes that complement host resistance mechanisms. Finally, clones may compete for susceptible hosts or nutrients within hosts. Those with the highest rates of transmission and/or proliferation should be competitively superior.

Forces also exist that counteract the mechanisms of isolation described above. Historically, oystermen and fishery management programs have transplanted oysters within and between estuaries. These actions have probably moved infected oysters, mixing what may have once been isolated populations of *P. marinus*. Despite such movements, some semblance of the overall population structure has probably remained due to the extensive range of the parasite and the limited movements of oysters. Oysters have not been spread up and down the coasts haphazardly. Rather, most transplantation has occurred within and between nearby estuaries in a somewhat controlled effort to restock dwindling populations, or to protect surviving oysters by moving them into areas less favorable for *P. marinus* (i.e., lower salinity tributaries). Most states now restrict or prohibit importation of oysters. Movement of oysters within or between adjacent estuaries may have destroyed any local population genetic structure, but there is a good possibility that population structure at a larger, regional scale (e.g., Gulf versus Atlantic or mid-Atlantic versus south-Atlantic) has remained intact. The population genetic structure of *P. marinus* is currently under investigation (NOAA 1995).

EVIDENCE FOR RACES OF *P. MARINUS*

Several field observations indicate that races may vary in environmental tolerance, virulence, or both. Despite the positive correlations between salinity and infection prevalence and intensity (Mackin 1956, 1962, Soniat 1985, Soniat and Gauthier 1989), high levels of infection persist in some low-salinity bays along the Gulf Coast of the United States (Craig et al. 1989, Wilson et al. 1990). In Chesapeake Bay, *P. marinus* spread from high- to low-salinity areas during the 1980s (Burreson and Andrews 1988). The persistence of *P. marinus* in low-salinity bays in the Gulf and its spread up Chesapeake Bay may indicate the existence of races tolerant to low salinity. Burreson and Andrews (1988) also reported that the rate *P. marinus* spread from one bed to another increased in Chesapeake Bay during the 1980s. This may denote the occasion of a more virulent race. Finally, the range of *P. marinus* has expanded northward. Prior to 1990, accounts of *P. marinus* in Delaware Bay were rare (Ford 1992, 1996), with outbreaks of disease usually associated with the importation of southern oysters. Generally, the parasite disappeared after winter, presumably because it could not tolerate the colder winters of the more northern latitude. The recent spread of *P. marinus* into Delaware Bay, and further north along the Atlantic Coast, may represent a cold-tolerant race. All of this information is, however, circumstantial. As an alternative explanation for the northward spread of *P. marinus* (and perhaps the other range expansions), Ford (1992) hypothesized that climatic changes, specifically a warming trend, may have created a more hospitable environment for the parasite. Differentiating between these hypotheses will require identification of the genetic population structure of *P. marinus* and characterization of isolates from distinct locales with respect to virulence and environmental tolerance.

To date, only two studies have compared geographically distinct isolates of *P. marinus*. The primary reason for the lack of work in this field has been the inability to obtain pure isolates of the parasite that can be manipulated under laboratory conditions. Recent development of *in vitro* culture methods for *P. marinus* (La Peyre et al. 1993, Kleinschuster and Swink 1993, Gauthier and Vasta 1993) has overcome this problem.

Before *P. marinus* could be cultured, Perkins and Menzel (1966) compared isolates of *P. marinus* directly. They found no difference in the ability of biflagellated zoospores from two isolates to infect excised tissue. Because of their motility and possession of an apical complex, biflagellated zoospores are possibly the primary infective stage during water-borne transmission, but naturally occurring zoospores remain undescribed. They are formed after infected tissue has been incubated in Ray's fluid thioglycollate media (RFTM) and the enlarged parasites transferred to sterile seawater (Perkins and Menzel 1966). Interestingly, Perkins and Menzel (1966) reported detecting few foci of infection in excised tissues exposed to biflagellated zoospores even though explants probably lack many of the defenses which a parasite must overcome. Apparently, the zoospores were uninfective under the conditions of the experiment. The role of zoospores remains unclear and recent attempts to produce *P. marinus* zoospores using identical or similar techniques have failed (Bushek 1994 and personal communications with F-L. Chu, C. F. Dungan, S. J. Kleinschuster, and F. O. Perkins). Similar techniques have worked well to produce infective zoospores in other *Perkinsus* spp. (Goggin and Lester 1987, Azevedo 1989), yet consideration of potential races is absent from the literature.

Bushek (1994) attempted to differentiate 12 geographically distinct isolates of *P. marinus* by comparing enlargement during RFTM incubation of infected host tissue. Replicate individuals from the same stock of uninfected Maine oysters were transfected

with *P. marinus* isolates to eliminate potential differences due to host physiology. Northern Atlantic isolates (above Georgia) generally enlarged more than southern or Gulf isolates (Figure 1). The significance of enlargement in RFTM is unclear, however, and infection intensities in host tissues could not be controlled. Nevertheless, the results suggested differences among the isolates of *P. marinus*.

The development of *in vitro* culture methods for *P. marinus* (La Peyre et al. 1993, Kleinschuster and Swink 1993, Gauthier and Vasta 1993) permits the comparison of isolates under highly controlled conditions. Pure cultures of *P. marinus* may be grown in commercially available media, and several isolates are available from the American Tissue Culture Collection. Races can be identified based on their virulence or their response to varying culture conditions. Using slightly modified methods of La Peyre et al. (1993), Bushek and Allen (1996) compared the virulence among four geographically distinct isolates of *P. marinus*. Two isolates were from the Atlantic Coast (Delaware Bay, NJ, and Mobjack Bay, VA) and two were from the Gulf Coast (Barataria Bay, LA, and South Bay Laguna Madre, TX). Over 500 uninfected oysters, offspring that represented four distinct oyster populations (Maine, New Jersey, Virginia, and Texas) and had been reared in a common environment, were divided equally among eight experimental tanks and then individually inoculated with one of the isolates. Oysters in the same tank were inoculated with the same isolate by injecting 5×10^5 parasites per gram wet oyster weight into the shell cavity of each oyster. Each isolate was used to inoculate oysters from two replicate tanks. Three months later, body burdens were significantly higher in oysters inoculated with Atlantic isolates compared to those inoculated with Gulf isolates (Figure 2). These results provide a clear demonstration of differences in virulence among isolates of *P. marinus*, indicating the likely existence of races. Furthermore, it may be possible to relate specific biochemical properties of these isolates to their virulence. Faisal et al. (1994) detected extracellular proteases, which may represent virulence factors, in the supernates of *P. marinus* cultures. It would be interesting to relate protease production among isolates of *P. marinus* to their infectivity. Undoubtedly, many future studies will use *in vitro* cultured parasites to compare and characterize distinct isolates of *P. marinus*.

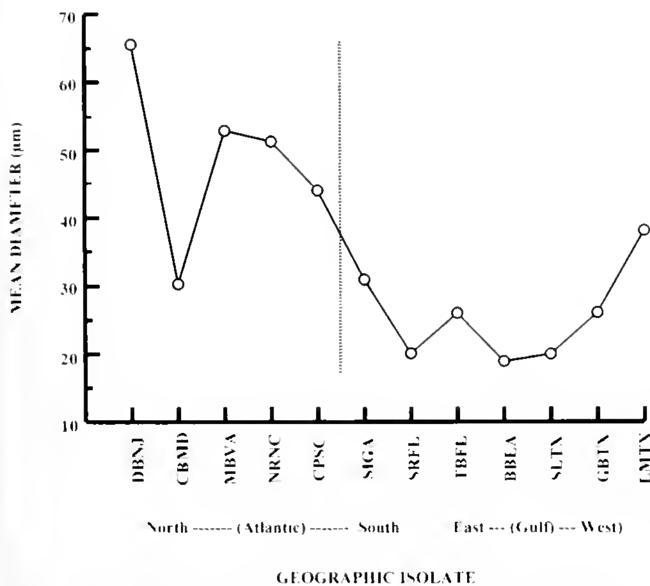

Figure 1. Effect of parasite origin on enlargement of *P. marinus* isolates in RFTM (Ray 1966) at 20°C. The x-axis indicates isolate of the parasite southward along the Atlantic Coast and westward along the Gulf Coast. Dashed line represents apparent clinal break. Isolate abbreviations: DBNJ = Delaware Bay NJ; CRMD = Choptank River MD; MBVA = Mobjack Bay VA; NRNC = Neuse River NC; CPSC = Cherry Point SC; SIGA = Skidaway Island GA; SRFL = Sebastian River FL; TBFL = Tampa Bay FL; BBLA = Barataria Bay LA; SLTX = Sabine Lake TX; GBTX = Galveston Bay TX; LMTX = Laguna Madre TX. Reproduced from Bushek (1994).

INTERACTIONS AMONG HOST-PARASITE RACES

We have considered the formation of *P. marinus* races, but races of *C. virginica* that vary in their response to *P. marinus* must also be considered (Bushek 1994). Parasite virulence and host resistance are dependent upon each other, i.e., the genetic basis of one cannot be determined without considering the other (Nelson 1973, Day 1974). For example, apparent differences in virulence of *P. marinus* from different regions may actually reflect differences in host resistance. To be certain that differences are due to parasite virulence, hosts of the same or similar genetic makeup must be used and exposed to parasites under identical conditions. The converse may also be true; apparent differences in host resistance may actually reflect differences in parasite virulence. The interaction can be further complicated by environmental effects.

Only one study has examined racial interactions between *P. marinus* and *C. virginica*. By separately exposing offspring from four oyster populations to four isolates of *P. marinus*, Bushek and Allen (1996) demonstrated variation in resistance among oyster populations and variation in virulence among parasite isolates. The experimental design also enabled them to detect any genetic interaction. Intuitively, one may expect a "race-specific" interaction where each host race is resistant to a specific parasite race and each parasite race is virulent to a specific host race. For example, Galveston Bay oysters may be more resistant to Galveston Bay *P. marinus* than Chesapeake Bay *P. marinus* because they have had more time to respond to Galveston Bay *P. marinus*. However, Bushek and Allen (1996) found no interaction. Resistance and virulence were "general," not "race-specific." In other words,

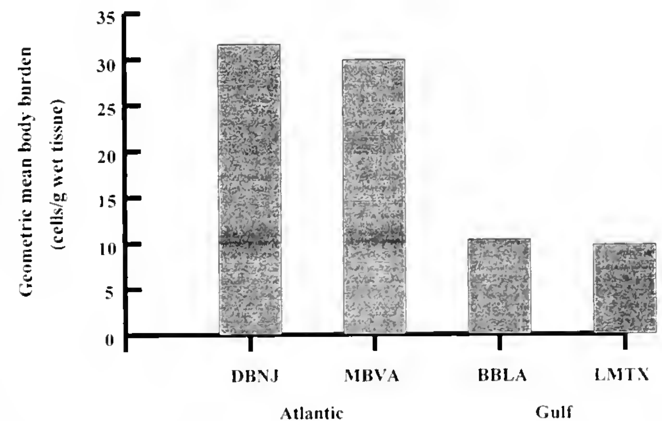

Figure 2. Geometric mean infection intensity of oysters 94 days postinoculation with four different isolates of *in vitro*–cultured *P. marinus*. A planned comparison between Atlantic and Gulf isolates indicated that the Atlantic isolates produced significantly heavier infections ($p = 0.013$). Isolate abbreviations as in legend to Figure 1. Reproduced from Bushek (1994).

the most virulent parasite isolate in one oyster population remained the most virulent across all oyster populations. Similarly, the most resistant oyster population to any isolate of *P. marinus* remained the most resistant to all parasite isolates. This experiment was conducted under one set of environmental conditions (27°C and 25 ppt salinity) and there is a possibility that changes in these environmental conditions may lead to a different outcome. Particularly, we note that different environmental conditions include the variation in the isolation and culture of the parasite itself. That is, these results may have been specific to the changes in virulence that may have occasioned the *in vitro* culture of *P. marinus*. Further investigations are needed to determine the interaction between environmental parameters and virulence.

MANAGEMENT IMPLICATIONS

The existence of *P. marinus* races that vary in virulence or environmental tolerance has important management implications. Management programs should be sensitized to the potential dangers of spreading *P. marinus* races when relaying oysters, restocking oyster beds, or regulating effluents from shucking houses. Certainly, spreading virulent races should be avoided. Spreading races with varying environmental tolerances can be equally harmful by producing epizootics in areas that are inhospitable to indigenous parasite races.

Perhaps the most important implication of parasite races pertains to the development of resistant oyster stocks. Regardless of the strategy employed (i.e, traditional breeding, chromosome set manipulation, hybridization with resistant species, and introduction of resistant species), the existence of races complicates the evaluation of resistant stocks. Will a resistant stock developed against one race be resistant to other races? The lack of a race-specific interaction between *P. marinus* and *C. virginica* (Bushek and Allen 1996) indicates that the answer is yes. It is worth noting that the "resistant" Texas population in Bushek and Allen's (1996) study continues to experience epizootic mortalities (Craig et al. 1989) despite nearly 50 years of natural selection.

The population genetic structure of *P. marinus* is currently unknown but should be a high priority for basic and applied research, particularly with respect to management of oyster populations. At this point, we do not know whether researchers currently studying *P. marinus* in different locales are working with similar or distinct races. Their results may not be comparable or transferable. An even worse possibility is that *in vitro* isolates may represent single clones that happen to be well suited to *in vitro* proliferation and therefore may represent a small fraction of the genetic variation present in wild populations.

Understanding the population genetic structure of *P. marinus* will help lead to a better understanding of the mechanisms that enable *P. marinus* races to spread. For example, combined with molecular tools, knowledge of the population genetic structure will help discriminate among the hypotheses commonly offered to explain the recent northward migration of *P. marinus* (Ford 1992, 1996). A continuous cline of genotypic frequencies in the population genetic structure from Chesapeake Bay northward would implicate natural range expansion, possibly permitted by recent regional climate warming. Alternatively, association of *P. marinus* isolates from the south with new epizootics in the north would imply that importation of southern oysters to northern habitats is responsible for the range expansion. Such an implication has been made regarding the origin of *Haplosporidium nelsoni*, which causes MSX disease in oysters on the East Coast of the United States. Recent studies (Friedman and Hedrick 1995) have described a Haplosporidian parasite in Pacific oysters (*Crassostrea gigas*) from Japan and California that is morphologically similar to *H. nelsoni*. Using a recently developed molecular probe for *H. nelsoni* (Stokes and Burreson, 1995), Stokes, Burreson, and Friedman (unpublished data) demonstrated genetic identity among parasites from each area. The presumption is that *H. nelsoni* was introduced to both coasts of the United States via the importation of infected oysters from Japan. Population genetic data on *P. marinus* will help identify the geographic source of those now in the northeast. Finally, the evolution of new strains of *P. marinus* with, for example, increased tolerance to cold temperatures or low salinity may be implicated by population genetic data if northern isolates are genetically unique and exhibit enhanced environmental tolerances compared to other isolates. These hypotheses are not mutually exclusive, but lacking information on *P. marinus* population structure, any are difficult to rule out.

Despite nearly 50 years of research on *P. marinus*, the studies by Perkins and Menzel (1966) and Bushek and Allen (1996) are the only investigations that have attempted to compare isolates of *P. marinus*. Although Bushek and Allen (1996) demonstrated that races of *P. marinus* vary in virulence, confirmation of these results and the intricacies of genetic population structure (e.g., are there more than two races?) await the precision of molecular tools and additional studies employing larger sample sizes. Once these have been developed, managers should be able to use molecular markers as a forensic tool to identify source populations of epizootics and as a preventative tool to stop the spread of virulent races.

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A WHOLE-OYSTER PROCEDURE FOR DIAGNOSIS OF *PERKINSUS MARINUS* DISEASE USING RAY'S FLUID THIOGLYCOLLATE CULTURE MEDIUM

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ABSTRACT Diagnosis of *Perkinsus marinus* disease of eastern oysters *Crassostrea virginica* has been routinely accomplished by incubating oyster tissues in a fluid thioglycollate medium described by Ray in the early 1950s. At least three modifications of the technique are available with applications to different diagnostic needs. Of these, the quantitative whole-oyster technique is potentially the most valuable because it includes all oyster tissues and does not rely on subjective estimates of intensity. A variety of protocols and approaches were examined in an attempt to develop a standardized procedure for quantitative whole-oyster diagnosis that optimizes sensitivity, specificity, precision and accuracy. A recommended procedure, with possible variations, is presented here with the expectation that its presentation will foster further refinement and improvement. We conclude that the recommended whole-oyster diagnostic technique is capable of providing reliable quantifications of prevalence and intensity and has great potential for examining correlations of total *P. marinus* body burdens with measurements of oyster biology and for evaluating or calibrating other diagnostic techniques.

KEY WORDS: Oyster diseases, *Crassostrea virginica*, *Perkinsus marinus*, disease diagnosis

INTRODUCTION

The discovery of *Perkinsus marinus* (=*Dermocystidium marinum*) as a pathogen of eastern oysters (*Crassostrea virginica*) was made from oysters collected in the Gulf of Mexico through examination of histological sections (Mackin et al. 1950). Soon after, Ray (1952a, 1952b) described a new technique that reduced the time, labor and equipment required for diagnosis and was more sensitive to detection of light infections. The technique was based on incubation of oyster tissue in (Ray's modified) fluid thioglycollate medium (RFTM) to enlarge the parasites for greater visibility. The standard fluid thioglycollate medium was modified by rehydration with sea water rather than fresh water and by addition of antibiotics and antimycotics to reduce bacterial and fungal contamination. Samples from infected oyster tissues formed well-developed and enlarged parasites that were "conspicuous and readily identifiable" (Ray 1952a) after 10–18 hr incubation in RFTM. Parasites were visible in whole mounts (tissue squashes) and recognized by their thin-walled, spherical, cyst-like bodies measuring up to 35 µm in size. After 72 hr incubation the size increased to 90 µm and could reach 150 µm after a week. As they enlarged, the parasites were observed to have thicker cell walls, increased vacuolization and greater lipid drop accumulation (Ray 1952a). Detection of infections, including light infections with smaller, less-developed parasites, was enhanced by staining with a [25-fold] aqueous dilution of Lugol's iodine. The cell walls of parasites incubated in RFTM for 18 hr, 36 hr and a week were progressively stained faint blue, a distinct dark blue, and blue-black. The ability of detect light infections using iodine stain was dramatically improved over unstained tissue squashes.

Early evidence indicated that parasite numbers did not increase during incubation in RFTM, even after several months (Ray 1952b), and further studies corroborated these findings (Stein and Mackin 1957). Since parasites evidently did not multiply in culture, the RFTM technique could be used to estimate infection intensity as well as prevalence. Thus, a relative intensity scale was established to delineate light, moderate and heavy infections (Ray et al. 1953). A rating system, devised by Mackin and first described by Ray (1954a), assigned values to six subjective intensity categories, then divided the total by the number of diagnosed individuals to obtain a "weighted incidence," i.e., an average infection intensity of the population sampled.[1] This method of determining average infection intensity, further characterized by Mackin (1962), has been used widely to describe the epizootiology of *P. marinus* disease (Andrews 1988). Although semi-quantitative, the rating system has also been successfully used to profile disease progression, to demonstrate the strong relationship of high-intensity infections with mortalities and to correlate infection intensity with physiological changes (Soniat and Koenig 1982).

Lewis et al. (1988) and Choi et al. (1989) provided an important alternative for the RFTM procedure. A major obstacle to enumerating parasites had been the obstruction of parasites by oyster tissue, i.e., they were not sufficiently isolated for quantification. These researchers used NaOH to digest oyster tissues without damaging the integrity of the prezoosporangia cell wall. Choi et al. (1989) used the method on dissected tissues to demonstrate that the commonly used Mackin scale was exponential. They could then retrospectively use Mackin scale ratings as an estimate of total parasite numbers. The digestion of tissues led Gauthier and Fisher (1990) and Bushek et al. (1994) to introduce two new quantitative assays using RFTM.

Consequently, there are now three modifications of RFTM culture to characterize *P. marinus* infection intensity in individual oysters: (1) an exponential estimate from selected tissues using the semi-quantitative Mackin ratings (Ray 1954a, Mackin 1962), (2) a quantitation of parasite density in oyster hemolymph (Gauthier and

[1]Although "incidence" was once used interchangeably with "prevalence," the current definition for incidence is the rate of occurrence of new cases of a particular infection in a population (Overstreet 1978, Margolis et al. 1982). "Weighted prevalence," as is currently used, is a more acceptable term for this calculated value.

Fisher 1990), and (3) a quantitative whole-oyster diagnosis (Bushek et al. 1994). The value of these different techniques depends on their application (Bushek et al. 1994). For example, the tissue squash technique has proven invaluable in epizootiological studies (Andrews 1988, 1996, Burreson and Ragone Calvo 1996, Ford 1996, Soniat 1996) and the hemolymph biopsy technique can be used when animals cannot be sacrificed (Fisher et al. 1992). Yet the whole-oyster technique is potentially the most valuable because it is quantitative and incorporates all oyster tissues. These attributes make it the standard by which other techniques should be evaluated and the most suitable technique for correlation of disease intensity with measurements of oyster biology. Researchers are now seeking to relate infection intensity to measurements of oyster physiology, immunology, reproduction and growth, in attempts to understand susceptibility of oysters and sublethal effects of *P. marinus* disease (Anderson 1996, Chu 1996, Paynter 1996). The most supportable correlations will stem from reliable whole-oyster quantifications of disease prevalence and intensity.

The value of any diagnostic technique depends on attaining certain standards of sensitivity, specificity, precision and accuracy (Bushek et al. 1994). These standards must be continually balanced with cost, ease, technical capability and purpose. The following section reviews some inherent assumptions when using RFTM methodology for diagnosis of *P. marinus* and the potential impact of those assumptions on diagnostic standards.

ASSUMPTIONS OF RFTM DIAGNOSIS

All diagnostic tests are encumbered with certain assumptions, the validity of which will affect the reliability of the test (Bushek et al. 1994). Assumptions of RFTM methods for diagnosis of *P. marinus* disease of eastern oysters involve at least the following issues: retrieval of parasites from the host tissue; detection; quantification; stability of parasite numbers during processing; and the representativeness for the tissue, organism or population sampled.

Retrieval of Parasites From Host Tissue

One assumption of the RFTM diagnostic test is that all parasites, including different life stages, are retrieved from the host. If oysters are being examined for possible introduction into a disease-free area, this becomes a precarious assumption; any false-negative diagnoses could introduce the parasite into a naive or uninfected oyster population. Single-tissue techniques (such as the tissue squash and hemolymph diagnoses) cannot adequately ensure the absence of parasites in other tissues. Obviously, diagnosis of the whole oyster avoids loss of parasites from unsampled tissue.

The possibility also exists that preparation or processing of oyster tissue for diagnosis destroys or masks certain parasite life stages. This possibility was difficult to test when alternative methods of diagnosis were lacking. Until recently, only histological examination of paraffin sections was available for comparison with the RFTM technique: Ray (1954b) and Stein and Mackin (1957) provided evidence that all known or identifiable stages of *P. marinus* did enlarge in RFTM. Although reassuring, these studies lacked a strong comparable method to disprove the potential presence of unidentified life stages.

Recent development of an immunoassay technique (Dungan and Roberson 1993) has provided an alternative means to detect *P. marinus*. Moreover, the technique is founded on molecular recognition that should identify any stage of *P. marinus* that does not present unique epitopes. Using the immunoassay technique, Ragone Calvo and Burreson (1994) did not detect previously undescribed cryptic stages of *P. marinus* in winter samples of oysters although they were able to detect light infections in oysters diagnosed as negative by RFTM culture of tissue and hemolymph. If further research verifies these findings, then there will be reasonable assurance that RFTM culture techniques retrieve and enlarge all forms of *P. marinus*.

Detection of Parasites

It must also be assumed for any RFTM test that all *P. marinus* retrieved from the host are detectable by the procedures employed. Techniques using RFTM have two significant attributes that enhance recognition and quantification: enlargement of the parasite and enhanced uptake of iodine stain. When enlarged, the parasites are distinguishable by morphological characteristics (double cell wall, signet ring appearance) (Perkins 1988, 1993, 1996); when enlarged and stained with iodine, the parasites become so easily recognized that quantification becomes feasible.

Enlargement is an important attribute of any RFTM technique. Ray (1952a, 1952b) noted that increasing numbers of parasites were detectable in RFTM culture over the first 18 hr of incubation, but not thereafter. This was interpreted to mean that it took 18 hr incubation for all parasites present to become sufficiently large for visual detection. Consequently, he recommended that a minimum incubation period of 48 hr would ensure enlargement of all parasites to a detectable size. However, after surprisingly low counts were recorded in field studies (Ray 1966a), he suggested that the incubation time be increased to 1 wk (Ray 1966b).

Ray (1966b) also changed antibiotic components of RFTM based on the need to enlarge the parasites. In studies comparing different antibiotic regimes, he found that penicillin, streptomycin and chloromycetin inhibited parasite enlargement, but this effect was "spared" by addition of the antifungal agent mycostatin. Trials showed infection intensities as much as 25 times higher with mycostatin in the formulation. The lower counts (without mycostatin) were believed to be due to a lack of parasite enlargement, since higher magnification revealed small, otherwise unidentifiable cells in the preparation. Thus, mycostatin was included in the RFTM formulation, not only as a useful broad-spectrum antifungal agent, but because it allowed the parasites to enlarge in the presence of other antibiotics. In a number of recent studies, mycostatin was inexplicably deleted from the formulation; it is unknown whether this practice affects the number of prezoosporangia detected.

Enlargement of *P. marinus* is due to their uptake of RFTM, and uptake occurs only if the parasites are living. Thus, accidental diagnosis of dead parasites is unlikely since they would remain too small for detection. It is presumed that all living parasites placed in RFTM survive the incubation period, although this has not been examined experimentally. Techniques that employ NaOH to dissolve oyster tissue (and, perhaps, kill parasites) do not impact enlargement because RFTM incubation precedes NaOH digestion.

Staining is particularly important for research efforts that attempt to quantify or estimate infection intensities. Ray (1952b) stated that the blue color of parasites after exposure to Lugol's iodine was not due to reaction with starch, but rather to a glucoside, such as saponarin, in the cell wall (using fungal terminology). Even with this diagnostic aid, quantitative precision and accuracy are not guaranteed. Since staining with Lugol's iodine requires enlargement in RFTM (Mackin 1962), unstained parasites

or those that are too small to be seen could cause artificially low counts. Artificially high counts may result if the iodine is too concentrated; parasite morphology becomes less distinguishable from stained debris and false-positives can result from precipitated iodine.

The specificity of the stain for *P. marinus* is another important consideration. To be completely effective, the stain must bind with all forms of *P. marinus* and with no other parasite. There is reasonable evidence that all known life stages of *P. marinus* can be stained with Lugol's iodine after enlargement in RFTM, but it is not clear whether *P. marinus* is the only parasite being stained. There has been concern over possible confusion with members of the family Thraustichitriidae, but these do not closely resemble *Perkinsus* and the stain appears brownish (F. Perkins, personal communication). There are some fish myxosporean parasites (Schmidt and Roberts 1977) that stain with iodine, but these have not been observed in bivalves. The greatest potential for confusion is with other species of *Perkinsus* (Lester and Davis 1981, Goggin and Lester 1987, Perkins 1993). Certainly *Perkinsus* sp. (*Perkinsus atlanticus?*) normally found in *Macoma balthica* could be mistaken for *P. marinus*, and considering the results of cross-infection experiments performed by Goggin et al. (1989), it would not be extraordinary if they occurred in *C. virginica*. Enlargement and staining by the RFTM technique are specific for *Perkinsus* only at the genus level. The polyclonal antibody developed by Dungan and Roberson (1993) is also specific for *Perkinsus* only at the genus level; thus, confirmation at the species level must await development of techniques such as genetic probes (Marsh and Vasta 1995).

Recent quantitative techniques have generated additional assumptions related to staining. The use of 2M NaOH to digest oyster tissue does not appear to affect staining of the parasites as long as samples are washed free of NaOH, but no studies have confirmed this. Basic conditions cause iodine staining to fade quickly (D. Bushek, personal communication), but again no studies have compared counts of stained *P. marinus* with and without treatment in NaOH. Overall, the use of NaOH should reduce errors from stained debris and should improve staining specificity since most nontarget parasites will not withstand the harsh NaOH treatment.

Quantification of Parasites

The semi-quantitative tissue squash has been used only as an *estimate* of infection intensity. Even so, the subjective nature of the rating scale predisposes the technique to researcher bias. Ray (1966b) recognized the potential problem:

> Since intensity rating is a subjective procedure . . . there may be a tendency to rate an infection higher for tissues with large (parasite) cells than for tissues with a similar concentration of small cells (p. 64)

Other variables, such as stain intensity and tissue thickness, can also influence the assigned ratings.

Lewis et al. (1988) recognized that the primary obstacle to quantification of *P. marinus* was the inability to separate prezoosporangia from oyster tissues. They found that digestion of oyster tissue in 0.5% trypsin followed by 2M NaOH provided a preparation of parasites free of oyster tissue and readily quantifiable. Choi et al. (1989) used only 2M NaOH, but accomplished the same objective as they were able to enumerate *P. marinus* from individual tissues after digestion. Quantification after NaOH digestion has now been used for several studies (Gauthier and Fisher 1990, Fisher et al. 1992, 1995, Bushek et al. 1994).

Tissue digestion in the protocol does not alleviate all of the problems associated with quantifying *P. marinus*. Some stained particles may still not be recognized as *P. marinus* because they are much smaller than neighboring particles. Even after digestion, *P. marinus* are not necessarily distributed uniformly in the preparation (Choi et al. 1989, Gauthier and Fisher 1990). Accurate counting is also a source of error, particularly since parasites are usually too numerous to quantify without diluting the sample. Prezoosporangia can adhere to each other, forming clumps and chains that will distort results even when the most careful dilutions are made.

Stability of Parasite Numbers

For any estimate or quantification of parasites, it is critical that the numbers of parasites do not increase or decrease after the sample is collected. The greatest concern has been that parasites increase in number during incubation in RFTM. Yet, Ray (1952b) found only four cases in several hundred where parasite numbers increased, and incubation times for these ranged from 23 d to 7 months. Parasite multiplication may have occurred prior to enlargement or may have been due to enlarged parasites that produced budding hyphae (fungal terminology). In later studies, Ray (1954b) concluded that the number of all microscopically identifiable stages of *P. marinus* could increase slightly during 8–18 hr incubation in RFTM, but not thereafter.

Mackin and Boswell (1956) and Mackin (1962) found multiplication of *P. marinus* in very dilute thioglycollate medium during studies to describe the saprophytic cycle of *P. marinus*. The parasite was found to multiply in dilutions ranging from [10:1] to [100:1] RFTM, with the best multiplication found in a [50:1] dilution. No multiplication was found in full-strength RFTM. In cases where multiplication did occur, it was never resolved whether the nutrient source was dilute RFTM or remnant pieces of oyster tissue that were added with the inoculation. Other attempts to culture *P. marinus* using full-strength RFTM have also generally failed (Ray 1954b, Mackin 1962, Prokop 1950, Mackin and Boswell 1956). Stein and Mackin (1957) monitored *P. marinus* closely to identify all life stages during RFTM incubation. They found that all known forms of the parasite were enlarged and that reproduction was minimal.

Although no studies have specifically addressed the issue, dying parasites that are unable to enlarge in RFTM could artificially lower the measured intensity. However, results from many studies showing relative stability of counts from the same sample over time indicate that parasite mortality during incubation is not a critical concern.

Representation of Tissue, Organism, and Population

One of the most challenging diagnostic issues is whether values obtained with RFTM diagnoses adequately reflect the true infection intensity of a given tissue, oyster or population of oysters. Early histological studies (Mackin 1951) and RFTM diagnoses (Ray 1952a) illustrated the fact that *P. marinus* are not evenly distributed among different oyster tissues or even in different sections of the same tissue. Consequently, techniques employing only one tissue, or one section of a tissue, could easily misrepresent the actual intensity. This is an important concern for both prevalence and intensity measurements.

Ray (1952b) recognized that different applications of the RFTM technique would require different approaches. He suggested that four tissues (gill, mantle, heart and rectum) should be examined for a thorough diagnosis of light infections, but allowed that only the rectum was necessary for survey work involving large numbers of oysters. In comparative studies, Ray (1966a) showed a slightly higher prevalence in rectal than in mantle tissue, but he favored using a section of the anterior mantle due to ease of dissection, which resulted in less opportunity for contamination. He also noted (Ray 1966b) that use of rectal tissue could be a disadvantage when oysters have a well-developed gonad, because of difficulty in separating tissues and interference from adductor muscle fragments. Choi et al. (1989) demonstrated differences in infection intensity among digestive gland, mantle and gill tissues from the same oysters. Bushek et al. (1994) compared rectal and mantle tissue squashes throughout an annual period and found essentially no differences in overall sensitivity, but combining results from both tissues lowered the likelihood of false-negative diagnoses. Ray (1966b) also concluded that using rectum, gill and mantle as a composite preparation probably gave a better indication of infection than a single tissue.

Some results (Ray 1966b) using the RFTM tissue squash technique found light *P. marinus* infections in oyster gills but not in rectal and mantle tissues. He suggested that at least some oysters may be invaded by way of the gills rather than the digestive epithelia as indicated by the histological studies of Mackin (1951). In more advanced infections, mantle and rectal tissues had higher parasite ratings than did gill tissues. It is likely that localization of parasites in tissues may be partly dependent on the route of infection and subsequent progression of the disease.

Gauthier and Fisher (1990) employed RFTM to diagnose *P. marinus* from oyster hemolymph samples. The principal purpose of using hemolymph was the ability to enumerate parasites without sacrificing the animal, thereby providing a means to study progression of disease in living animals. Results indicated that the density of parasites in 1-mL hemolymph samples was comparable to the subjective ratings of mantle tissue from the same individuals with the added advantage of detecting many light infections that were negative with the mantle tissue squash (Gauthier and Fisher 1990). However, Bushek et al. (1994), using 250-μL hemolymph samples, found no difference in sensitivity between hemolymph diagnosis and the combined ratings of mantle and rectal tissue squashes. These authors noted that differences between the two studies may have been due to the lower hemolymph volume analyzed and the combined ratings of rectal and mantle tissue used in their study (Bushek et al. 1994).

Uneven parasite distribution in tissues is probably the strongest argument for development of a whole-oyster diagnostic technique. Quantification of whole oysters would not have been possible without the procedures of Lewis et al. (1988) and Choi et al. (1989) to extract *P. marinus* prezoosporangia from obstructing oyster tissues. Capitalizing on this technique, Bushek et al. (1994) published a quantitative whole-oyster diagnostic protocol in their evaluation of tissue squash and hemolymph diagnosis. The whole-oyster technique served well as a baseline for the comparison because, as expected, it was the most sensitive of the three protocols. Separately, a quantitative whole-oyster protocol was generated at the Environmental Protection Agency's Gulf Ecology Division (EPA/GED) to assess the effects of tributyltin on disease susceptibility (Fisher et al. 1995) and to examine annual intensity fluctuations of *P. marinus* in three bays in North America (Oliver et al. 1996). The basic principles of the Bushek et al. (1994) and EPA/GED protocols were the same, but several technical details varied. These details were considered in the following development of a protocol that should, at least temporarily, standardize the approach and serve as a template for further refinement.

QUANTITATIVE RFTM DIAGNOSIS USING WHOLE OYSTERS

The benefits of a quantitative whole-oyster diagnostic procedure may not offset the time and cost of performance for most monitoring (epizootiological) studies that require average population intensity and prevalence estimates. It is, however, a technique that has great potential for correlating total body burdens with measurements of oyster biology and may be particularly useful as a standard for evaluating other RFTM techniques. Recognizing the potential of these and other applications for a quantitative whole-oyster diagnostic technique, we have examined different procedural components to develop a protocol that would optimize sensitivity, specificity, precision and accuracy. The protocol is based on that of Lewis et al. (1988) and Choi et al. (1989) and incorporates procedures of Bushek et al. (1994) and those developed at EPA/GED. A similar protocol was appraised and performed by E. Burreson and L. Ragone Calvo at Virginia Institute of Marine Science, College of William and Mary, and by S. Ford and J. Gandy at Haskin Shellfish Research Laboratory, Rutgers–The State University. Some of their results are reported here. Also, valuable recommendations have been made by Drs. Burreson, Ford and D. Bushek (Belle W. Baruch Institute for Marine Biology and Coastal Research, University of South Carolina) during the preparation of this text.

A summary of the recommended protocol is provided (Table 1). Minced or homogenized oyster tissues are placed into RFTM for enlargement of prezoosporangia. After RFTM incubation, preparations are placed into 2M NaOH, which digests oyster tissue but does not destroy parasites. After centrifugation and washing, the extracted parasites are stained with iodine, diluted, and aspirated onto a filter paper for counting at low magnification. Various aspects of this scheme require clarification and qualification, and some recommended steps have valid alternatives. These considerations are presented under the following sections on sample preparation, RFTM incubation, NaOH digestion, storage of extracted parasites, iodine staining and parasite dilution, identification and counting.

Sample Preparation

Oysters should be kept cold after collection to reduce proliferation of the parasite prior to processing. When oysters are shucked, care must be taken to retain all hemolymph with the sample to ensure that *P. marinus* associated with hemolymph is included. Oyster tissues should be diced or minced so that RFTM can easily penetrate tissues to uniformly reach all parasites. Also, finer pieces will enhance subsequent tissue digestion with NaOH. In most studies, sample replication is not performed until the end of the diagnostic process, i.e., with replicate counts of aliquots taken from the same tube. In such a case, it is not necessary to homogenize oyster tissues into a slurry before incubation in RFTM. However, some studies, such as interlaboratory comparisons, quality assurance exercises or evaluations of reproducibility, may require sample replication earlier in the process. In such cases, sample homogeneity is critical and tissues should be mixed in a blender and tissue grinder.

TABLE 1.

Summary of protocol for whole oyster diagnosis.

Reagent preparation

A. RFTM

Heat 242.5 mL of distilled water to a boil, stir in 7.3 g of thioglycollate and 5 g of NaCl. Remove from heat and dispense into 50-mL culture tubes. Autoclave for 15 min at 15-lb pressure and 250°C (color should be orange/brown). Store in the dark at room temperature.

B. Antibiotics

Mix 0.25 g chloromycetin (Sigma No. C-0378) with 10.0 mL of distilled water, shake well and refrigerate.

Mix 0.01 g mycostatin or nystatin (Sigma No. N-3503, 5160 USP units/mg) with 10 mL of distilled water, shake well and refrigerate.

C. Lugol's iodine

Stock solution: Mix 6.0 g of potassium iodide with 4.0 g of iodine and add to 100 mL of distilled water. Working solution = 1 mL of Lugol's stock solution mixed in 25 mL of distilled water.

Tissue sample preparation and incubation

A. Shuck the oyster carefully and obtain a wet weight. Dice the tissues into 2–5 mm sections and, if desired, homogenize in a blender and/or tissue grinder.

B. Place tissue homogenates into 50-mL tubes, add 20 mL of RFTM and 100 μL of chloromycetin and mix, then gently layer 2 mL of nystatin onto media. Do not mix.

Incubate tubes in the dark for 7 d at room temperature.

C. Centrifuge at 1,500 × *g* for 10 min, aspirate and discard the RFTM supernate.

Add 20 mL of 2M NaOH to the tubes and incubate in a 60°C water bath or oven for 2–6 hr, depending on tissue degradation.

Centrifuge at 1,500 × *g* for 10 min, aspirate to remove NaOH supernate.

Wash 3 times with 10 mL of deionized water, mixing well and centrifuging at 1,500 × *g* for 10 min, washed samples may be stored in refrigeration in 0.2% NaN_3.

D. Resuspend pelleted parasites in 1 mL of Lugol's [25:1] working iodine solution.

Mix vigorously, place 100-μL aliquot on 0.22-μm-pore-size filter paper and aspirate.

Use an ocular grid and 100× magnification to count the dark blue parasites:

If <20 prezoosporangia are found in 100 μL, then count the entire (1-mL) sample. If >200 prezoosporangia are found in 100 μL, serially dilute by transferring at least 100 μL of sample into less than 10-fold dilutions.

Multiply the means of three 100-μL aliquots by the appropriate dilution factor and record infection intensity as $\log_{10}$ prezoosporangia g^{-1} wet weight tissue.

In one such exercise performed at EPA/GED, a heavily infected oyster was placed into a blender and homogenized for 30–45 sec. The resulting chopped tissue was further processed using a tissue grinder with 0.1-mm clearance, producing a fine slurry. This was subdivided into 10 equal volumes and added to RFTM in 10 separate tubes for a 1-wk incubation period. After incubation, a technician processed five subsamples through NaOH digestion (2 hr), staining and counting. The remaining five subsamples were identically processed by a different technician. Results of these counts exhibited a significant difference (Student's t-test, $p < 0.05$) between the means obtained by each technician ($\log_{10}$ 7.05 vs. $\log_{10}$ 7.26, range = $\log_{10}$ 6.86–$\log_{10}$ 7.36). The significant difference in this limited exercise illustrates the potential bias attributable to different individuals performing the diagnostic test, even though the same protocol, equipment and reagents were used. If possible, it appears highly desirable that sample processing and counting for a given study be conducted by the same technician.

Sample processing and counting variability was examined through efforts with researchers at Virginia Institute of Marine Science and Haskin Shellfish Laboratory. At EPA/GED, slurries of four individual oysters were prepared as described and duplicate 1.0-mL aliquots were dispensed into sterile test tubes with thorough mixing between each aliquot removal. Each tube was labelled with a blind code, placed on ice in a cooler and shipped overnight to the collaborating laboratories. Blind coded samples that remained at EPA/GED were refrigerated overnight. Protocols for RFTM incubation, NaOH digestion and counting of prezoosporangia were performed by each laboratory according to a written protocol similar to that described in Table 1. Several comparisons were performed during 1994.

In one interlaboratory comparison, good agreement in infection intensity was attained (Table 2a), as reflected by coefficients of variation below 10%. These data clearly support the *potential* reproducibility of the diagnostic procedure among researchers at different laboratories with different equipment and reagents, at least for samples containing moderate to high levels of infection. Another comparison (Table 2b) demonstrated an apparent weakness of the RFTM technique that may not be overcome by technical improvements. Quantification of infection intensity was reasonably reproducible for oysters with moderate infection intensi-

TABLE 2.

Total number ($\log_{10}$) of prezoosporangia per sample counted during two interlaboratory comparisons, labelled (a) and (b), of oyster tissues infected with *P. marinus*. Four oysters (1–4) were tested in each comparison with duplicate samples of homogenate analyzed by each laboratory. The values recorded for each duplicate are the averages of three counts made from 100-μL aliquots. Overall means were calculated from counts of all six duplicates. Coefficients of variation (CV%) were calculated as standard deviation divided by the mean times 100.

Oyster No.	Laboratory A	Laboratory B	Laboratory C	Mean	CV%
(a)					
1	3.14	3.24	3.75		
	2.91	3.44	3.72	3.37	9.9
2	5.24	5.31	5.92		
	5.62	5.56	5.62	5.52	4.5
3	5.62	5.56	5.84		
	5.26	5.48	5.58	5.56	3.4
4	3.80	3.07	3.30		
	3.38	3.13	3.63	3.39	8.4
(b)					
1	0.90	1.90	1.48		
	0.00	2.10	0.78	1.19	65.9
2	3.72	3.61	3.95		
	3.62	3.73	4.01	3.78	4.5
3	3.56	3.27	1.30		
	2.10	3.33	3.72	2.88	33.4
4	0.00	1.58	0.85		
	0.00	1.97	0.85	0.87	92.0

ties (oysters #2 and #3) but highly variable for those with low infection intensities (oysters #1 and #4). This weakness was previously noted by Bushek et al. (1994) who found both hemolymph and tissue assays to lose sensitivity below 10^3 parasites g^{-1} wet tissue weight. In our comparisons of whole body burdens, replication within each laboratory (i.e., between duplicate samples) was not particularly poor at low intensities (Table 2b), but coefficients of variation were high among laboratories. This pattern may indicate that to accurately quantify low-intensity infections, other protocols, such as specific antibody (Dungan and Roberson 1993) or DNA probe (Marsh and Vasta 1995) techniques, may be required.

RFTM Incubation

P. marinus prezoosporangia progressively enlarge from around 10 μm diameter (Mackin et al. 1950, Ray 1954b) to 35, 90 and up to 150 μm diameter after 18 h, 72 h and 1 wk incubation in RFTM (Ray 1952a). If insufficient time or medium is available for all parasites to enlarge to a detectable size, lower counts will result. This protocol recommends that oyster tissues be placed into 50-mL tubes containing 20 mL RFTM (Table 1). The constant volume in all tubes allows RFTM to be prepared in advance and should not create discrepancies unless there is insufficient medium for all parasites to enlarge to a detectable level. However, Bushek et al. (1994), who placed 25 mL RFTM into each tube, found the size of *P. marinus* prezoosporangia in whole-oyster diagnoses inversely correlated to infection intensity. Although it was not investigated, parasite enlargement in high-intensity samples may have been curtailed by competition for limited resources in the medium. If so, then we must question whether enlargement can be so inhibited that some parasites will be too small for detection. To address this question, experiments must be performed that resolve the discrepancies of sample volume : RFTM volume noted by Bushek et al. (1994). Although other factors, such as biological cycles of parasites related to their seasonal intensity, could create the small size of prezoosporangia in high-intensity samples, it should be considered that greater volumes of RFTM may be needed. If so, the ratios of antibiotics added to RFTM in the culture tubes must be maintained.

Ray (1952a, 1952b) originally suggested that a minimum 48-hr RFTM incubation period would ensure enlargement of all parasites present to a detectable size, but later altered this opinion to 1 wk (Ray 1966b). Most studies have reported incubation times from 5 to 7 d, but Bushek et al. (1994) used a 2-wk incubation period for whole-oyster diagnosis. A longer incubation period may serve to diminish concerns over insufficient medium.

Centrifugation of the sample after RFTM incubation must reliably pellet all of the parasites before removal of the supernate. For hemolymph samples, Bushek et al. (1994) found no parasites remaining in RFTM supernate after centrifugation at $1,000 \times g$ for 20 min. The EPA/GED protocol for whole oysters recommended $1,500 \times g$ for 10 min, although no studies were completed to evaluate speeds and durations. As an introductory experiment, we examined duplicate RFTM supernates of whole-oyster homogenates after centrifugation speeds of 280, 570, 1,118, 1,460 and $2,282 \times g$ for 10 min. Supernates were counted after aspiration through a filter paper and iodine staining. Parasite intensity over all samples averaged 1.1×10^6 parasites g^{-1} oyster tissue (s.d. = 0.08×10^6) and percentages of parasites remaining in the supernate for increasing speeds were, respectively, 0.011, 0.015, 0.008, 0.004 and 0.004%. Obviously, centrifugation at speeds as high as $2,282 \times g$ for 10 min did not retrieve all of the parasites from RFTM, but at the density tested, the number of parasites lost would not appreciably affect the count (0.004%). The importance of this finding at other parasite densities is not yet known. High centrifugation speeds can pellet the parasites so tightly that they are hard to disperse for subsequent processing. Perhaps a longer duration would be the best procedure if these minor losses are unacceptable to the investigator. To avoid loss of pellet material, supernates should be removed by gentle aspiration rather than pouring.

NaOH Treatment

The whole-oyster technique was made possible when researchers at Texas A&M University (Lewis et al. 1988, Choi et al. 1989) described a "hypnospore separation method" that dissolved oyster tissues from *P. marinus* prezoosporangia. Tissue digestion removed oyster debris to allow easier identification and counting of parasites. Choi et al. (1989) digested oyster tissues for 1 hr at 50°C in 20 mL 2M NaOH per gram of wet tissue weight and then centrifuged them at $1,600 \times g$ for 15 min, removed the supernate, and washed them four times in buffered saline before resuspending the parasites. The generation of this protocol was not detailed but has been used by others without apparent complication. Bushek et al. (1994) used only 10 mL 2M NaOH per gram of wet tissue weight.

It was not clear from the published texts of Lewis et al. (1988) or Choi et al. (1989) whether NaOH digestion degraded or in any way altered the detectable number of parasites. This may have been difficult to examine in a controlled experiment because without digestion, enumeration of parasites from tissues can be highly variable. To shed some light on this question, an experiment was performed to compare parasite numbers after increasing durations of digestion. Both whole oysters and hemolymph were examined. Hemolymph samples were included because there is little tissue debris in hemolymph that can interfere with counts in the control (no digestion) samples. In fact, Bushek et al. (1994) suggested that hemolymph samples could be counted without NaOH digestion. Hemolymph (2 mL) from three oysters was withdrawn from the adductor muscle and placed in three individual tubes containing RFTM. The oysters were then shucked and tissues were finely homogenized and placed into three separate tubes containing RFTM. After incubation for 1 wk at room temperature, each hemolymph sample and each whole-oyster sample was divided into eight subsamples and centrifuged to remove RFTM. Duplicate subsamples were then digested in 10 mL 2M NaOH for 0, 2, 6 and 24 hr at 60°C. Following digestion, samples were washed and quantified according to the protocol (Table 1).

Results of this experiment (Table 3) indicated no effect of NaOH on *P. marinus* counts. This was evidenced by the lack of change in the hemolymph samples and from the lack of diminution in the whole-oyster samples. As implied by Bushek et al. (1994), it does not appear from this experiment that digestion of oyster hemolymph is necessary for reasonable precision. However, digestion of whole-oyster tissues does appear necessary for quantitative applications. Undigested whole-oyster samples were difficult to count because of interference by oyster tissues, and counts were generally not as high as digested samples. Also, longer digestion periods provided lower coefficients of variation in the whole-oyster samples, probably due to greater separation of pre-

TABLE 3.

Mean numbers of prezoosporangia ($\log_{10}$) and coefficients of variation (CV%) for three oysters analyzed for both tissue (g^{-1}) and hemolymph (mL^{-1}) levels. Means were derived from triplicate counts of duplicate samples.

Oyster No.	NaOH Digestion Period (hr)			
	0	2	6	24
Tissue samples				
1	5.01 (8%)	5.14 (41%)	5.17 (21%)	5.07 (6%)
2	4.79 (67%)	4.31 (1%)	5.36 (2%)	5.38 (4%)
3	5.30 (10%)	5.40 (6%)	5.43 (1%)	5.48 (6%)
Hemolymph samples				
1	3.01 (21%)	3.09 (5%)	3.11 (5%)	3.05 (30%)
2	2.40 (6%)	2.44 (13%)	2.33 (3%)	2.31 (3%)
3	2.88 (29%)	2.91 (1%)	2.67 (2%)	3.06 (29%)

zoosporangia from oyster tissues. For these reasons, a 2–6 hr digestion period is recommended in the protocol (Table 1), dependent on tissue degradation. Highly homogenized tissue samples need less digestion time. Even longer periods could be used if necessary: Although some artifacts were observed in samples digested for 24 hr, it does not appear that long digestion periods will reduce the parasite count. It also seems reasonable to redigest samples with remnant tissues without fear of altering the counts.

Centrifugation and washing after NaOH digestion are important steps that must be completed with care. This may be more critical than centrifugation after RFTM incubation where parasites are less dense. If centrifugation is too light, then parasites are lost in the supernate. If too harsh, the parasites can form clumps that are difficult to disperse and interfere with counting. Also during NaOH digestion, parasites become sticky and may adhere to the walls of centrifuge tubes (D. Bushek, personal communication). Yet the parasites must be washed free of base or the iodine stain fades very quickly. Since available equipment varies among laboratories, it is important that researchers optimize post-RFTM and post-NaOH centrifugation speeds and times to balance these factors.

Storage of Extracted Parasites

Once *P. marinus* prezoosporangia are extracted from oyster tissues and washed free of NaOH, they may be stored for several weeks before counting. The EPA/GED procedure resuspended parasites in 1 mL of 10 mg/mL NaCl and stored them in the refrigerator. Bushek et al. (1994) stored extracted parasites at room temperature in a phosphate-buffered solution containing 2% paraformaldehyde and 0.04% sodium azide (NaN_3). Currently at the Haskin Shellfish Research Laboratory, parasites are resuspended in 0.2% NaN_3 and samples are stored in the refrigerator (S. Ford, personal communication). Sodium azide is primarily used to reduce bacterial contamination but may also prevent clumping.

Parasite clumping is a serious problem that can create high variability in replicate counts. Clumping is compounded by poor washing, high centrifugation speeds and prolonged storage. Most often, vortex mixing is employed to resuspend samples but is not always successful. It is possible that the addition of a soap could reduce the aggregation of hypnospores prior to staining. However, different concentrations of Tween 80 applied to prezoosporangia suspensions did not reduce their clumping and caused the iodine stain to fade more quickly. The possibility of using other soaps, such as Triton-X, should be investigated. Sonication for 1–2 min has been found to successfully disrupt clumps (S. Ford, personal communication) and is recommended for routine processing. In all cases, preparations should be examined before counting to determine whether parasite clumping will cause misrepresentation of the sample.

Iodine Staining

As previously noted, incomplete digestion of oyster tissue could lead to obstruction of parasites during counting. Alternatively, remnant tissues that stain with iodine could artificially increase parasite counts. Ample digestion of oyster tissues is therefore going to reduce problems associated with staining and counting of prezoosporangia. Even so, morphological identification of parasites is an important aspect of any RFTM diagnostic technique. Proper staining with iodine greatly improves this capability, but overstaining can lead to poor identification. Ray (1952a) originally recommended a [25:1] aqueous dilution of Lugol's iodine for tissue squash diagnoses. Since then, different studies have used a variety of iodine dilutions, but most increased the strength of iodine to better visualize parasites in tissues. Because NaOH digestion removes most obstructing tissue, iodine strength was reexamined for the whole-oyster protocol.

Six aqueous dilutions of Lugol's working solution, ranging from [3:1] to [300:1] (water to iodine), were compared using prezoosporangia extracted from the same oyster after incubation in RFTM. More dilute staining increased the resolution of parasite morphological features and reduced the likelihood of miscounting stained artifacts as positive. However, parasites stained with more dilute solution tended to fade faster. With heavily infected samples that require more time to count, aliquots on filter paper must sometimes be restained. Staining intensity appears to depend on the number of prezoosporangia, the amount of residual oyster tissue, the working stain concentration and the duration of staining. Researchers should test a variety of stain concentrations and examine representative stained parasites for the double wall and signet ring appearance, even though many parasites may not exhibit internal characteristics after NaOH digestion. Experiments at EPA/GED found that a [25:1] dilution, Ray's original recommendation, was light enough to determine structural features, yet remained dark enough during the counting period to distinguish prezoosporangia from the filter paper background.

It should also be noted that working solutions of iodine can precipitate, especially if high concentrations are being used. Bushek et al. (1994) noted that false-positives could occur from iodine precipitates and suggested that working iodine solutions should be filtered intermittently and/or periodically examined for precipitate. Refrigerator storage of iodine or stained parasite samples is contraindicated because cold temperature increases precipitation.

Parasite Dilution, Identification and Counting

In most cases, sample replication will occur at the counting stage of the protocol so homogeneity of parasite suspensions is critical. Samples should be sonicated for 1–2 min if parasite clumps are present and mixed on a vortex between each replicate aliquot. Since dilutions of the sample will be required (in all but the lightest infections) to achieve countable prezoosporangia densities, the dilution process must be accurate and consistent. Dilu-

tions should be performed such that larger volumes are transferred in several serial dilutions rather than a smaller volume in a single-step dilution. Tenfold (or less) dilutions and transfer volumes of no less than 100 μL are recommended to avoid errors due to lack of homogeneity.

Immediately before counting, a 47-mm-diameter, 0.22-μ-pore-size filter paper (mixed cellulose ester composition) is placed on a borosilicate filter fitted to a vacuum aspirator flask and moistened with distilled water. If triplicate counts are not required, a smaller diameter (25-mm) filter paper may be used. Filters that stain with iodine (i.e., that contain starch) should not be used. The parasite sample must be mixed vigorously before a 100-μL aliquot is placed onto the filter paper and aspirated with a small pump. Replicates may be filtered onto the same filter paper, which is then "mounted" on two microscope slides taped together for examination (no coverslip). Prezoosporangia appear dark blue against the white filter paper background using a light microscope at 100× magnification. An ocular grid should be used to avoid re-counting microscope fields. If <20 prezoosporangia are found in 100 μL, then the entire (1-mL) sample should be counted. This can be accomplished by filtering the entire sample or by concentrating the sample with centrifugation and resuspending in a small volume of stain. If the 100-μL aliquot contains >200 prezoosporangia, the sample must be diluted (as noted above) and a new aliquot counted. A minimum 100-μL sample volume is recommended for each serial dilution because of the tendency of prezoosporangia to clump.

The use of filter paper for counting parasites has some advantages over hemacytometers (Choi et al. 1989), glass slides (Bushek et al. 1994) or tissue culture plate wells (Gauthier and Fisher 1990). Not only do the stained parasites contrast sharply with the white filter paper background, but they are coplanar. This reduces problems associated with parasite clumps dislodging hemacytometer and microscope slide coverslips and problems associated with locating suspended parasites or parasites attached to tissue culture well walls.

It is recommended that at least three counts of 100-μL aliquots are made and the mean multiplied by the appropriate dilution factor. Infection intensity can be recorded as prezoosporangia g^{-1} wet weight tissue, based on the starting weight of the entire oyster. Counts should be $\log_{10}$ transformed for statistical analyses since actual counts are generally highly variable among individual oysters and will most likely not fit the assumptions of an analysis of variance model.

FINALE

The described protocol for whole-oyster diagnosis should provide a more accurate and precise quantitation of *P. marinus* infection intensity than do single-tissue, semi-quantitative protocols. Sensitivity and reproducibility, especially at lower infection intensities, remain questionable, but this could be improved by further technical refinement. The generation of cultured lines of *P. marinus* (Gauthier and Vasta 1993, Kleinschuster and Swink 1993, La Peyre et al. 1993) could be creatively applied to RFTM technology to verify count precision and sensitivity. Although this protocol is recommended as a standard, different steps within the procedure can be or may need to be modified for specific scientific objectives, available equipment, time and personnel, personal preference, or even oyster size and tissue composition. Researchers will want to adapt incubation periods, NaOH digestion periods and stain concentrations to increase the homogeneity of suspensions and enhance morphological recognition of the parasites. Rationales for selected steps were discussed here so that modifications and options can be more easily considered.

Whether or not this protocol is modified, researchers will want to generate quality assurance objectives. Some provisions will undoubtedly become routine during the diagnostic process, such as checking iodine solution for precipitation and digested samples for parasite clumps. Others need to be included into the experimental design. At least once in a project, samples should be subdivided prior to RFTM incubation to characterize the variability of the entire diagnostic procedure. Similarly, dilution consistency of stained parasites should be verified periodically. Centrifugation effectiveness after RFTM incubation and NaOH digestion should be confirmed by adding stain to supernates, aspirating them onto filter paper and observing for parasites.

Whole-oyster diagnoses may best be used for correlation with oyster physiological measurements or for standardizing and verifying other techniques. It is, however, more time intensive and labor intensive than are other techniques. We believe, as did Bushek and co-workers (1994), that it will become the most defendable technique for many scientific applications. Determination of disease-free status may be more reliable using nucleic acid recognition techniques, such as those being developed by Marsh and Vasta (1995).

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THE EFFECTS OF *PERKINSUS MARINUS* INFECTION ON PHYSIOLOGICAL PROCESSES IN THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA*

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ABSTRACT Although *Perkinsus marinus* infections have been associated with high mortalities in populations of the eastern oyster *Crassostrea virginica* for several decades, the pathological mechanism(s) by which death is induced is unclear. Physiological changes in the oyster associated with *P. marinus* infection are not well studied. Infections typically cause significant reductions in growth rate and several studies have shown reductions in condition index as well. The reduction in condition index may be the result of a perturbation in free amino acid metabolism caused by infection. Further, the effects on free amino acid metabolism may be associated with parasite-induced changes in mitochondrial function. A significant acidosis has also been shown to occur in the hemolymph of infected oysters which may affect general tissue functions. However, changes in oxygen consumption and clearance and assimilation rates of whole oysters have not been correlated with increasing infection. Finally, reproductive capacity may be reduced by *P. marinus* infection.

KEY WORDS: *Perkinsus marinus, Crassostrea virginica,* physiology

INTRODUCTION

Most of the research on the sublethal effects of diseases on bivalves has pertained to growth and mortality. Even though physiological data are essential to determine the effects of diseases on oyster populations, very little research regarding the sublethal, physiological effects of diseases has been conducted until recently. However, in the past few years several studies have appeared that provide a beginning to understanding the physiological consequences of *Perkinsus marinus* parasitism in *Crassostrea virginica*.

Oysters are typically stricken with *P. marinus* infections in high-salinity (>15 ppt) waters from the Chesapeake Bay south throughout the Gulf Coast states. However, recently, *Perkinsus* infections have been found in oysters as far north as Massachusetts (Ford 1992) and in Maryland oyster beds at low salinity (<10 ppt) (Smith and Jordan 1992). Shortly after infection oyster growth usually ceases or is greatly reduced (Andrews 1961, Paynter and Burreson 1991) but large-scale mortality in a population typically does not occur until the second summer of infection. Mortality is associated with high summer water temperatures and is greatest in August or September, depending on the latitude. The typical scenario of disease infection, lengthy progression, and eventual death suggests that infection produces significant initial sublethal effects and causes cumulative physiological damage which becomes most acute at high temperatures when metabolic demand in both oyster and parasite is likely highest.

The physiological effects of *P. marinus* infection are most apparent as a reduction in growth rate. However, little is known about the ultimate causes of oyster mortality. Since the protozoan was first described, scientists have observed histological effects which lead to postulation that lytic and cytotoxic agents are produced by the parasite (Mackin 1962, Mackin and Ray 1954), but these results do little to identify the effects on physiological functions of the various host tissues and how they may be impaired. Importantly, Mackin (1962) observed that infected oysters could not maintain valve closure as long as uninfected oysters. The inability to maintain valve closure makes oysters much more vulnerable to predation and limits their ability to tolerate rapid changes in salinity since valve closure is the first defense against osmotic shock. Furthermore, much of the oyster's ability to survive in the estuary is based on cellular adaptation to the environment. How these sophisticated cellular mechanisms are affected has only been recently examined.

Oysters such as *C. virginica* are physiologically complex organisms. They dwell in an estuarine habit that exposes them to a wide range of environmental stresses including high and low temperatures, very little or no oxygen for extended periods of time, and rapid salinity changes of 10 ppt or more. To survive these extremes, *C. virginica* has evolved a sophisticated series of cellular and metabolic mechanisms which either neutralize or avoid potentially fatal environmental changes. One of the most well-studied mechanisms is cellular volume regulation in response to a change in salinity (Lynch and Wood 1966, Paynter et al. 1995). When the ambient salinity increases, oyster cells accumulate free amino acids (FAA) to offset the increasing extracellular osmotic pressure. With osmotic pressures nearly equal on both sides of the cell membrane the cell does not lose water and shrink, and can therefore remain functional. Similarly, when oxygen is depleted from ambient water, the oyster consumes less oxygen, lowering its own metabolic rate (Hammen 1980). It makes the chemical energy necessary for survival through alternative metabolic processes which require less oxygen. In this way the cells can continue to function at a low rate in the absence of oxygen.

Recent research has focused on the possible effects of *P. marinus* on these sophisticated mechanisms and other physiological characteristics of *C. virginica*. In the following section I shall review some of the advances made over the last few years including general effects on growth and condition as well as the effects of infection on FAA concentrations in oysters, differences in mitochondria isolated from infected and noninfected oysters, the acid-base physiology of oyster hemolymph, and the relationship between *P. marinus* infection and physiological energetics. These important studies shed light on the mechanisms through which *Perkinsus* infests and kills oysters and allow us to better understand how to prevent or control infections and mortality.

Growth, Condition and Mortality

Andrews (1961) used an underwater weighing technique to show that individual oysters that acquired *P. marinus* infections exhibited greatly reduced growth. The underwater weighing technique measured shell deposition so that somatic tissue growth, or the effects of infection thereon, could not be assessed. Paynter and Burreson (1991) reported very similar observations on whole populations of oysters; growth of the whole population was greatly reduced even though less than 100% of the population were diagnosed as infected. Similar to the study by Andrews, shell height (a measure of shell production rather than somatic tissue) was measured. The difficulties in assessing effects on somatic growth lie in the seasonal variation of tissue weight in oysters; typically dry tissue weight declines in late summer even without infection. Furthermore, Hilbish (1986) and Borrero and Hilbish (1988) have shown that shell and soft tissue growth in mussels is not always similar and that temporal differences exist between shell and soft tissue growth periods. It is clear, however, that shell deposition is greatly reduced by *P. marinus* infection, subsequently limiting somatic tissue growth.

Most studies measuring the effects of *P. marinus* have used a condition index, dry tissue weight per unit shell volume, as a measure of somatic growth or health (Menzel and Hopkins 1955, Craig et al. 1989, Gauthier et al. 1990, Crosby and Roberts 1990, Burreson 1991, Paynter and Burreson 1991, Chu and La Peyre 1993a, Dittman 1993). This measure typically declines after spawning and during hot summer months in most regions and most studies have shown a negative correlation between condition index and *P. marinus* infection (Gauthier et al. 1990, Crosby and Roberts 1990, Burreson 1991, Paynter and Burreson 1991, Dittman 1993, Volety and Chu 1994). However, other studies (Chu and La Peyre 1993b, Chu et al. 1993, Newell et al. 1994) have revealed no relationship between *P. marinus* infection and condition index. Laboratory exposures of relatively short duration may not allow enough time for condition to become reduced, but they nevertheless reveal that reduction in condition is not always directly associated with increasing infection intensities.

Free Amino Acid Metabolism

C. virginica is a euryhaline species capable of acclimating to wide changes in ambient salinity. Cell volume is controlled by regulating a large, intracellular FAA pool and the quaternary ammonium compound glycine betaine to offset changes in extracellular osmotic pressure, i.e., this oyster is an osmoconformer (Pierce et al. 1992). The time course of amino acid accumulation has been measured in oysters exposed to increased salinity (Paynter et al. 1995) and appears to be typical of other bivalves. In ribbed mussels, for instance, alanine rapidly accumulates and reaches high levels immediately after a hyperosmotic stress. As acclimation proceeds, the glycine concentration rises and within a few days replaces alanine as the major osmotic effector. During the next several days to weeks at high salinity, proline typically appears as a transient peak, beginning to rise slowly after the alanine accumulation peaks and declining as taurine accumulates. Taurine usually becomes the major osmotic effector, often comprising as much as 70% of the FAA pool (Baginski and Pierce 1977). The ability to regulate intracellular amino acids in this way allows *C. virginica* to inhabit estuaries such as Chesapeake Bay.

While the physiological effects of protozoan parasitism have been addressed by a few studies (Newell 1985, Barber et al. 1988a, 1988b, Ford and Figueras 1988, Newell and Barber 1988), none have examined salinity tolerance. Heavily infected oysters appear wasted, watery, and translucent, and the ratio of whole wet tissue weight to dry tissue weight is increased (Paynter and Burreson 1991). In addition, oysters from various locations within Chesapeake Bay had smaller intracellular free amino acid pools, almost no glycine betaine, and reduced salinity tolerances compared with conspecific oysters from several locations along the Atlantic Coast from Georgia to Cape Cod (Pierce et al. 1992). It is likely that all of the Chesapeake Bay oysters in that study were parasitized with *P. marinus*. These observations, together with the observed reductions of morbidity and mortality amongst parasitized oysters in lower salinities (Andrews 1988, Burreson and Andrews 1988, Paynter and Burreson 1991), suggest that *P. marinus* infection may have an impact on the salinity tolerance mechanisms of the oyster, a hypothesis suggested many years ago by Soniat and Koenig (1982).

Paynter et al. (1995) examined the effects of both *P. marinus* parasitism and environmental salinity on intracellular FAA concentrations of oyster tissues during a cycle of infection in the field.

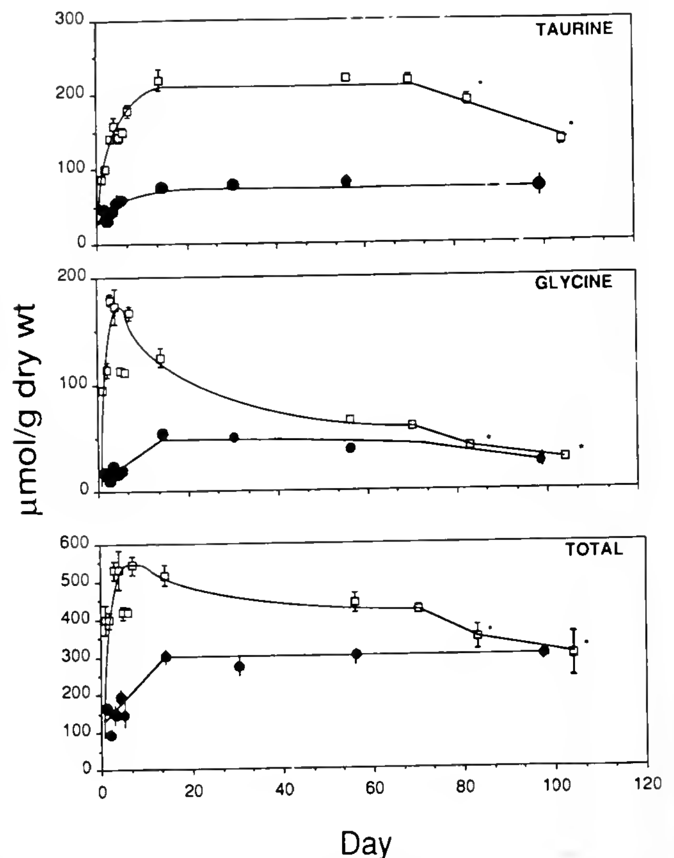

Figure 1. Concentration of taurine, glycine, and total FAA in gill tissues from oysters held at high (20 ppt, open square)- and low (8-12 ppt, closed circle)-salinity sites. Transfer of oysters from low to high salinity occurred on day 0. An increase from 8 to 12 ppt occurred at the low-salinity site between days 10 and 20. Asterisks denote infection of oyster groups by *P. marinus* at high salinity. From Paynter et al. 1995.

In that study, the FAA levels in gill tissues changed after transfer to the high-salinity site (Fig. 1), essentially as predicted by earlier laboratory studies on other bivalve species. Overall, the total FAA increased to a peak around 500 μmol/g dry wt within 3 d and remained constant until the day 85 sample when protozoan infections were first detected and total FAA levels declined. Taurine concentrations at days 85 and 105 were significantly lower than the levels exhibited before infection. Remarkably, glycine and total FAA declined to levels that were not different from those of the low-salinity oysters. The amino acid levels in tissues from the oysters held at low salinity stayed constant after the period between days 10 and 20 when the total FAA level went up, presumably in response to a 4 ppt salinity increase at the site. The mean levels of glycine, taurine, and total FAA remained constant at the low-salinity site for the remainder of the study period.

The levels of FAA in the oysters transferred to high salinity in this study were significantly different among three phases (uninfected, lightly infected, and heavily infected) following salinity acclimation. Mean levels of the major amino acids of the FAA pool—taurine, glutamate, and glycine—and the minor components—threonine, serine, and β-alanine—were all lower in the infected groups compared to the uninfected groups. All of the FAA, except taurine and glutamine, were significantly lower even with light infection intensity, producing a 24% decline in the total FAA pool. Glycine and β-alanine levels declined further as infection intensity increased and taurine levels were substantially reduced in the heavily infected oysters. Both glutamine and alanine levels increased in the heavily infected group. Overall, the total FAA pool in the oysters at the high-salinity field site declined by 40% between the acclimated uninfected phase and the heavily infected phase.

In summary, intracellular FAA levels were much lower in the gills of the groups of high salinity–adapted oysters infected by *P. marinus*. As the infection intensified in the group, the amino acid pool declined by 40% of its original level largely due to taurine decreases in all of the oysters, including those scored as uninfected by our fluid thioglycollate–based diagnostic method. Since taurine levels in oysters or mussels acclimated to a particular salinity remain at the acclimated level unless a subsequent salinity change occurs (Soniat and Koenig 1982, Baginski and Pierce 1977, Bishop et al. 1983, Pierce et al. 1992) and given the lack of a similar decline in taurine in the low-salinity oysters, the reductions in taurine and other FAA in the oysters are likely related to protozoan infection rather than some type of seasonal change. Since oysters are osmoconformers, a reduction of 33% in intracellular FAA must be compensated for by the elevation of other intracellular solutes. At present, these solutes are not known but inorganic ions such as Na^+, K^+, and Cl^- are obvious possibilities. An increase in intracellular ion concentration of this magnitude is likely to produce negative physiological effects (Yancey et al. 1982). The decreased FAA concentration might be caused by perturbations either in the synthesis of FAA important for salinity tolerance or in the membrane characteristics which keep the FAA inside the cell once they are synthesized. The intracellular amino acids that are utilized for salinity tolerance are synthesized largely by the mitochondria (Paynter et al. 1984, Pierce et al. 1992). Once synthesized, the amino acids are transported to the cytosol where their intracellular concentration is regulated by the permeability control mechanisms of the cell membrane (Pierce and Politis 1992). Therefore, the presence of *P. marinus* might affect the permeability control mechanisms of the cell membrane or synthetic mechanisms in the mitochondria. In addition to the effects on the osmolytes, impaired mitochondrial function could result in a reduction in ATP production, which could account for the reduction of growth observed in the presence of the parasite.

Mitochondrial Metabolism

Pierce et al. (1992) found that oysters from a variety of locales within Chesapeake Bay had salinity tolerances that were more narrow than the tolerances of oyster populations elsewhere along the Atlantic Coast. The basis of this difference was that the FAA pool of Bay oysters was smaller and composed of different amino acids than that of the Atlantic oysters, agreeing with the results reported by Paynter et al. (1995) summarized above. In addition, the Atlantic oysters had substantial intracellular concentrations of glycine betaine not present in the cells of Bay oysters. These differences in osmolyte composition between Chesapeake and Atlantic oysters no doubt account for the salinity tolerance differences. Furthermore, since both the amino acids used in cellular osmoregulation and glycine betaine are synthesized in the mitochondria (Paynter et al. 1984), the differences between Chesapeake and Atlantic oysters may reside in the mitochondria. In addition, since all of the Bay oysters studied have been parasitized with *P. marinus*, it is possible that the differences are due to the parasite rather than to genetics.

The respiratory control ratios (RCRs) of mitochondria from Bay oysters are often higher than those of mitochondria from Atlantic oysters (Fig. 2). In addition, the Bay oyster mitochondria give highest RCRs with malate as a substrate while the Atlantic mitochondria prefer α-ketoglutaric acid. The basis of this coupling ratio difference is not clear at present, but at least suggests that the energy metabolism of Bay and Atlantic animals is different and that the difference may lie with the control of the kinetics of various steps in the Kreb's cycle.

In addition, Pierce et al. (1995) have shown that the uptake of choline, a precursor of the osmotically active compound glycine betaine, by mitochondria isolated from Chesapeake Bay oysters is significantly lower than that by mitochondria from Atlantic conspecifics. While infection levels were not part of this study, all of the Chesapeake oysters tested from the experimental groups were

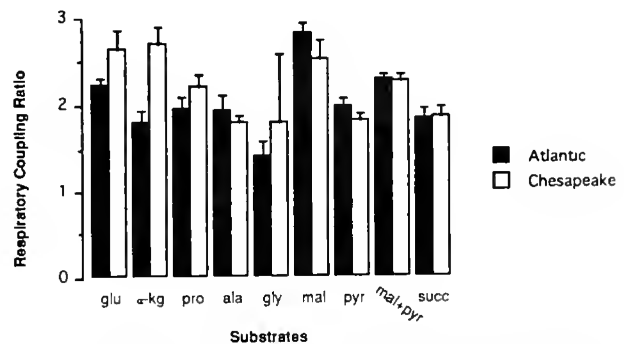

Figure 2. Respiratory coupling ratios of mitochondria isolated from Atlantic and Chesapeake Bay oysters acclimated to low salinity. Histogram bars are the means of at least 10 measurements. Error bars indicate standard errors. From Pierce et al. 1992.

infected with *P. marinus* while most of the Atlantic oysters were not. This suggests that mitochondrial metabolism may be affected by *P. marinus* infection.

The initial accumulation of FAA in response to hyperosmotic shock is the result of a complex biochemical regulatory process that allows oyster cells to route carbon and nitrogen in a very specific way (Bishop et al. 1983). Since the biochemical pathways used to respond to hyperosmotic stress may be the same as, or very similar to, the pathways involved in hypoxic tolerance (Baginski and Pierce 1975), it is possible that the ability of oysters to tolerate hypoxia, which is common in Chesapeake Bay (MacKiernan 1987), might also be diminished by *P. marinus* infection. A reduced tolerance of hypoxia could lead to the large-scale mortalities that occur in Chesapeake Bay oysters during exposure to hypoxia, which occurs frequently during the summer months over many oyster bars.

Hypoxia Tolerance and Acid-Base Balance

Hypoxia or anoxia causes not only the obvious stress associated with the lack of oxygen but also causes a general decrease in tissue pH caused by an increase in CO_2. These changes can have harmful effects on the general well-being of organisms and affect many aspects of normal physiological performance. When oysters are air exposed, they close their valves and the oxygen contained in the water trapped between the valves is quickly exhausted. The oyster tissues then become hypoxic and hypercapnic. Infected oysters cannot hold their valves closed for as long as uninfected oysters when aerobically exposed (Fig. 3). This is thought to be due to the stress induced by infection, but the exact nature of the stress has never been addressed. Dwyer and Burnett (1996) have studied the acid-base physiology of oysters and the effects of *P. marinus* on acid-base balance and discovered important correlations between infection and acidosis in oysters.

Dwyer and Burnett (1996) showed that the normal pH of oyster hemolymph is around 7.7 while oysters infected with *P. marinus* show significant acidosis (pH 7.2; Fig. 4). Furthermore, they showed that minimal hypoxic stress caused a large decline in hemolymph pH in both infected and noninfected oysters but that

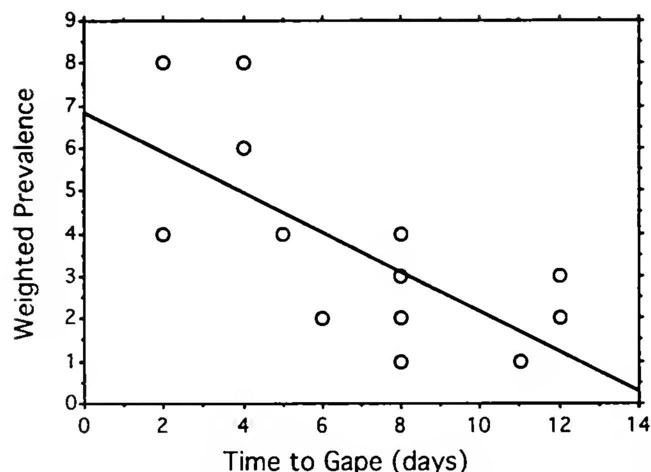

Figure 3. Relationship between weighted prevalence of *P. marinus* infection and time to gape of oysters held undisturbed out of water at room temperature. Regression analysis revealed a strong negative correlation between weighted prevalence and time to gape ($P > 0.001$).

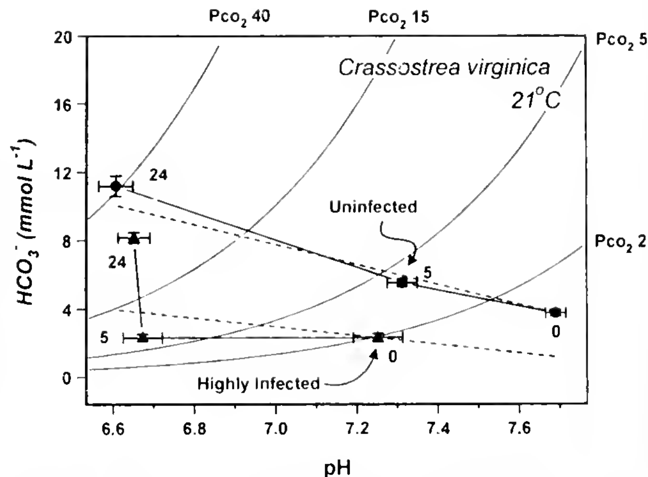

Figure 4. A pH-HCO_3^- diagram showing the acid-base status of oyster hemolymph at 0, 5, and 24 hr of air exposure at 21°C. PCO_2 isopleths (curved lines) are given in torr. In vitro buffer lines are shown as dashed lines. Circles represent uninfected oysters; triangles represent oysters with high infections. Values are means ± SE. N for each experiment ranged from 22 to 56. From Dwyer and Burnett 1996.

infected oysters incurred a steeper decline. The pH of hemolymph of infected oyster dropped to 6.7 after 5 hr of hypoxic stress compared to a decline to 7.3 in healthy oysters. This response could result in large differences in nutrient absorption or retention, blood cell function (see Anderson 1996), oxygen consumption, and metabolic efficiency between infected and uninfected oysters. Indeed, it could be associated with the loss of FAA from cells or an increased rate of parasite growth. It is likely that this acidosis is associated with the inability of infected oysters to remain closed as long as uninfected oysters. In fact, Dwyer and Burnett (1996) have shown that adductor muscles of infected oysters contain significantly lower amounts of glycogen than do uninfected oysters and that heavily infected oysters have less glycogen than lightly infected individuals. This suggests a direct correlation between disease and an oyster's ability to perform ecologically critical tasks such as keeping its valves closed (see Fig. 3). These kinds of studies bring us to a closer understanding of the nature of mortal injury inflicted by the parasite.

Physiological Energetics

Newell (1985) reported that the feeding rates of eastern oysters were significantly reduced when they were infected by the parasite *Haplosporidium nelsoni* (MSX). Such reduced feeding activity resulted in lower amounts of glycogen being sequestered (Barber et al. 1988a), resulting in a reduction in condition index. Energy allocation by MSX-infested oysters to gametogenesis was also disrupted, resulting in significantly inhibited gametogenesis during the spring (Ford et al. 1990). The effects of *P. marinus* infections on the physiological functions of feeding, metabolic energy expenditure, and assimilation efficiency in eastern oysters have only recently been studied.

Using a combination of field and laboratory experiments to study the effects of *Perkinsus* infections on *C. virginica*, Newell et al. (1994) have shown that *P. marinus* infection has a surprisingly small effect on most aspects of feeding physiology and metabolism. In that study, oxygen consumption, clearance rates, condi-

tion index, and assimilation rates were measured over a 2-year cycle of growth and infection at three sites in Chesapeake Bay. When the oysters were transferred from low to higher salinity sites at the initiation of the experiments, feeding rates increased sharply and remained high for 1 to 2 months but declined rapidly in concert with the first detection of *P. marinus* infections in the experimental groups. Surprisingly, however, further declines in the clearance rates of the oysters did not occur in association with progression of disease. In contrast, Mackin and Ray (1954) showed that fecal and psuedofecal production in moderate and heavily infected oysters was less than half that of uninfected oysters.

Other aspects of the physiological energetics were unaffected by *P. marinus* infection. Oxygen consumption did not change between uninfected and infected oysters, even in oysters heavily infected and within weeks of succumbing to the disease. Condition index has been reported to decline with infection (Craig et al. 1989, Gauthier et al. 1990, Crosby and Roberts 1990, Paynter and Burreson 1991, Dittman 1993) but was not associated with infection level in Newell et al. (1994). Most surprisingly, assimilation rates remained unchanged by *P. marinus* infection even though the primary portal of entry by this parastie is thought to be through the intestine. Clearly, the nature of physiological damage caused by *P. marinus* infections cannot yet be fully understood.

Reproductive Capacity

Parasitism by *H. nelsoni* has been shown to significantly affect reproduction in oysters. Gametogenesis is apparently inhibited by infection-induced disruptions in carbohydrate metabolism (Barber et al. 1988b, Ford et al. 1990) which lead to a reduction in fecundity (Barber et al. 1988a). Given these observations one might expect that *P. marinus* infections would also have significant deleterious effects on reproduction in the oyster. However, the effects of *P. marinus* infection on gamete production seem to be much less direct.

Although fecundity or reproductive condition has not been directly studied in relation to *P. marinus* infection as it has with *H. nelsoni* (Barber et al. 1988a), Cox and Mann (1992) have shown that reductions in reproductive activity in oyster populations in the James River, VA, have coincided with increases in *P. marinus* prevalence over the last few years. It also seems likely that *Perkinsus* infection may cause perturbations in gonad development since Ragone Calvo and Burreson (1994) showed that *P. marinus* parasites survive overwintering and can develop into substantial infections soon after temperatures increase, which may coincide with gonadal maturation and spawning.

Kennedy et al. (1995) showed that *P. marinus* infections acquired during the previous year did not have a deleterious effect on reproduction the following year. Similarly, Dittman (1993) showed that oysters with light first-year infections had percent gonad areas that were similar to those of uninfected oysters. However, percent gonad areas in oysters with heavy infections were significantly lower, indicating a significant negative impact of *Perkinsus* infection on reproductive capacity. Ray et al. (1953) and Kennedy et al. (1995) reported significant reductions in reproductive output, in terms of numbers of eggs produced, of oysters heavily infected with *P. marinus*. In contrast, eggs from *P. marinus*–infected individuals were not smaller than eggs from uninfected animals and the lipid content of eggs from infected oysters was no different from that of eggs of uninfected oysters (Kennedy et al. 1995). Thus, it appears that heavy *P. marinus* infection may have some deleterious effect on reproduction but that perhaps the oyster can shunt energy from growth (which is reduced even with light infections) to gametogenesis to minimize the effects of infection on egg quality.

SUMMARY

Infection of the eastern oyster by *P. marinus* induces a number of significant changes in the physiology of the oyster. Given the changes in the hemolymph pH associated with infection, one would expect nearly all cell-mediated functions, including ciliary beating, respiration, absorption of nutrients, and excretion of waste products, to be altered. Certainly acidosis could inhibit calcification and shell deposition, accounting for the cessation of shell growth associated with infection. It could also alter membrane characteristics to the point where amino acid uptake was retarded and it may account for changes in blood cell function as described by Anderson (1996). However, given the expected response of general acidosis, the observations of Newell et al. (1994), which show little or no effect on oxygen consumption, clearance rate (a measure of ciliary action of the gills), or food assimilation, are startling. These contradictory observations only serve to demonstrate the need for a better understanding of the biochemical, pharmacological, and physiological effects of *P. marinus* infections on oysters.

La Peyre and Faisal (1996) have shown that *P. marinus* cells in culture produce extracellular proteases. These proteases are thought to play a role in damaging host tissue, protecting the parasite from host immune response, and perhaps enhancing the parasites' ability to replicate within the host. General changes in the host physiology such as a decline in hemolymph pH may enhance the cytotoxic activity of such parasite-produced chemical agents.

It is important to note that the physiological effects of *P. marinus* infection on oysters may differ between physiological races of *C. virginica*. Several studies (Bushek and Allen 1996, Paynter and Burreson 1991, Pierce et al. 1992, Brown et al. 1994) have shown that different intraspecific populations of oysters respond differently to *P. marinus* infections. Therefore, a level of infection that would induce mortality in one population may not induce mortality in another population (Brown et al. 1994). This makes it more important to understand the mechanisms of pathology that result in mortality in oysters.

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INTERACTIONS OF *PERKINSUS MARINUS* WITH HUMORAL FACTORS AND HEMOCYTES OF *CRASSOSTREA VIRGINICA*

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ABSTRACT *Perkinsus marinus* can exist in huge numbers in the hemolymph of its host, *Crassostrea virginica*. It is readily recognized and phagocytized by the hemocytes, however, these putative immunocytes seem unable to destroy this parasite, since the infection is rarely controlled. Some of the defense responses known to be available to the oyster are discussed in relation to *P. marinus* infection. The picture that emerges is far from clear, and it seems likely that new approaches to studying oyster defense physiology will be required before the mechanisms underlying susceptibility/resistance to dermo disease will be defined. The situation is further complicated by the fact that ambient environmental conditions exert profound effects on both the parasite and the activity of the oyster's defense system. Perhaps the parasite plays a direct role in suppressing the host's defenses by not triggering or by scavenging the production of reactive oxygen species, by regulating immune responses by excretory/secretory factors, or by releasing proteolytic enzymes. The dynamic interplay of other factors of host and/or parasite origin may also influence the progression of dermo disease.

KEY WORDS: Dermo disease, *Perkinsus marinus*, *Crassostrea virginica*, hemocyte, defense mechanisms, humoral defense molecules, reactive oxygen species, lysozyme, proteases, acid phosphatases, iron-binding proteins, stress proteins, agglutinins

BRIEF OVERVIEW OF THE DEFENSE MECHANISMS OF *CRASSOSTREA VIRGINICA*

In order to focus on the subject of this chapter, the putative involvement of *Crassostrea virginica* defense mechanisms in the host response to *Perkinsus marinus* infection, no attempt will be made to present a complete review of the literature on immunity in other bivalve species. Instead, the protective mechanisms available to the eastern oyster will be mentioned briefly, while most of this contribution will describe what is currently known about the effects that these physiological mechanisms have on *P. marinus*, and vice versa. Although this parasite has had devastating effects on *C. virginica* on the East Coast of the United States, surprisingly little is known about specific interactions between it and the defense reactions of its host. Throughout this chapter the terms "immune mechanisms" and "defense mechanisms" will be used interchangeably. Specific or adaptive immunity characterized by the presence of lymphocytes and immunoglobulins cannot be found in oysters; however, cellular and humoral components analogous to those typical of the nonadaptive (nonspecific) immune systems of higher animals are present. Phagocytes, antimicrobial molecules, agglutinins, lysins, and other humoral components comprise the immune systems of invertebrates.

Humoral Components

A number of lysosomal hydrolases not only participate in the intrahemocytic destruction of microorganisms but also find their way into the cell-free hemolymph of the oyster. Degranulation of hemocytes probably is a major contributing mechanism for the extracellular presence of these enzymes (Foley and Cheng 1977, Cheng et al. 1975, Cheng 1992). These factors are thought to function in the control of infection, and lysozyme is probably one of the most studied examples (McDade and Tripp 1967a, Cheng and Rodrick 1975, Hardy et al. 1977, Steinert and Pickwell 1984). Lysozyme levels show considerable natural variation among oysters, as well as variation due to seasonal effects, the presence of parasites, and exposure to xenobiotics (Feng and Canzonier 1970, Cheng and Rodrick 1974, Pickwell and Steinert 1984, Chu and La Peyre 1989). Other lysosomal enzymes including acid phosphatase, aminopeptidase, beta-glucuronidase, and lipase have also been detected in hemolymph (Cheng 1976b, 1978, Cheng and Rodrick 1975, Yoshino and Cheng 1976, Fries 1984, Pipe 1990). The roles of these enzymes in antimicrobial responses are less studied than lysozyme; however, they may degrade surface integrity of microorganisms contributing to their recognition and/or destruction by the host.

Another widely studied class of oyster humoral factors is the agglutinins (lectins) directed against various saccharide moieties on cell surfaces. These molecules react avidly with mammalian erythrocytes and have been shown to facilitate their subsequent phagocytosis (Tripp 1966, McDade and Tripp 1967b). It is postulated that their normal *in vivo* functions may involve extracellular recognition and opsonization of bacteria and protozoans (Renwrantz 1983, Fisher and Dinuzzo 1991), and they may also serve as non-self-receptors associated with the hemocyte surface (Vasta et al. 1982, Renwrantz and Stahmer 1983, Vasta et al. 1984, Vasta 1991). There is evidence that naturally occurring oyster agglutinins, as well as plant lectins, (Mullainadhan and Renwrantz 1986), can form bridging molecules between hemocytes and non-self-material. However, the lectin-mediated recognition of foreignness is variable, dependent on the determinants available on a given foreign particle or cell. The requirement for opsonization in phagocytosis by molluscan hemocytes is not absolute; recognition and uptake can proceed in the absence of serum factors, or in hemolymph with no apparent ability to agglutinate the foreign material (Renwrantz and Stahmer 1983).

Cellular Components

The hemolymph of the oyster contains several hemocyte types that can be identified based on morphology (Cheng 1981; Fisher 1986). The main phagocytic hemocytes are often referred to as granulocytes and hyalinocytes. This distinction is based on relative degrees of granularity and is rather subjective but will have to suffice until functional subsets with unequivocal surface markers

are identified. The granular hemocytes are more avidly phagocytic in oysters. The numbers of hemocytes in the circulation of *C. virginica* increase during *P. marinus* infection (Anderson et al. 1992c); similar increases have been noted in other bivalves stressed by toxicants or infections (Cheng 1988, Renwrantz 1990, Oubella et al. 1993, Coles et al. 1994). The hemocytes are thought to be of central importance in resisting and controlling infections in bivalves by virtue of their ability to ingest and destroy microorganisms. In addition to the presence of cidal and digestive hydrolases, oyster cells can generate antimicrobial reactive oxygen intermediates (ROIs) (see reviews by Adema et al. 1991, Anderson 1994b). It is likely that *C. virginica* hemocytes contain myeloperoxidase (MPO) which is involved in the production of the potent antimicrobial factor hypochlorous acid. MPO has been demonstrated in mussel hemocytes (Schlenk et al. 1991). Zymosan-stimulated, luminol-augmented chemiluminescence (CL) is strong in oyster hemocytes, indicating the presence of the MPO/H_2O_2/halide antimicrobial pathway. Phenoloxidase may also be present in oyster hemocytes, as it is in the cells of *Mytilus edulis* (Coles and Pipe 1994). Involvement of this enzyme in microbicidal, antiparasitic, recognition, opsonization, and cellular communication aspects of arthropteran immunity has long been recognized (Söderhäll 1982, 1992). The hemocyte-mediated immune mechanisms of *C. virginica* in relation to *P. marinus* infection will be detailed in subsequent sections.

AMBIENT ENVIRONMENTAL EFFECTS ON IMMUNE PARAMETERS AND *P. MARINUS* INFECTION

Obviously, the ambient temperature might be expected to influence hemocyte functions and defense-related activities of oysters and other ectothermic organisms. Earlier literature indicated that elevated temperatures were associated with increased pinocytosis, migratory activity, and phagocytosis (Feng and Feng 1974, Feng 1965b, Foley and Cheng 1975). Fisher and Tamplin (1988) reported that hemocyte rate of locomotion and foreign particle–binding capacity showed positive correlation with temperature. Phagocytosis rates in oysters acclimated to various temperatures (Chu and La Peyre 1993b) also rose as temperatures approached 25°C. These authors found that both the total hemocyte count and the percentage of granulocytes in circulation increased with temperature. This could result from concomitant higher heart contraction rate (Feng 1965a) or some other means of mobilization from noncirculating hemocyte pool(s). It is hypothetically possible that increased cell counts result from hematopoiesis, but histological areas of blood cell proliferation have not as yet been described in oysters. Oysters collected in Virginia, and more northern sites, had serum lysozyme levels that were generally higher during winter months than in the summer (Feng and Canzonier 1970, Chu and La Peyre 1989); however, Fisher et al. (1993) reported higher lysozyme levels in summer months in Gulf Coast oysters, suggesting possible regional differences in temperature influences. Lysozyme levels in laboratory-held oysters were also lower at elevated temperatures, but hemagglutinin titers were unchanged (Chu and La Peyre, 1993b).

The relationship between environmental temperature and *P. marinus* incidence in field- and laboratory-infected *C. virginica* has been known since the earliest reports of the parasite. Generally, the parasite requires temperatures ≥20°C to multiply in oysters (Andrews 1988), but it can persist through the winter. Fisher et al. (1992) showed that elevated temperature increased the mortality of infected oysters. The direct correlation of disease prevalence (and intensity) with increasing temperature has been shown in many laboratory studies (e.g., Chu and La Peyre 1993b, who also showed that total hemocyte count increased with higher temperatures [10–25°C]). Elevated total hemocyte counts in *P. marinus*–infected oysters at 25°C were noted, as seen during development of moderate-heavy infections by Anderson et al. (1992b). According to Chu and La Peyre (1993b), both lysozyme levels and condition index were negatively correlated with this disease, unlike the percent granulocytes, phagocytosis, and hemagglutinin levels. *P. marinus* infection apparently did not alter temperature effects on the various defense-related activities measured; the positive and negative correlations between these activities and temperature seen in uninfected oysters were identical in infected oysters.

Ambient salinity is also reported to affect oyster immune parameters as well as prevalence and intensity of *P. marinus* disease (La Peyre et al. 1989, Gauthier et al. 1990, Chu and La Peyre 1989, Chu et al. 1993). Increasing salinity suppresses hemocyte spreading and locomotion (Fisher and Newell 1986) and may affect hemolymph lysozyme concentration, as described below. Low salinity has an inhibitory influence on *P. marinus* infection; transmission and progression can occur at 10 ppt (Chu and La Peyre 1993a), but sporulation is inhibited at 6 ppt (Chu and Greene 1989). *P. marinus* infection progression in oysters is retarded at 12 ppt and is stopped at ≤9 ppt (Ragone and Burreson 1993). Low salinity can delay or reduce oyster mortality caused by this disease (Scott et al. 1985, Ragone 1991). *P. marinus*–infected oysters, when placed in low salinity water, have lower serum lysozyme levels and show increased survivorship (La Peyre et al. 1989, Ragone 1991, Chu et al. 1993). La Peyre et al. (1989) reported a weak correlation between total hemocyte count and salinity, but hemolymph total protein levels and hemagglutinin levels showed no relationship to salinity. The effect of salinity on lysozyme level in *C. virginica* is uncertain. For example, Chu and La Peyre (1989) found no correlation between salinity and lysozyme levels in a 1-year study but subsequently reported that lysozyme concentrations decrease with elevated salinity (Chu et al. 1993).

HEMOCYTIC DEFENSE AGAINST *P. MARINUS*: ROIs

In mammalian leukocytes, appropriate membrane perturbations, caused by phorbol myristate acetate binding or phagocytosis, trigger the uptake of O_2, generation of NADPH, activation of NADPH oxidase, and generation of superoxide anions (O_2^-). A number of other toxic ROIs such as hydrogen peroxide (H_2O_2), singlet oxygen, hydroxyl radicals, hypochlorous acid, etc., can be subsequently derived from O_2^- (see reviews by Babior 1984; Klebanoff 1985). Similar reactions seem to take place in molluscan hemocytes (Adema et al. 1991; Anderson 1994b). The ROIs are thought to play significant roles in phagocyte-mediated killing of microorganisms. They can also have deleterious effects when generated at levels that exceed the antioxidant mechanisms available to the host.

One of the most commonly used assays for the quantitation of ROIs is luminol-augmented CL, in which ROIs generated by the hemocytes activate the probe luminol. Upon relaxation, the luminol molecules emit photons which can readily be measured in a luminometer or a liquid scintillation spectrometer adapted for single-photon counting. The CL activity of mammalian leukocytes has been directly linked to their antimicrobial activity (Horan et al.

1982). This association of CL activity with defensive mechanisms has often been assumed in the case of molluscan hemocytes.

Current data suggest that strong luminol-augmented CL is produced when oyster hemocytes bind and phagocytize yeast cells or zymosan particles (Larson et al. 1989, Fisher et al. 1990, Anderson et al. 1992a). However, luminol-augmented CL induction by yeast or zymosan may not be seen in hemocytes of other bivalve species, such as *Mercenaria mercenaria* (Cheng 1976a, Anderson 1994a). The CL assay with luminol actually measures a MPO-dependent antimicrobial pathway (DeChatelet et al. 1982, Dahlgren and Stendahl 1983) in mammalian blood cells. MPO activity is present in bivalve hemocytes (Schlenk et al. 1991), and treatment with specific MPO inhibitors will greatly reduce zymosan-stimulated, luminol-dependent CL of oyster hemocytes (Anderson, unpublished). Therefore, oyster hemocytes probably have a mechanism analogous to the H_2O_2/MPO/halide antimicrobial system first characterized by Klebanoff (1968).

What is the physiological significance of luminol-augmented CL to molluscan defensive capacities against infectious agents, especially *P. marinus*? Several authors report that exposure to several classes of environmental contaminants can result in decreased hemocyte CL responses (Larson et al. 1989, Fisher et al. 1990, Anderson et al. 1996, 1994, Coles et al. 1994). Exposure of oysters to chemical stressors can enhance latent and/or experimental *P. marinus* infections (Winstead and Couch 1988, Chu and Hale 1994, Anderson et al. 1996, Fisher et al. 1995), but it is very difficult to use CL to probe the underlying mechanism(s) involved. It is easy to dismiss studies of CL, or any other defense-related activity of *C. virginica* hemocytes, as irrelevant to understanding this disease, because of the obvious inability of hemocytes to control ultimately the progression of the infection. However, there could be limited ROI-mediated anti-*Perkinsus* capability involved in the elimination of the parasites during early stages of infection. There is electron microscopic evidence of limited intrahemocytic *P. marinus* destruction (La Peyre 1993, Bushek et al. 1994). Perhaps the expression of this minimal ability to cope with initial, very light infections is important to determining resistance. Eventually our understanding of the pathogenesis of this disease will be more complete, but the use of CL to fill in the details may be complicated. For example, it could be hard to interpret changes in zymosan- or yeast-induced, luminol-augmented CL in hemocytes from a study designed to evaluate the effects of an environmental contaminant on *P. marinus* progression. As mentioned above, many chemical stressors will produce dose-dependent inhibition of CL, which could be considered the basis of immunosuppression and enhanced disease susceptibility. However, CL of *C. virginica* hemocytes has been shown to significantly increase with elevated levels of *P. marinus* infection (Anderson et al. 1995). Therefore, it is difficult to sort out the simultaneous and opposite effects of chemical stress and infection in an oyster experiencing both situations. In addition, some chemicals that suppress CL at higher doses are actually stimulatory at low doses (Fisher et al. 1990, Anderson et al. 1994). The relationship between high levels of *P. marinus* infection and increased CL is interesting in that it suggests a form of hemocytic activation produced by the intracellular presence of the parasite. We speculated that although this apparent activation did not provide effective anti–*P. marinus* protection, it might serve to explain some of the pathogenic effects of the disease via increased ROI-mediated tissue damage (Anderson et al. 1992b). However, one cannot extrapolate data from zymosan-induced CL to those from *P. marinus*–induced CL. In fact, *P. marinus* ingestion seems to be ineffective in stimulating CL in hemocytes withdrawn from oysters, regardless of the level of infection in the host (La Peyre et al. 1992, Anderson, unpublished).

Clearly, the putative activation of hemocytes during *P. marinus* infection has little effect on the parasite in vivo, if zymosan is required to induce CL (ROI) generation. It is possible that phagocytosis of many kinds of foreign particles (including *P. marinus*) can prime or activate *C. virginica* hemocytes, but the expression of elevated CL responses depends on subsequent stimulation by specific agents (such as zymosan, but not *P. marinus*). If this is true, CL could prove to be a more useful mechanistic probe to study infectious diseases of *C. virginica* other than that caused by *P. marinus*. The inability of certain other bivalve parasites to elicit CL in zymosan-responsive hemocytes of their host has been previously reported (Hervio et al. 1989a,b, LeGall et al. 1991, Bachère et al. 1991). *Leishmania* and other parasites of vertebrates can enter host macrophages by interacting with receptors mediating internalization without triggering the respiratory burst (McNeely and Turco 1987, Russel and Talamus-Rohana 1989).

CLASSIC OYSTER DEFENSE MOLECULES AND *P. MARINUS*

Agglutinins

The presence of lectins, or natural agglutinins, has been noted in many bivalves including oysters. Hemagglutinins can have opsonic properties, as shown by their ability to enhance phagocytosis of foreign erythrocytes by hemocytes (McDade and Tripp 1967b). Bacterial agglutinins have also been described (Arimoto and Tripp 1977, Tamplin and Fisher 1989, Fisher and Dinuzzo 1991); in some cases these lectins can be induced by bacterial infection and may facilitate their immobilization and destruction by the host (Olafsen et al. 1992). Infection of *C. virginica* by *Haplosporidium nelsoni* (the causative agent of MSX) can produce alterations in the levels of serum agglutinins (Ling 1990, Chintala and Fisher 1991). For example, *Vibrio cholerae* agglutinin titers were seasonally elevated in MSX-resistant strains of oysters (Chintala and Fisher 1991). In a study of *P. marinus* infections in oysters from the Gulf of Mexico held under different laboratory conditions, no association was found between antimammalian erythrocyte hemagglutinin levels and parasite infection intensity (Fisher et al. 1992). Recently Chintala et al. (1994) have studied the relationship of oyster serum agglutinins to the protozoan parasites *P. marinus* and *H. nelsoni*. No relationship was shown between initial baseline levels of hemagglutinins or bacterial agglutinins and postinfection survival times of the oysters. Agglutinin levels were also not correlated with parasite densities during disease progression. Therefore, the lectins measured in this study probably play little or no role in defense reactions against these protozoan parasites.

Lectins from various plant sources will bind to oyster hemocytes; concanavalin A has received considerable study in this regard (Yoshino et al. 1979). Recently, Cheng et al. (1993) reported that *Lathyrus odoratus* (sweet pea) lectin will bind and agglutinate *C. virginica* hemocytes. Subsequently, it was shown that hemocytes from *P. marinus*–infected hosts were less subject to *L. odoratus* lectin agglutination than cells from uninfected animals (Cheng and Dougherty 1994). This was explained by competition for the lectin by common saccharides on the extracellular parasites and the hemocytes. Sharing of these as yet unidentified saccharides was suggested to account for the failure of the hemocytes to

recognize and phagocytize those meronts existing free in the serum.

Lysozyme

Oysters from an area of the James River with comparatively low salinity (10 ppt) showed higher *P. marinus* survival rates than those from higher salinity sites (20 and 32 ppt). The lysozyme concentration in the James River oysters' hemolymph was also higher than that seen in other groups, prompting the authors to suggest a cause-and-effect relationship (Chu and La Peyre 1993a, Chu et al. 1993). A positive correlation between lysozyme concentration and *C. virginica* survival had been previously reported by La Peyre et al. (1989). Since both disease intensity and hemolymph lysozyme activity are negatively correlated with salinity, the relative importance of lysozyme as a determinant in the outcome of *P. marinus* infections is not clear. Its bactericidal activity is well documented, but its effect on *P. marinus* has yet to be directly assessed.

Prophenoloxidase-Activating System

The capability of phenoloxidase to mediate the conversion of phenolic substrates, such as L-dopa, to melanin has been demonstrated in leukocytes from human beings and other vertebrates. The enzyme has also been detected in the hemocytes and hemolymph of many marine invertebrates (Smith and Söderhäll 1991). Melanization occurs mainly in arthropods, where it plays a prominent role in many aspects of defense responses including non-self-recognition, opsonization, cellular communication, antibacterial activity, wound healing, and encapsulation (Söderhäll 1982, 1992). Phenoloxidase can be released by activation of the corresponding proenzyme by well-known immunostimulators such as β1,3 glucans (Pye 1974) and bacterial lipopolysaccharides (Söderhäll 1982).

A detailed description of phenoloxidase activity in the hemocytes and serum of *M. edulis* (Coles and Pipe 1994) shows that this defense system can also be found in bivalves. The enzyme was localized in large granules of the eosinophilic hemocytes and showed significant seasonal variability. *M. edulis* hemocytes showed phenoloxidase activation after preincubation with zymosan supernate, a treatment already known to cause a similar effect in crayfish hemocytes (Unestam and Söderhäll 1977). The precise role of phenoloxidase in bivalve immunity has not as yet been elucidated; however, it clearly merits more study, particularly in light of presently existing uncertainties about the significance of more commonly investigated putative immune mechanisms to defense against *P. marinus* and other pathogens.

OTHER OYSTER- AND *P. MARINUS*–ASSOCIATED FACTORS

Proteases

P. marinus infections inhibit growth in *C. virginica* (Paynter and Burreson 1991) and cause severe mortalities. Surprisingly, no major alterations in feeding rate, metabolic rate, and assimilation efficiency of *C. virginica* accompany heavy infection by *P. marinus* (Newell et al. 1994). Virulence factors associated with this parasite and mechanisms of pathogenicity seem to be poorly understood at this time. A number of *P. marinus*–derived proteins can be detected in spent culture media, including proteases that could participate in necrotic reactions in infected oysters (Faisal et al. 1994, La Peyre and Faisal 1995). Some of these enzymes have been shown to be serine proteases and have been purified from *P. marinus* media by affinity chromatography (La Peyre et al. 1995). Five distinct bands could be eluted with molecular weights ranging from 35 to 55 kDa.

Acid Phosphotase

Acid phosphatase associated with parasites can play a role in avoidance of host defense reactions by disruption of phosphoproteins and/or inhibition of superoxide anion production. Both *P. marinus* meronts and *C. virginica* hemocytes have been shown to contain acid phosphatase (Volety and Chu 1994). The intrahemocytic activity varied markedly depending on the geographic sources of the oysters, and both intrahemocytic and intrameront activities were positively correlated with ambient temperature. Living *P. marinus* meronts significantly inhibited zymosan-stimulated oxyradical (CL) release by *C. virginica* hemocytes, whereas heat-killed *P. marinus* did not produce this effect (Volety and Chu 1995). Whether this is due in part to acid phosphatase–mediated ROI suppression by the viable parasites has yet to be determined. In this regard, it is interesting that estuarine water in which *P. marinus* had been maintained slightly suppressed zymosan–stimulated CL; it was also shown to contain acid phosphatase, which was not detected in the appropriate control water. Characterization of extracellular products from *P. marinus* that suppress CL and other defense mechanisms of *C. virginica* will probably be an active area of research in the near future. Acid phosphatase is one such product that blocks O_2^- in leukocytes, permitting intracellular survival of parasites such as *Leishmania* (Remaley et al. 1984), as well as molluscan parasites such as *Bonamia ostreae* in *Ostrea edulis* (Hervio et al. 1991).

Iron-Binding Proteins

P. marinus has a strong requirement for soluble iron (Gauthier and Vasta 1994). Soluble iron in the culture medium enhances *P. marinus* replication, and the addition of natural iron chelators such as lactoferrin, transferrin, or desferrioxamine to the medium reduces parasite proliferation in a dose-dependent manner. It was suggested that shifts in iron availability to the host and parasite during ongoing infection might affect the iron pools required for O_2^- and ·OH production, which in turn might allow *P. marinus* to avoid intracellular oxidative damage. The characterization of the iron-binding proteins of oysters and their modulation during infection should provide valuable insight with regard to antimicrobial defense mechanism(s). Nonimmunological mechanisms that involve simply withholding iron from the pathogen may be important in controlling microbial infections.

Stress Proteins

In ectothermic animals like oysters the hemocytes must function over a considerable temperature range; it has already been reported that environmental factors such as salinity and temperature can influence certain hemocyte defense responses (Fisher 1988). As mentioned previously, increased mortality among oysters infected with *P. marinus* is associated with high ambient temperature and salinity. A highly conserved biological response to hyperthermia, hypoxia, toxicants, and other noxious stimuli is the synthesis and accumulation of heat shock proteins and other stress proteins (SP). These proteins ensure the survival, normal functioning, and recovery of cells during and after stressful circumstances. Among their numerous activities, SP have been shown to be important in the functioning of mammalian immune cells (DeNagel and Pierce 1993, Young et al. 1993). Many SP

classes are present in aquatic organisms, including bivalves (Sanders 1993), but their possible role(s) in oyster hemocyte-parasite interactions are only recently being investigated.

The level of the 70-kDa heat shock protein (SP70) in oyster hemocytes was reported to increase with increasing *P. marinus* infections (Brown et al. 1993). The SP70 level in mantle tissue was elevated during the late summer and fall, times when *P. marinus* infection is high. More recently the SP of both oyster hemocytes (Tirard et al. 1995a) and *P. marinus* (Tirard et al. 1995b) have been characterized. Cold shock had no significant effect on protein synthesis by hemocytes, but heat shock produced by temperatures >20°C above ambient triggered synthesis of 32-, 34-, 37-, 70-, and 85-kDa SP. The hemocytes withstood the acute temperature increment well, since most remained intact and viable. It is possible that the induced SP played an important role in protecting key cellular proteins against denaturation, thus permitting the hemocytes to maintain their surveillance and effector functions during periods of thermal stress. *P. marinus* also will produce heat shock proteins, but only at temperatures somewhat higher than those used to elicit SP in *C. virginica* hemocytes. The molecular masses of *P. marinus* SP were about 29, 63, 79, and 86 kDa, clearly distinguishable from those of the host. These differences suggest the possibility of studying the SP responses of both host and parasite simultaneously in a mixed culture. The higher thermal threshold for the parasite's SP response may mean that it can retain normal function at temperatures that are very stressful to the hemocytes. Although the relevance of these observations to host-parasite interactions under environmental conditions encountered in the field needs to be carefully evaluated, clearly this is an exciting area for future research.

CONCLUSIONS AND FUTURE RESEARCH DIRECTIONS

Based on our knowledge of host-parasite interactions, it is reasonable to speculate that the functional capacity of the immune system of *C. virginica* plays a pivotal role in the response elicited by the protozoan *P. marinus*. It has been suggested by several authors (e.g., Cheng 1987) that the virulence of *P. marinus* increases when its host becomes immunologically compromised; however, this conclusion is often based on indirect evidence. We are still limited by an insufficient knowledge of oyster defense mechanisms as related to *P. marinus* infections and/or incomplete understanding of the mechanisms underlying the pathogenicity of this parasite. The picture is further complicated by the difficulty of interpreting defense activities during progression of the disease. It is difficult to know to what extent the changes observed indicate factors determining resistance/susceptibility or merely represent responses to the presence of the parasite. In addition, a number of hemocyte activities can be modulated by environmental factors such as temperature and salinity; these same conditions can have profound direct effects on survival and multiplication of *P. marinus*.

Perhaps the most direct link between *C. virginica* immunocompetency and resistance to *P. marinus* has come from recent toxicity studies. In studies of the acute toxicity of the carcinogen n-nitrosodiethylamine to oysters, this compound was found to enhance *P. marinus* infections (Winstead and Couch 1988). Oysters receiving sublethal doses developed locally heavy infections which triggered atypically light hemocytic responses, suggesting chemically induced immunosuppression relevant to *P. marinus*. In vitro exposures to other environmental contaminants, such as tributyltin (TBT), were known to reduce the ability of oyster cells to produce ROIs (Fisher et al. 1990). Subsequent studies have shown that in vivo TBT exposure of oysters will accelerate the progression of experimental *P. marinus* infections (Fisher et al. 1995, Anderson et al. 1996). In addition to these single-toxicant experiments, exposure of *C. virginica* to water-soluble fractions of polyaromatic hydrocarbon (PAH)-contaminated sediments enhanced preexisting *P. marinus* infections and increased susceptibility to experimental infection (Chu and Hale 1994).

The above-mentioned data are interpreted by the investigators involved as likely examples of contaminant-induced reductions of oyster defensive capacity that are expressed as diminished resistance to latent or experimentally initiated *P. marinus* infections. However, the possibility that the chemicals produce direct stimulatory effects on the parasite, or act on some nonimmunological parameter of disease resistance, is also raised. There seems to be little doubt that, under typical conditions, *C. virginica* has little ability to control the multiplication and progression of *P. marinus* once it becomes established in the hemolymph or other tissues. There is only circumstantial evidence that the best characterized putative antimicrobial defense mechanisms of *C. virginica*, such as lysozyme or ROIs, have significant deleterious effects on *P. marinus*. The question of the relative protective importance of these factors in other bivalve species less seriously affected by this parasite needs to be resolved. Clearly, the basis for the pathogenicity of *P. marinus* in minimally stressed *C. virginica* is poorly understood and the role of immunity in the process is hard to evaluate. However, since chemical and other stressors are able to accelerate the progression of this parasitic disease, perhaps xenobiotic exposure–based models will contribute useful knowledge of physiological disease control mechanisms. In-depth studies of *C. virginica:P. marinus* interactions at the cellular and molecular levels are still needed. Such work should have interest to immunologists and parasitologists and will hopefully find practical application in managing and/or controlling this major disease of oysters.

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GENETIC ASPECTS OF DISEASE RESISTANCE IN OYSTERS

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ABSTRACT Like other phenotypic traits, resistance to disease is generally subject to underlying genotypic variability. This may come about indirectly, as a result of variation in overall physiology or in life history characteristics, or may be more directly attributable to variation in cellular or biochemical mechanisms. We summarize here our current understanding of genetic influences on physiological and life history variation in *Crassostrea* and review the evidence available to date on intra- and interspecific genetic variation in disease resistance, with emphasis on *Perkinsus marinus* and MSX. We also describe our current view of population structure in *Crassostrea virginica*, and how it may affect the evolution of disease resistance. Finally, we explore approaches to the development of disease-resistant oysters that capitalize on the genetic variability inherent in *C. virginica* and within the genus *Crassostrea*.

KEY WORDS: *Crassostrea virginica, Crassostrea gigas, Perkinsus marinus, Haplosporidium nelsoni,* MSX, disease, polyploidy, genetics, heterozygosity, physiology, population structure

INTRODUCTION

The resurgence of infectious human diseases in modern times serves as a sobering reminder that host-pathogen relationships are rarely static; rather, they are dynamic on both ecological and evolutionary time scales. This view is colorfully expressed by the "Red Queen hypothesis" of Van Valen (1973), in which both parties are engaged in an incessant evolutionary arms race. (In Lewis Carroll's *Through the Looking Glass*, the Red Queen said to Alice, "here, you see, it takes all the running you can do to keep in the same place.") In this chapter, we focus on the role that genetic variability in the oyster host may play in determining its response to *Perkinsus marinus*.

Physiological Variation

Physiological variation in the oyster may influence both the acquisition of disease and its subsequent development. For example, ventilation and clearance rates in bivalves vary considerably in response to a number of environmental factors such as season, salinity, temperature, nutrition, and turbidity; they also vary during the course of development and with respect to body size. If the likelihood of infection is a function of the rate of exposure to infective particles, then any physiological variation affecting pumping and clearance rates may alter disease risk. It also seems likely that physiological status will affect the progression of disease following infection, although it is difficult in this case to separate cause and effect.

Certainly a large proportion of physiological variation in natural populations may be attributed to local environmental influences, as demonstrated by reciprocal transplant experiments (Widdows et al. 1984). It seems likely that a substantial fraction of differences in disease resistance among populations that occupy geographically proximate but physically different environments (e.g., Chu and La Peyre 1993) may represent physiological acclimation rather than genetic differences, but definitive data are lacking.

At the same time, physiological functions may also be influenced by genotype. For example, standard metabolic rate (measured as oxygen consumption by nonfeeding animals) in *Crassostrea virginica* was found to be inversely related to heterozygosity at a set of five enzyme-coding (allozyme) loci (Koehn and Shumway 1982). In a similar vein, more heterozygous oysters appeared to lose weight less rapidly when starved (Rodhouse and Gaffney 1984). Other studies have failed to detect associations between allozyme heterozygosity and physiological traits, and it remains unclear whether the association is genuinely sporadic or simply weak and therefore difficult to detect consistently (Gaffney 1986, 1990).

Studies of the mussel *Mytilus edulis* demonstrate a positive relationship between multilocus allozyme heterozygosity and fitness that appears to result from lower protein turnover (and thereby a lower energetic demand for maintenance metabolism) in more heterozygous individuals (Hawkins et al. 1989). Although this suggests a metabolic basis for allozyme-associated heterosis in bivalves, the mechanism(s) responsible for the connection between genotype and physiology is still unknown. Regardless of the actual mechanism, genotypic variation in whole-body metabolism may translate into genotypic variation in resistance to pathogens, if the latter depends on net energy balance or rate of protein turnover.

In general, physiological variation, whether due to environmental influences, heredity, or both, may result in differences in susceptibility to infection. For example, individuals with higher filtration rates may enjoy an energetic advantage from greater food consumption, but at the same time could experience increased risk of exposure to infection with *P. marinus*. We are unaware, however, of any experimental data testing this hypothesis and note that the stress of spawning does not appear to accelerate the development of MSX infections in *C. virginica* (Ford and Figueras 1988). Likewise, *P. marinus* infection intensity appears to be independent of sex and reproductive stage (Burrell et al. 1984, Wilson et al. 1990).

Response to selection is a traditional method of demonstrating genetic variability in a trait. Although several studies have documented differences in resistance to MSX between wild oysters and selected lines (Haskin and Ford 1988, Ewart et al. 1988, Hawes et

al. 1990, Matthiessen et al. 1990, Paynter and DiMichele 1990), no detailed physiological investigations have been conducted on selected lines.

Life History Variation

Variation in life history traits may be regarded as a higher order expression of physiological variation, in which differences in integrated physiological functions lead to variability in features of primary ecological importance, e.g., growth rate, fecundity, age at sexual maturation, or timing of reproduction. Two types of evidence point to considerable genetic variability in life history traits in the oyster.

Numerous studies have demonstrated a positive correlation between heterozygosity at allozyme loci and growth rate in natural oyster populations (reviewed by Zouros and Foltz 1987). One might anticipate that greater somatic growth would also result in greater fecundity, given the generally positive relationship between body size and fecundity. Although this has not been examined in oysters, a study of *M. edulis* showed that more heterozygous individuals tended to have greater fecundities (Rodhouse et al. 1986). Less is known about the relationship between heterozygosity and viability. Heterozygosity was positively associated with survival in *C. virginica* (Zouros et al. 1983) but negatively associated with survival in *Ostrea edulis* (Alvarez et al. 1989). Studies of other bivalve species generally point to positive associations (e.g., Blot and Thiriot-Quiévreux 1989, Borsa et al. 1992, Pecon Slattery et al. 1993), but sometimes no relationship is apparent (e.g., Gaffney 1990, Fevolden 1992).

Although the physiological correlates of increased heterozygosity are generally thought to result in enhanced fitness, this need not always be so. In the flat oyster *O. edulis*, a negative correlation between allozyme heterozygosity and viability was attributed to the enhanced susceptibility of larger (more heterozygous) oysters to the pathogen *Bonamia ostreae* (Alvarez et al. 1989). However, no direct evidence on the relationship between host genotype and rates of parasitic infection was available.

A second, more indirect indication of genetic variation in life history traits is the existence of considerable latitudinal variation in growth rate and reproductive cycles. Although the majority of this variation may be environmental in origin, it appears that at least some is genetic. Barber et al. (1991) found that a hatchery stock of *C. virginica* derived from Long Island Sound initiated gonadal development and spawned 1 month earlier than native (Delaware Bay) controls, even after 5-6 generations of rearing in Delaware Bay. Hatchery lines of Virginia origin likewise spawn later than Delaware Bay lines (Ford et al. 1990).

The relationship between life history traits and susceptibility to disease in oysters is purely conjectural at this point. However, given that gametogenesis and spawning are major physiological events, requiring the mobilization of energy reserves and imposing a substantial physiological load, it seems plausible that they may alter the oyster's susceptibility to infection. It is also possible that biochemical or metabolic changes associated with reproduction may affect the process of infection or disease development. Available data on this point are discussed below.

Variation in life history parameters may also represent an evolutionary adaptation to disease pressure. High disease-induced mortality may favor reproduction at an earlier age, before the onset of disease (or before its effects become debilitating). Sexual maturation has been observed to occur in *C. virginica* at 3 months of age in New Jersey (Bushek and Allen, pers. comm.) and at 1 month of age in Gulf Coast oysters (Hopkins 1954), indicating that early reproduction is a feasible strategy. However, there is no evidence that oysters have taken this course. In the absence of solid data on population structure and demography in the eastern oyster, we can only speculate on the factors responsible for the apparently limited ability of natural populations of *C. virginica* to develop resistance to *P. marinus* and MSX.

Intra- and Interspecific Variation

Any species that possesses an adequate store of genetic variability can be expected to exhibit genotypic variation in response to a pathogen. Differences in susceptibility may result from overall physiological or metabolic variation, as discussed above, or from more specific biochemical or cellular responses. Genetic variation may exist at several levels, affecting the probability of exposure to pathogens, the uptake of pathogens in contact with tissue surfaces, and the progression of disease following infection.

Different species may or may not differ considerably with respect to the same traits that vary within species; often interspecific differences may be far greater than intraspecific ones. This truism is central to agricultural breeding programs, in which within-species selection is often supplemented by efforts to move genetic elements from one species to another. Currently available data (presented below) suggest that this is also true for *Crassostrea*.

EVIDENCE FOR GENETIC VARIATION IN DISEASE RESISTANCE IN *CRASSOSTREA*

To date, studies of genetic variation in disease resistance in oysters have involved direct challenges of candidate oysters with pathogens, either in field or laboratory settings, followed by assays of disease prevalence and intensity. Studies of intraspecific variation have focused primarily on evaluations of hatchery strains, whereas interspecific comparisons have generally compared the Pacific oyster (*Crassostrea gigas*) to wild or susceptible hatchery populations of *C. virginica*. In addition, intraspecific genetic variation in disease susceptibility is indirectly demonstrated by the evolution of resistance in disease-challenged natural populations. The rate of evolution may be modest in some cases (e.g., resistance to MSX) and dramatic in others (e.g., rapid evolution of resistance to Malpeque Bay Disease (Logie et al. 1960)). However, the extent to which this apparent evolution represents genetic change in the parasite vs. genetic change the host is usually unknown.

Intraspecific Variation

Clearly, there is evidence for genetic variation in physiology and life history traits in *C. virginica*. It is not clear, however, to what extent this is matched by variation in the ability of the American oyster to resist or tolerate infection with *P. marinus*.

Because the study of intraspecific variation in oyster resistance to *P. marinus* has been limited, it may be instructive to consider the better-studied case of MSX (*Haplosporidium nelsoni*). Decades of selective breeding of *C. virginica* for resistance to MSX have resulted in several oyster lines that show a markedly enhanced ability to tolerate infection with this pathogen (Haskin and Ford 1979). This response to selection argues strongly for the existence of useful genetic variation within populations. At the same time, the fact that tolerance is not complete, and that the ability to actually *resist* (rather than merely tolerate) infection has

not noticeably increased, suggests that the oyster's repertoire of genetic variability is not sufficient to provide complete protection against MSX. It is also worth noting that MSX-resistant oyster lines do not appear to have enhanced resistance to *P. marinus* (Chintala and Fisher 1989).

Although parasitic infection appears to exert manifold physiological effects on its host, not all physiological variation in the host directly affects susceptibility to parasites. In a common-garden experiment, Ford et al. (1990) noted that the timing and intensity of MSX infection were comparable for three strains of *C. virginica*, despite marked differences in their reproductive state. Conversely, Barber et al. (1991) found that selected and unselected oysters differed dramatically in degree of susceptibility to MSX but showed no detectable differences in metabolic rates prior to infection.

Unfortunately, it appears unlikely that genetically mediated resistance to MSX will also confer resistance to *P. marinus*: genetic variation in resistance to one pathogen often has little bearing on resistance to other pathogens. In rainbow trout, artificial selection for high and low stress response (as measured by blood cortisol levels) was conducted, in the hope that trout with lower stress response would prove generally less susceptible to disease. Trout selected for low stress response showed lower mortality than a high stress response line when challenged with *Aeromonas*, but significantly higher mortality when infected with *Vibrio* (Fevolden et al. 1992). In *C. virginica*, Chintala and Fisher (1989) reported that oysters from an MSX-resistant line showed reduced susceptibility to MSX and higher serum lectin concentrations compared to native oysters, but were no less susceptible to *P. marinus* than native animals.

Only recently has attention been given to intraspecific variation in susceptibility to *P. marinus*. In a set of field trials in Chesapeake Bay, Burreson (1991) reported that two strains of *C. virginica* selected for increased resistance to MSX were highly susceptible to *P. marinus* infection, failed to reach market size, and suffered 99% mortality over the course of the study. In contrast, three unselected (native) lines showed lower susceptibility to *P. marinus* and lower mortality (80%) and grew to market size. A second inbred strain of Chesapeake Bay origin, selected for growth rate but not disease resistance, also showed greater susceptibility to *P. marinus* than native Chesapeake Bay or Delaware Bay oysters (Paynter and Burreson 1991). When cultured in North Carolina, this same selected strain showed faster growth than native oysters but ceased growing and showed higher mortality than native oysters when *P. marinus* infection reached high levels. It is not clear whether the enhanced susceptibility to *P. marinus* in stocks selected for high growth rate or resistance to MSX represents a genetic trade-off, or merely the negative consequences of inbreeding during the selection process, but there is clearly intraspecific variation in response to *P. marinus*.

Bushek (1994) found that oysters from different geographical regions show distinct differences in their response to infection with *P. marinus* (Figure 1). Following shell-cavity injections with *P. marinus* cultured *in vitro*, oysters originating from populations with previous exposure to the pathogen (Virginia and Texas) showed lower body burdens than oysters originating from less exposed populations (Maine and New Jersey). In addition, Atlantic Coast isolates of *P. marinus* caused heavier infection levels than Gulf Coast isolates. No interaction between virulence of the isolate and resistance of the oyster host population was observed, suggesting that the mechanism(s) of resistance to *P. marinus* may be general, rather than specific to particular strains of the pathogen.

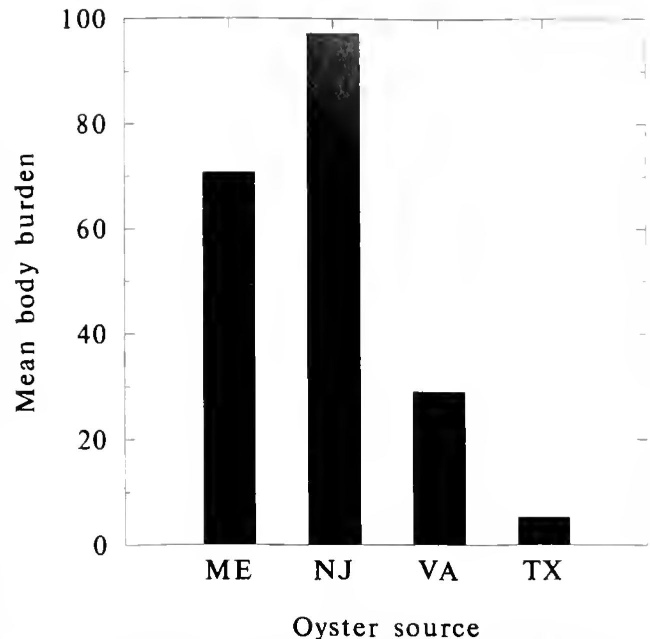

Figure 1. Geometric mean infection intensity (cells per g wet tissue) of oyster populations 94 days after inoculation with *in vitro*–cultured *P. marinus*. Populations with a long history of exposure to *P. marinus* (Virginia and Texas) showed significantly (P = 0.004) lower infection intensity than populations with little or no history of exposure (Maine and New Jersey). From Bushek (1994).

La Peyre and Chu (1988) found that *C. virginica* from Mobjack Bay, VA (a site exposed to heavy MSX pressure), showed higher hemocyte chemotaxis activity and agglutination titers than oysters from James River, VA (a source of oysters susceptible to both MSX and *P. marinus*). It is not known whether these differences reflect genetics, environmental influences, or both.

One indirect way to examine the interaction between physiology and disease is to alter physiology by changing the number of chromosome sets an individual possesses (ploidy manipulation). Individuals with three sets of chromosomes (triploids) typically differ from their normal diploid counterparts by displaying reduced gametogenesis and spawning. Do the physiological changes accompanying inhibited reproduction alter susceptibility to disease in *Crassostrea*?

Triploidy did not appear to decrease susceptibility to *P. marinus* in either *C. virginica* or *C. gigas* exposed to the pathogen in flow-through seawater systems (Meyers et al. 1991). Field trials in Virginia also showed no effect of triploidy on susceptibility of *C. virginica* to *P. marinus*, but triploids did show higher growth rates (Barber and Mann 1991). Chu et al. (1993) reported that although *C. gigas* challenged with *P. marinus* trophozoites generally showed lower infection prevalence than *C. virginica*, triploid *C. gigas* held at 10°C showed a higher prevalence than either diploid *C. gigas* or *C. virginica*.

At a commercial grow-out site in Massachusetts, Matthiessen and Davis (1991) found that triploid *C. virginica* showed higher rates of infection with MSX, but significantly lower mortality, than diploids. These differences may reflect the significantly larger size of the triploids rather than triploidy *per se*. In a large-scale field study, triploids exposed to both MSX and *P. marinus*

suffered consistently greater mortality than diploids, although the relative contributions of the two diseases have not yet been assessed (Allen and Gaffney, unpubl.).

Interspecific Variation

The limited data presently available suggest that differences between oyster species in resistance to pathogens such as *P. marinus* (and MSX) are far greater than intraspecific differences. Upon exposure to both MSX and *P. marinus* in a running seawater system in Maryland, native *C. virginica* died from both diseases, while *C. gigas* acquired no MSX infections and low levels of *P. marinus*, which did not intensify (Farley et al. 1991). In a similar study, Meyers et al. (1991) reported that 40% of *C. gigas* exposed to *P. marinus* became infected after 83 days, compared to 100% of *C. virginica*. Infections in *C. gigas* were light compared to those in *C. virginica*, and the low mortality observed in the former was attributed to causes other than *P. marinus*. Comparable results were obtained were obtained by Barber and Mann (1994).

POPULATION STRUCTURE AND GENETIC VARIATION IN OYSTERS

Subdivided Versus Panmictic Populations

A panmictic population is one in which mating is random. Natural species may be subdivided into local subpopulations or demes, within which breeding may usually be treated as random. In the case of organisms with the capacity for extensive dispersal, such as oysters, it is difficult to determine the spatial boundaries of demes. Because it is virtually impossible to directly track the dispersal and fate of oyster larvae, we are focused to rely on indirect measures of population structure, i.e., genetic markers. Although *C. virginica* has been the subject of numerous genetic analyses of population structure, our understanding is far from complete. Patterns of mitochondrial DNA (mtDNA) variation suggest that *C. virginica* may be divided into distinct Atlantic Coast and Gulf Coast assemblages (Reeb and Avise 1990). In addition, allozyme surveys suggest that populations at the extremes of the species range (Nova Scotia and southern Texas) are genetically distinct from the main assemblages (see Gaffney 1996 for review).

It is now becoming clear that these assemblages are further divided into subpopulations or "races," as many oyster biologists have long suspected. Barber et al. (1991) showed that at least some of the physiological differences among latitudinally separated Atlantic populations are genetic, i.e., there are genetic differences in reproductive schedules between Long Island Sound oysters and Delaware Bay oysters. Preliminary data on mtDNA sequence variation likewise suggested that genetically distinct subpopulations may exist within the Atlantic and Gulf assemblages (O'Foighil et al., 1995). In a large-scale population survey of sequence variation in a 0.4-kb fragment of the small-subunit (16S) mitochondrial ribosomal DNA gene by denaturing gradient gel electrophoresis, Gaffney and Wakefield (unpubl.) found three major haplotypes, each of which was restricted to a single region (Gulf Coast, southern Atlantic, northern Atlantic). Within any region, haplotype diversity was negligible, with the exception of Prince Edward Island, which exhibited unusually high diversity. These results point to significant population subdivision, suggesting limited gene flow despite the potential for widespread larval dispersal. Further work is needed to characterize these genetically distinct oyster populations, particularly with regard to their response to disease.

Population Structure and the Evolution of Disease Resistance

From an evolutionary perspective, the interplay between host and pathogen is now realized to be more complex than originally thought and is the subject of continuing theoretical development. One important element is the population structure of the host species, i.e., whether isolated subpopulation (demes) exist, and to what extent gene flow occurs among them. If gene flow via larval dispersal is extensive, local adaptation may be retarded by the influx of genes from other areas subject to different selection pressures. This phenomenon is often invoked to explain the failure of *C. virginica* to develop effective resistance to pathogens such as MSX and *P. marinus:* despite locally intense disease pressure, the continued influx of immigrants from relatively uninfested source populations (and the export of locally produced offspring) limits the evolution of resistance in the host. The same argument was invoked to explain the failure of mainland populations of *C. virginica* in Canada to develop increased resistance to the enigmatic Malpeque Bay Disease, despite introductions of resistant oysters from Prince Edward Island (Logie et al. 1960).

DEVELOPMENT OF DISEASE-RESISTANT OYSTERS—PROBLEMS AND PROSPECTS

We conclude, not surprisingly, that genetic variation for resistance to *P. marinus* is found within *C. virginica*, both within local populations and among geographic regions. An even greater degree of variation is found among *Crassostrea* species. It is possible to capitalize on this variability, to produce more disease-resistant oysters for aquaculture, or to replenish natural stocks?

Developing resistant lines of *C. virginica* for aquaculture is relatively straightforward, at least in principle. Hatchery lines may be initiated with founders drawn from diverse sources, including areas with a long history of exposure to *P. marinus*. Selection may be imposed by culturing animals at known sites of heavy disease pressure. Appropriate breeding plans designed to increase disease resistance without compromising other production traits can be employed. Although years may be required to develop high-performance lines, it can be done with presently available technology.

Challenging oysters in the field with naturally occurring pathogens is technically simple and is most likely to provide a realistic selection regime. This approach suffers, however, from unpredictable interannual variation in disease pressure and the confounding effects of other variables (e.g., other diseases, temperature and salinity variation). More controlled selection regimes can be employed by deliberately infecting oysters in the laboratory.

Ray (1954) demonstrated that uninfected oysters could be challenged with *P. marinus* by sharing a tank with infected animals. This permits control of physical factors (e.g., temperature, salinity) but does not allow control of dose and exposure to other pathogens. This approach also provides little control over the genetic (clonal) composition of the pathogen population. Use of *in vitro*–cultured *P. marinus* (La Peyre et al. 1993, Kleinschuster and Swink 1993, Gauthier and Vasta 1993) overcomes these problems but has other shortcomings. Bushek (1994) and Chintala et al. (1995) found *in vitro*–cultured cells to be relatively uninfective when ingested by oysters (presumably the most common mode of infection). Injection either into the shell cavity or directly into tissue will produce infections (Gauthier and Vasta 1993, La Peyre et al. 1993, Bushek 1994). However, these methods bypass natural barriers to infection (e.g., gill and palp sorting, tissue epithelia, etc.), which may contribute to differences in resistance among

oyster populations. At this point, field exposure to naturally occurring disease appears to be the best method for large-scale selection programs, while controlled laboratory infection will probably prove valuable for investigating and more precisely evaluating mechanisms of resistance in selected oysters.

An alternative approach to traditional artificial selection involves attempting to move genetic elements that confer disease resistance from one species to another. For example, *C. gigas* appears to be highly resistant to both *P. marinus* and MSX. Can we move the gene(s) responsible from *C. gigas* to *C. virginica?* In principle, this is possible, even though we have no idea what genetic elements are responsible. A time-honored method is hybridization and subsequent backcrossing under selection for the desired trait. To date, this approach does not appear to be feasible, as hybrids between *C. gigas* and *C. virginica* are inviable (Allen et al. 1993).

Modern genetic engineering techniques provide a means of moving genes between species that cannot be hybridized. This approach may be feasible once methods have been developed for producing transgenic bivalves and the gene(s) conferring disease resistance is identified. While the former barrier may not be difficult to breach, the identification of disease-resistance genes is not a trivial matter.

A simpler form of genetic engineering, involving the transfer of chromosomes or chromosome fragments from *C. gigas* to *C. virginica* by means of partial gynogenesis, is currently under investigation. When lightly irradiated *C. gigas* sperm are used to fertilize *C. virginica* eggs, the developing zygote will possess a single set of maternal chromosomes, plus any paternal chromosomes or fragments that are not completely inactivated by irradiation. Application of cytochalasin B to the newly fertilized egg blocks extrusion of the second polar body, resulting in a diploidized gynogenetic embryo—i.e., one that contains two sets of maternally derived chromosomes, plus any paternal genes contributed by the irradiated sperm. If the paternal fragments contain elements that provide disease resistance, and if these are incorporated into the genome and stably expressed, the result may be a disease-resistant oyster. Efforts are currently underway to develop methods for partial gynogenesis in *C. virginica* (Guo, pers. comm.).

None of these approaches to the development of disease-resistant *C. virginica* can be implemented quickly or simply. One alternative is to grow a disease-resistant *Crassostrea* species in areas now decimated by *P. marinus* (or MSX). *C. gigas* has often been suggested as an alternative (Mann et al. 1991), in view of its worldwide use in aquaculture and resistance to both MSX and *P. marinus*. Introduction of *C. gigas* has been credited with rescuing the cupped oyster industry in France, which crashed when the Portuguese oyster (*Crassostrea angulata*, considered either a conspecific or a close relative of *C. gigas*) succumbed to disease (Grizel and Héral 1991). However, its suitability for culture in mid-Atlantic waters is debatable, as field trials have shown high unexplained mortality (Barber and Mann 1994) and heavy shell damage from the boring polychaete *Polydora* (Burreson et al. 1994). In any event, it appears unlikely that *C. gigas* will be deliberately cultured in Atlantic waters, given the level of concern about introducing exotic species.

A species of *Crassostrea* that exhibits good growth, viability, and disease resistance, yet fails to reproduce, is still a good candidate for aquaculture in disease-ravaged areas. One possibility is the hybrid of *C. gigas* and *Crassostrea rivularis*. Unlike *C. gigas*, the latter species is apparently well adapted to high temperature and low salinity (Nie 1990). Viable hybrids can be produced (Allen and Gaffney 1993). If they prove to be sterile, and show good performance, they may someday earn a niche in commercial oyster culture.

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MODELING DISEASED OYSTER POPULATIONS. II. TRIGGERING MECHANISMS FOR *PERKINSUS MARINUS* EPIZOOTICS

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ABSTRACT Densities of *Crassostrea virginica* remain high enough to support substantial fisheries throughout the Gulf of Mexico despite high mortality rates produced by the endoparasite *Perkinsus marinus*. The infrequency of epizootics in these populations suggests that controls exist on the disease intensification process. The progression of epizootics in oyster populations, the factors that trigger epizootics, and the factors that terminate epizootics once started were investigated with a coupled oyster population–*P. marinus* model.

The time development of a simulated epizootic was triggered by environmental conditions that occurred and disappeared as much as 18 months prior to the onset of mortality in the oyster population. Initiation of epizootic conditions was detected as an increase in infection intensity in the submarket-size adult and juvenile portions of the oyster population. Infection intensity of the market-size adults is maintained at a relatively stable level by the death of heavily infected individuals and the slow rate of *P. marinus* division at high infection intensities. Once started, most of the simulated epizootics resulted in population extinction in 2 to 4 years. Stopping an epizootic required reducing the infection intensity in the submarket-size adults and juveniles. The infection intensity of market-size adults does not need to be reduced to stop an epizootic nor must it be raised to start one.

The simulated oyster populations show that a reduction in ingestion rate (by reduced food supply or increased turbidity) can trigger an epizootic, especially if the reduction occurs during the summer. Increasing food supply or decreasing turbidity in the following year does not necessarily prevent the occurrence of an epizootic. Rather, the onset of the event is simply delayed. Additional simulations show that the relative combination of variations in salinity and temperature is important in determining the occurrence of an epizootic. A dry (high-salinity) summer followed by a warm winter produces conditions that favor the development of an epizootic. Conversely, a warm dry year followed by a cool wet year fails to produce an epizootic. Simulations that consider variations in the biological characteristics of oyster populations, such as changes in recruitment rate or disease resistance, show that these are important in regulating the occurrence of an epizootic as well as in terminating the event. In particular, increased recruitment rate dilutes the infected population sufficiently to terminate an epizootic.

One primary conclusion that can be obtained from these simulations is that epizootics of *P. marinus* in oyster populations are difficult to generate simply with changes in either temperature or salinity. Rather, the epizootics are triggered by some other factor, such as reduced food supply or reduced recruitment rate, that occurs prior to or coincident with high salinity or temperature conditions.

KEY WORDS: *Perkinsus marinus* disease, disease model, oyster disease, eastern oysters, *Crassostrea virginica*

INTRODUCTION

Throughout the southern extent of their habitat range, populations of the eastern oyster, *Crassostrea virginica*, are impacted greatly by the disease-producing endoparasite *Perkinsus marinus* (Quick and Mackin 1971, Wilson et al. 1990, Lewis et al. 1992). In the Gulf of Mexico, the market-size component of oyster populations generally suffers about 50% yearly mortality due to *P. marinus* (Mackin 1961, 1962, Hofstetter 1977). Only one Gulf of Mexico oyster population is known to be free of infection from *P. marinus* (Powell et al. 1992a). Typically, prevalence of this organism exceeds 60% in nearly all populations (Craig et al. 1989, Wilson et al. 1990). Similar conditions exist in oyster populations along the southeastern coast of the United States (Crosby and Roberts 1990, Hofmann et al. 1995). However, despite high disease prevalence and high mortality rates, *C. virginica* generally maintains healthy population densities, which support substantial fisheries throughout much of the range of this animal (Hofstetter 1990, NOAA 1991, Powell et al. 1995b).

Nevertheless, epizootics produced by *P. marinus* do occasionally occur throughout the range of *P. marinus*, although those occurring in the mid-Atlantic region have been more noteworthy in their areal extent and effect on the fishery (Mann et al. 1991, Sindermann 1993). Epizootics of most animal species follow a series of characteristic stages (Gill 1928), which are shown schematically in Figure 1. Most are triggered during a preepizootic phase when the host population appears to be at its healthiest (Gill 1928). The triggering mechanism does not need to remain after the initiation of epizootic conditions. In fact, most mortality usually occurs a significant time after the conditions triggering the epizootic have disappeared. Recovery occurs during the postepizootic phase and the population remains in quasiequilibrium with the disease during a potentially extended interepizootic phase during which time the host population abundance normally increases (e.g., Ross 1982, McCallum and Singleton 1989).

Most models of the factors that trigger and/or control epizootics stress the role of transmission rates and the relative proportion of the population that is susceptible to the disease (Ackerman et al.

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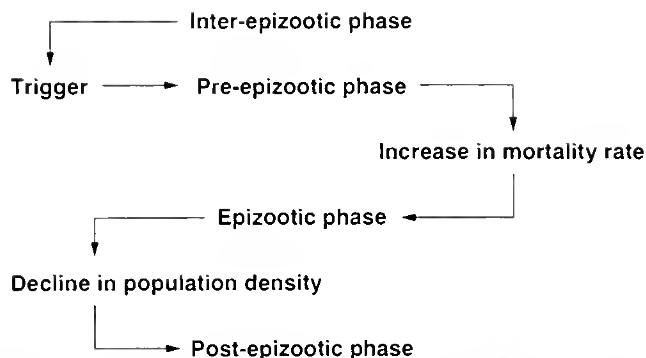


Figure 1. Schematic of the stages that are associated with an epizootic.

1984, Mollison 1987, Kermack and McKendrick 1991a,b, Anderson 1991, Dwyer and Elkinton 1993). Typical triggers for epizootics involve factors that vary contact rates, prevalences, and the behavior of susceptible individuals. *P. marinis*, however, presents a relatively unusual case. Disease prevalence in most oyster populations during the interepizootic phase is high. Thus, variations in disease transmission rates are of lesser consequence. Most oyster populations retain a balance between disease intensification and oyster population expansion that favors the oyster population and the breakdown of this balance initiates an epizootic.

The relative infrequency of epizootics in oyster populations routinely existing with disease prevalences of 80% or more suggests that controls exist that operate on the disease intensification process but not on the transmission process. One such control is suggested by experimental studies that have shown that the doubling time of *P. marinus* is reduced at high infection intensities. This permits oysters to survive at infection intensities that are only a few doublings from lethal levels (Saunders et al. 1993). Also, high fecundity rates, particularly in the spring and early summer when infection intensities are low in the younger, less heavily infected adults (Choi et al. 1994), result in the introduction of uninfected individuals into the population, which dilutes the mean disease intensity.

Under normal conditions, mortality from *P. marinus* infection does not exceed the rate of oyster population expansion and the oyster population remains healthy even though suffering substantial mortality from the disease. The infrequency of epizootics in *P. marinus*–infected oyster populations suggests that triggering mechanisms must exist that permit the rate of disease intensification to override the rates of oyster population expansion for a time. Once this threshold is crossed, the disease process is sufficient to reduce population growth and fecundity, and subsequently recruitment, so that the population slips inexorably toward extinction. Epizootics are generally associated with high salinities; however, other triggering mechanisms may exist. Generally, these should be factors that reduce oyster growth rate or fecundity or adversely affect the ability of the oyster to fight the disease.

Once started, an epizootic may prove to be difficult to stop because most progress until host mortality reduces the population density to a level that can no longer sustain the disease. This critical population density is normally determined by the transmission rate (Bartlett 1960, Black 1966, Mollison 1987, Anderson 1991). When transmission rate is high, as it is for *P. marinus*, this critical population density is usually very low. Thus, local extinction of the host population is a likely outcome of a *P. marinus* epizootic (e.g., Plowright 1982, Mollison 1987).

The objectives of this paper are to investigate the progression of *P. marinus* epizootics in oyster populations, the factors that trigger these epizootics, and the factors that can terminate an epizootic once it is started. These objectives are addressed using a coupled oyster population–*P. marinus* model. A series of simulations are presented that are designed to investigate the role of environmental factors, competition from other filter feeders, population recruitment rates, and disease resistance in triggering epizootics of *P. marinus*. Additional simulations consider the environmental and biological factors that can stop an epizootic. The simulations primarily use idealized time series of environmental variables designed to illustrate specific points. However, measured environmental conditions from Galveston Bay, Texas, a mid-latitude bay where *P. marinus* infects almost 100% of the oyster population, are used where appropriate.

THE OYSTER POPULATION—*PERKINSUS MARINUS* MODEL

General Characteristics

The host-parasite model (Fig. 2) consists of separate models for the dynamics of the postsettlement oyster population and the growth of *P. marinus*. The two models are coupled by relationships that describe the removal of oyster energy by the parasite to support its metabolic requirements and relationships that relate the rates of parasite division and mortality to host mortality. The oyster population model, described in detail by Hofmann et al. (1992, 1995) and Powell et al. (1992b, 1994, 1995a), consists of a size-structured model that considers the processes regulating the growth and death of the oyster from newly settled juveniles to adults. The description of this model will focus on only the modifications made to allow connection between the parasite and host components. The *P. marinus* model includes metabolic growth and loss processes as well as a component that describes the transmission of the disease. The parasite model, which uses infection level as the state variable, is described in detail following a brief review of the oyster population model.

Governing Equation

The time change in oyster standing stock ($O_{j,k}$) in each oyster size class (j) and *P. marinus* infection level (k) is the result of changes in net production ($NP_{j,k}$), which is the sum of the production of somatic ($Pg_{j,k}$) and reproductive ($Pr_{j,k}$) tissue, and the addition of individuals from the previous size class or loss to the next largest size class by growth. Oyster net production is assumed to be the difference between assimilation ($A_{j,k}$) and respiration ($R_{j,k}$), as discussed by White et al. (1988), and losses to *P. marinus* ($E_{j,k}$) and is expressed as:

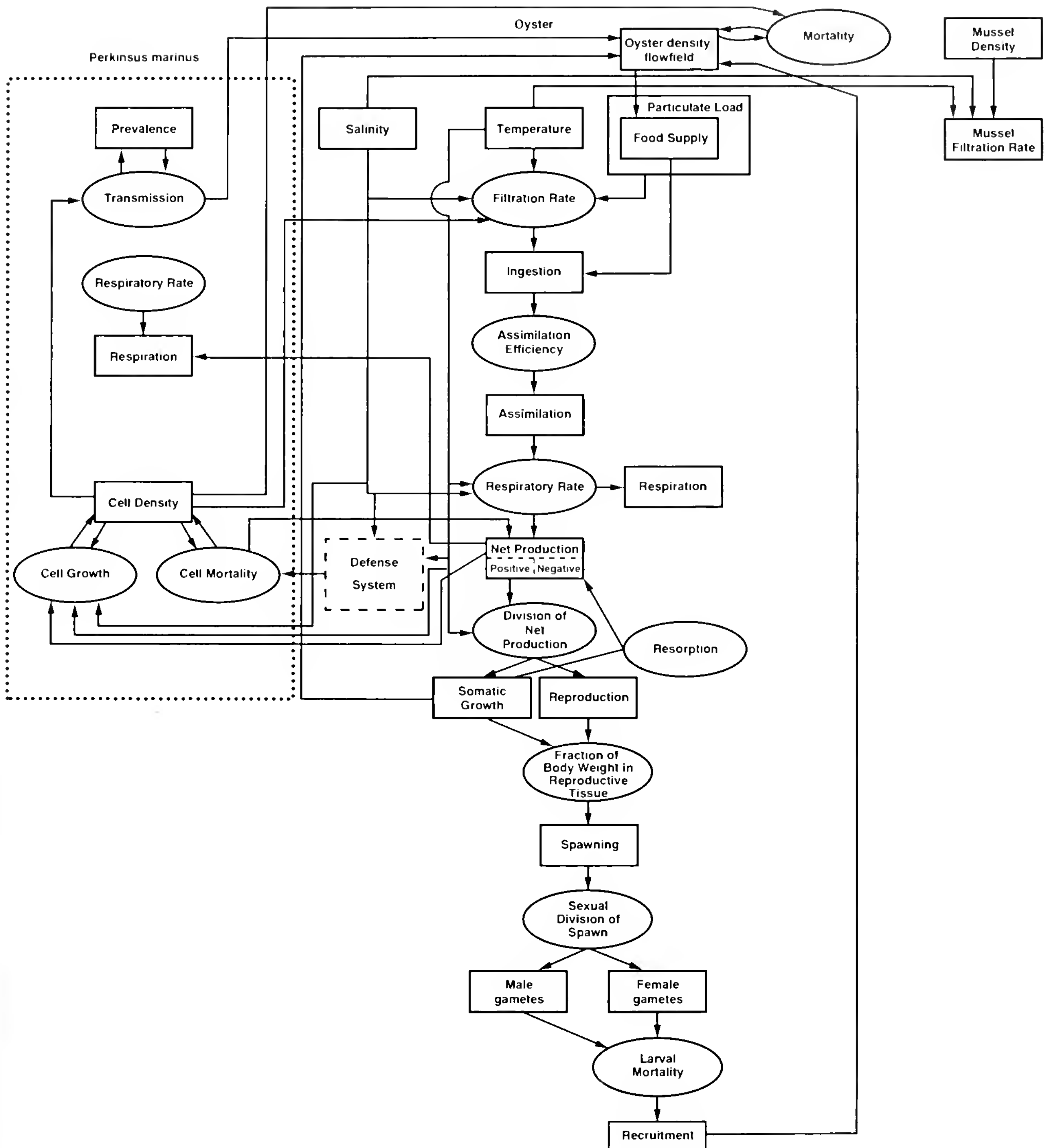


Figure 2. Schematic of the coupled oyster population–*P. marinus* population dynamics model.

$$NP_{j,k} = Pg_{j,k} + Pr_{j,k} = A_{j,k} - R_{j,k} - E_{j,k}. \quad (1)$$

The governing equation for the oyster population is then:

$$\frac{dO_{j,k}}{dt} = Pg_{j,k} + Pr_{j,k} + (\text{gain from } j - 1) - (\text{loss to } j + 1) \quad (2)$$

where $j = 1,11$, which represents the size class partitioning of the oyster life history. The last two terms on the right side represent gains and losses in a particular size class by individuals gaining biomass and moving to the next higher size class. Size is defined in terms of biomass (g AFDW) rather than length (Table 1). Reproductive tissue formation is zero for the first three size classes, which represent juveniles.

During suboptimal conditions, oysters can resorb gonadal or somatic tissue and hence lose biomass ($NP_{j,k} < 0$) and transfer into the next lower size class. Thus, biomass can change during periods of negative scope for growth, which is the basis for the use of

TABLE 1.

Biomass and length dimensions and lethal *P. marinus* density for the oyster size classes used in the model. Biomass is converted to length using the relationship given in White et al. (1988). Mortality level is defined as the number of *P. marinus* population doublings required to reach or exceed the lethal parasite density as calculated from equation (24).

Model Size Class	Biomass (g ash free dry wt)	Length (mm)	Mortality Level (population doublings required)
1	1.3×10^{-7}–0.028	0.3–25.0	21
2	0.028–0.10	25.0–35.0	23
3	0.10–0.39	35.0–50.0	24
4	0.39–0.98	50.0–63.5	25
5	0.98–1.44	63.5–70.0	26
6	1.44–1.94	70.0–76.0	26
7	1.94–3.53	76.0–88.9	26
8	3.53–5.52	88.9–100.0	27
9	5.52–7.95	100.0–110.0	27
10	7.95–12.93	110.0–125.0	27
11	12.93–25.91	125.0–150.0	27

condition index as a measure of health in oysters (e.g., Newell 1985, Wright and Hetzel 1985). To allow for this, the above equation is modified as:

$$\frac{dO_{j,k}}{dt} = Pg_{j,k} + Pr_{j,k} + (\text{gain from } j-1) - (\text{loss to } j+1) + (\text{gain from } j+1) - (\text{loss to } j-1) \quad (3)$$

The last two terms on the right side represent the individuals losing biomass and, thus, moving to the next lower size class.

The final modification to the oyster-governing equation allows oysters in any size class to increase or decrease in *P. marinus* infection intensity:

$$\frac{dO_{j,k}}{dt} = Pg_{j,k} + Pr_{j,k} + (\text{gain from } j-1) - (\text{loss to } j+1) + (\text{gain from } j+1) - (\text{loss to } j-1) + (\text{gain from } k-1) - (\text{loss to } k+1) + (\text{gain from } k+1) - (\text{loss to } k-1). \quad (4)$$

The last four terms represent changes in infection intensity of the oyster population as the *P. marinus* population increases or decreases in number. The model includes 28 predefined infection levels. Level 1 consists of uninfected oysters. The remaining 27 levels represent degrees of infection that correspond to the number of doublings of the *P. marinus* population beginning with one cell in level 2.

Three aspects of the model given by the above equation deserve note. First, settlement of juvenile oysters (as spat) occurs exclusively in the first size class ($j = 1$) and first infection level ($k = 1$). These newly recruited individuals are uninfected by *P. marinus*. Second, movement of oysters from the uninfected to the newly infected stage occurs by the acquisition of one infective cell (infection level 2) and occurs only in the positive direction, a gain of infection. Infections, once acquired by oysters, are never lost (Andrews 1988). Finally, once the oysters have reached the infection level defined as lethal (Table 1), they are classified as dead and both the *P. marinus* and the oyster biomasses are permanently lost from the population.

The gain, loss, or transfer of energy (or biomass) between size classes or across infection levels is expressed in terms of specific rates (d^{-1}), which are multiplied by the caloric quantity in the size or infection class. Transfers of oysters between size classes were scaled by the ratio of the average weight of the current size class (in g dry wt) to that of the size class from which energy was gained or to which energy was lost. This scaling, made necessary because the oyster size classes were unevenly distributed across the size-frequency spectrum, ensured that the total number of oyster individuals in the model was conserved, in the absence of recruitment and mortality, even though all calculations were done in terms of calories and biomass. A similar scaling is used for transfers between infection levels because these are not equivalent in dimension. Thus, each specific rate for each transfer was scaled by:

for transfers up: $W_j/(W_{j+1} - W_j)$
for transfers down: $W_j/(W_j - W_{j-1})$

where W is the median value for biomass (g AFDW) in the size class, or by the ratio of parasite number between infection classes:

for transfers up: $C_k/(C_{k+1} - C_k)$
for transfers down: $C_k/(C_k - C_{k-1})$

where C is the number of cells of *P. marinus* per individual. For simplicity, these scalings are not explicitly stated in the equations given in the following sections.

THE OYSTER MODEL

The model includes parameterizations for the processes that determine the production of somatic and reproductive tissue and thus the transfer between size classes. Specifically included are formulations for: assimilated ingestion as it depends on filtration rate, ambient food supply, and assimilation efficiency; filtration rate as a function of oyster size, temperature, salinity, turbidity, and current flow; respiration as it depends upon oyster size, temperature, and salinity; the apportionment of net production into somatic and reproductive growth as a function of temperature and time of year; the preferential resorption of gonadal tissue when $NP_{j,k} < O$; and spawning as a function of the total cumulative reproductive biomass and the male/female ratio. The relationships used for these processes are shown in Table 2.

The modifications made to the oyster population model so that it could be interfaced with the *P. marinus* model consisted of including the competing effect of mussels on oyster filtration and adding sources of natural mortality for the oyster populations. Larval mortality, mortality of the postsettlement population due to predation, and mortality due to low salinity were identified as the primary sources of natural mortality other than *P. marinus*. The mussel filtration effect and mortality sources were added to the model as follows.

Mussels

Under certain conditions, mussels can be an important competitor with oysters for available food resources (Medcof 1961). The degree of competition is a complex process which depends on population density, size frequency, food content, and current flow. In the oyster model, the effect of mussels is to increase total

community filtration rate, which is used in the calculation of available food supply as described in Table 2. Available food supply is determined by the initial food content, the community demand, and the rate of food replacement by current flow. Community filtration rate, α, is calculated as:

$$\alpha = \sum_{j=1}^{11} \sum_{k=1}^{28} F_{D_{j,k}} + \sum_{i=1}^{10} Fm_{T_i} \qquad (5)$$

where $\Sigma_{j=1}^{11} \Sigma_{k=1}^{28} F_{D_{j,k}}$ is the oyster population filtration modified by *P. marinus* as described below and $\Sigma_{i=1}^{10} Fm_{T_i}$ is the filtration of the mussel population summed over 10 mussel size classes, i, that ranged from 0 to 100 mm in 10-mm increments.

On Gulf of Mexico oyster reefs, *Brachidontes exustus* and *Brachidontes recurvus* are the principal mussel species. Few data exist for either species; therefore, the relationships used in the model to describe these species are based on those for bivalve molluscs and oysters. This approach assumes that the salinity and turbidity tolerances are somewhat similar for these co-occurring species.

Mussel weight is estimated from length using the bivalve length-to-biomass relationship given in Powell and Stanton (1985):

$$W_m = L_m^a \, 10^b \qquad (6)$$

where W_m is mussel dry weight in g and L_m is mussel length in mm. Coefficient values and definitions are given in Table 3. As was done for oysters, the relationship given in Doering and Oviatt (1986) for filtration rate was used with Hibbert's (1977) biomass-to-length relationship to obtain filtration rate as a function of biomass. Hence, the mussel filtration rate equation as a function of biomass is similar to the one shown in Table 2 for oysters. The primary difference is that the ash-free dry weight, in grams, used for each mussel size class is input to the above equation to obtain mussel length, which is then input to the filtration equation.

No data exist that describe the relationship between filtration rate of *Brachidontes* and salinity. Therefore, it was assumed that the relationship is similar to that for oysters. Thus, mussel filtration rate was specified using the salinity equations given in Table 2 for oysters. This allows filtration rate to decrease as the salinity drops below 7.5 ppt and to cease at salinities below 3.5 ppt.

Brachidontes spp. are rarely important at salinities above 14 ppt (Powell unpublished data for Galveston Bay, Baughman and Baker 1949). The observations from Galveston Bay further suggest that the salinity control on mussel population density is a step function at about 14 ppt, with little further effect as salinity rises above or declines below 14 ppt. Therefore, mussel density was set to zero at salinities above 14 ppt.

Total particulate content, at high concentrations, may also reduce mussel filtration rate. Again, data sufficient to describe this for *Brachidontes* are lacking. Therefore, the relationships for oysters, which are given in Table 2, were assumed to also apply to mussels.

Oyster Mortality

While in the plankton, oyster larvae undergo considerable mortality from a variety of sources, which reduces the number of individuals that are recruited to the postsettlement population from a spawn. Oyster larval mortality was included using a linear relationship of the form:

$$\text{number of larvae recruited spawn}^{-1} = s(\text{number of eggs spawned}) \qquad (7)$$

where s determines the rate at which individuals are lost per spawn. No attempt is made to differentiate among the many sources of planktonic mortality.

Natural mortality of the postsettlement population was also specified with a linear relationship of the form:

$$M_p = k_p(\text{number of living oysters}) \qquad (8)$$

where M_p is the number of individuals that die in a given time interval and k_p is the daily mortality rate (d^{-1}). As with larval mortality, this approach does not differentiate among the many sources of oyster mortality. For the simulations presented here, this relationship was used to produce mortality for the juvenile oyster size classes to complement the adult mortality produced by *P. marinus*.

Low salinity is a principal cause of catastrophic mortality of postsettlement oyster populations during some flood years (Hofstetter 1977, Ray 1987, Soniat and Brody 1988). Wells (1961) and Chanley (1957) provide survivorship data at low salinity for temperatures greater than 20°C, which show that salinities lower than 6 ppt produce mortality at summer temperatures and that the rate of mortality rises as salinity declines below 6 ppt. Additionally, observations given in Gunter (1953) and those from Galveston Bay (Powell unpublished data) show that oyster survivorship increases substantially at low salinity as temperature declines. Therefore, the temperature-dependent mortality produced by salinities lower than 6 ppt was modeled as:

$$M_s = k_s(\text{number of living oysters}) \qquad (9)$$

where M_s is the number dying per time and k_s in d^{-1} is given by

$$k_s = (\alpha_1 S + \beta_1)T + (\alpha_2 S + \beta_2). \qquad (10)$$

Salinity, S, is given in ppt and temperature, T, is in °C. Coefficient values for the above equations are given in Table 2.

THE *PERKINSUS MARINUS* MODEL

The *P. marinus* model includes processes that govern parasite growth and mortality, those that determine the energy demand of the parasite on the host, and those that affect the physiology of the host. The relationships used to describe these processes are given in the sections that follow.

Perkinsus marinus ***Growth***

Cell division time is the time between one cell division and the next for an individual cell. The population doubling time, however, depends upon the balance between the rate of cell division and the rate of cell mortality. For *P. marinus*, cell mortality is likely mediated in some way by the defense system of the oyster (Saunders et al. 1993). In the *P. marinus* population model, the biology of the parasite and the processes determining the rate of parasite division are treated separately from those that describe the oyster's defense system and the rate of parasite mortality.

Measurements of *P. marinus* division time are limited and the effects of temperature, salinity, and cell density are poorly known. However, information from Ray (1954) and Mackin and Boswell (1954) suggests that the parasite division times range from 7 to 60 hours. More recently, Choi et al. (1989) estimated a doubling time of 7 hours. The fastest rate of division, at low parasite density,

TABLE 2.

Equations and relationships used in the oyster population dynamics model. Complete discussions of these are given in Powell et al. (1992b, 1994, 1995a) and Hofmann et al. (1992, 1995).

Equations	Comments and Parameter Definitions
Filtration rate and water flow	Flow limitation on food supply is calculated using a volume of water over the bottom with length and width, L, and height, h
$\frac{\partial F}{\partial t} + \frac{\partial (uF)}{\partial x} + \frac{\partial (wF)}{\partial z} - \frac{\partial^2 (AF)}{\partial z^2} + \alpha FO = 0$	F, food u, horizontal advective velocity w, vertical advective velocity A, vertical diffusion coefficient α, total filtration rate summed over all oyster size classes, $\sum_{i=1}^{n} FR_{D,i}$ O, oyster biomass partial derivatives indicate changes in time (t) and in the horizontal (x) and vertical (z) directions
$\frac{\partial u}{\partial x} + \frac{\partial w}{\partial x} = 0$	Continuity equation
Characteristic velocity profile	
$u(x,z) = u_o(x)\ln(z/z_o)$	$u_o(x)$, a specific horizontal speed at height, $z = h$ z_o, bottom roughness parameter = 10% of height of oyster clumps
$u_o(x) = \bar{u}(x)/\ln(h/z_o)$	$\bar{u}(x)$, the specified speed
Food profile	
$F = F_o(t) + (x/L)F_1(t)$	Food assumed to be independent of height and a linear function of distance across the box. F_o, F_1 are food concentrations at the upstream and downstream boundaries of the volume of interest
Food calculation	
$F_o(t = 0) = F_o(t = \Delta t) = F_{oo}$	F is integrated over the volume, and the average amount of food in the box during one time step is calculated by differencing over time. F_{oo}, the specified food concentration
$F_1(t = 0) = 0$	
$F_1(t = \Delta t) = F_1^1 = [-2\alpha \Delta t F_{oo} 0^{\circ} + (\Delta t/hL)(D^1 + D^{\circ})]/$ $[1 + 0.5\alpha\Delta t 0^{\circ} + [(\bar{u}(L) + \bar{u}(0))(\Delta t/2L)][1 - 1/\ln(h/z_o)]]$	
Food reduction factor	
$F_{red} = (F_{oo} + 0.25F_1^1)/F_{oo}$	F_{red} is the fraction by which the food concentration is reduced
Food content	
$f^* = F_{red}F_{oo}$	f^* is the available food
Conditions for simulation	Box length, L = 1 m Thickness of bottom flow, h = 5.4 cm
Filtration rate as a function of temperature	
$FR_j = \frac{L_j^{0.96} T^{0.95}}{2.95}$	Filtration rate (FR_j) in ml filtered ind^{-1} min^{-1} by a particular oyster size, j, length (L_j) obtained from W_j, the ash-free dry weight in **g**; T, temperature
$L_j = W_j^{0.317} 10^{0.669}$	
Filtration rate as a function of ambient salinity, S	
$FR_{s_j} = FR_j$	at $S \geq 7.5$ ppt
$FR_{s_j} = FR_j(S - 3.5)/4.0$	at $3.5 < S < 7.5$ ppt
$FR_{s_j} = 0$	at $S \leq 3.5$ ppt
Filtration rate as a function of turbidity	
$\tau^* = \tau_{red}\tau_{oo}$	Calculated similarly to f^*
$\tau = (4.17 \times 10^{-4})(10^{0.0418x})$	τ, total particulate content (inorganic + organic) in g l^{-1}; x, the percent reduction in filtration rate
$FR_{\tau_j} = FR_{s_j}\left[1 - 0.1\left(\frac{\log_{10}\tau + 3.38}{0.0418}\right)\right]$	Filtration rate with turbidity effects
Ingestion	
$I_{j,k} = f^* FR_{D_{j,k}}$	Ingestion rate (I) as a function of food concentration and filtration rate
Assimilation	
$A_{j,k} = I_{j,k}A_{eff}$	A_{eff}, assimilation efficiency
Respiration as a function of temperature	
$R_j = (69.7 + 12.6T)W_j^{b-1}$	Respiration rate (R_j) for a particular oyster size class in µl O_2 consumed hr^{-1} g dry wt^{-1}; b = 0.75
Respiration as a function of salinity	
$R_r = 0.007T + 2.099$	at $T < 20°C$

TABLE 2.

continued

Equations	Comments and Parameter Definitions
$R_r = 0.0915T + 1.324$	at $T \geq 20°C$
$R_{T_j} = R_j$	at $S \geq 15$ ppt
$R_{T_j} = R_j/(1 + [(15 - S)(R_r - 1)/5])$	at 10 ppt $< S <$ 15 ppt
$R_{T_j} = R_j R_r$	at $S \leq 10$ ppt
Reproduction, R_f:	
Juvenile/adult boundary	0.39 g ash-free dry weight, about 50 mm
$Pr_{j,k} = R_{eff_j} NP_{j,k}$	Reproductive tissue development for a given oyster size class as a function of reproductive efficiency, R_{eff_j}, and total net production, $NP_{j,k}$
$R_{eff_{j,k}} = 0.054T(t) - 0.729$	Reproductive efficiency temperature dependence for January to June
$R_{eff_{j,k}} = 0.047T(t) - 0.809$	Reproductive efficiency temperature dependence for July to December
when $NP_{j,k} < 0$	Preferential resorption of gonadal tissue
$R_{f_{j,k}} = 0.20\ O_{j,k}$	Spawning occurs when the reproductive biomass exceeds 20% of total oyster biomass
$f_{ratio} = 0.021\ L_b - 0.62$	f_{ratio}, the ratio of females to males, L_b, length in mm
number of eggs spawned $= R_{f_{j,k}} \left(\frac{1}{C}\right) \left(\frac{1}{W_{egg}}\right)$	Number of eggs spawned, C is number of calories per egg, W_{egg} is egg weight
$W_{egg} = 2.14 \times 10^{-14}\ V_{egg}$	V_{egg}, oyster egg volume
Larval recruitment	Larval planktonic time assumed to be 20 days
Larvae mortality	
Number of larvae recruited spawn^{-1} = s (number of eggs spawned)	s, the mortality rate, in spawn^{-1}
Postsettlement population natural mortality	
$M_p = k_p$(number of living), for $j = k,l$	M_p, the number dying time^{-1} k_p, the daily mortality rate (d^{-1}), k and l, the inclusive size classes being affected by mortality
Postsettlement salinity mortality	
$M_s = k_s$ (number of living)	M_s, the number dying time^{-1}
$k_s = (\alpha_1 S + \beta_1)T + (\alpha_2 S + \beta_2)$	K_s, daily mortality rate (d^{-1}) $\alpha_1 = -0.000348$ $\alpha_2 = 0.00232$ $\beta_1 = 0.01764$ $\beta_2 = -0.3089$ S, ambient salinity (ppt) T, ambient temperature (°C)
Caloric conversions	
Oysters	6100 cal (g dry wt)$^{-1}$
Food	5168 cal (g dry wt)$^{-1}$
Oyster eggs	6133 cal (g dry wt)$^{-1}$

observed by Saunders et al. (1993) ranged between 4 and 10 hours at 30°C and 17 ppt.

Given the limited observations on *P. marinus* growth *in vivo*, this process was modeled using standard relationships for temperature and salinity dependencies which were calibrated by comparing the simulated growth of *P. marinus* to data sets that provide observations of the seasonal dependency of parasite infection intensity as salinity and temperature change. These data came from April Fools Reef in Galveston Bay, TX (Soniat 1985), Biloxi Bay, MS (Ogle and Flurry 1980), and North Inlet, SC (Crosby and Roberts 1990).

Temperature control on the specific rate of parasite division, $r_d(T)$, was assumed to follow a standard exponential form:

TABLE 3.

Coefficient definitions and values for the mussel model.

Coefficient	Definition	Value	Units
Fm_τ	Mussel filtration rate	Calculated	ml mussel^{-1} min^{-1}
W_m	Mussel weight	Calculated	g dry wt
L_m	Mussel length	Assigned	mm
a	Mussel weight scaling factor	−4.8979	No units
b	Mussel weight scaling factor	2.8734	No units

$$r_d(T) = r_{d0}\, e^{\alpha(T(t) - T_0)}. \tag{11}$$

To calibrate equation (11), a known division rate at a given temperature is needed. Observations of field populations suggest that infection intensity begins to rise in most populations when the temperature exceeds 20°C and the salinity exceeds 20 ppt. Therefore, the 20°C–20 ppt boundary was used to standardize parasite division and mortality rates. At 20°C and 20 ppt, parasite division should just balance loss (Ray 1954, Mackin 1962, Andrews 1988). The division time at 20°C and 20 ppt was set at 30 hours by comparing simulated distributions to those in Soniat (1985), Ogle and Flurry (1980), and Crosby and Roberts (1990). This division time is within the ranges of those reported from the limited laboratory and *in vivo* measurements. A Q_{10} of 2.0, which is consistent with measurements for *P. marinus* (Chu and Greene 1989), is used to calculate a parasite division rate at temperatures other than 20°C. The coefficients and their values thus determined for equation (11) are defined in Table 4.

The rate of parasite division is independent of salinity except at and below 10 ppt (Chu and Greene 1989, Ragone and Burreson 1993). Thus, for salinities (S) below 10 ppt, equation (11) is modified as:

$$r_d(T,S) = r_{d1}\left(\frac{S}{10}\right)e^{\alpha(T(t) - T_0)} \tag{12}$$

where coefficient definitions and values are given in Table 4. This relationship provides a decrease in parasite division rate at low salinity but retains the temperature relationship.

Simulations of *P. marinus* population dynamics using equation (12) in the oyster–*P. marinus* model resulted in parasite growth rates and densities that were too high relative to those suggested by field measurements in Soniat (1985), Ogle and Flurry (1980), and Crosby and Roberts (1990) under the appropriate environmental constraints (Hofmann et al. 1995). Most measurements of protozoa in culture show that parasite division rate decreases at high population densities as food becomes limiting (Hall 1967). A similar response by *P. marinus* is suggested by *in vivo* experiments in which the rate of DNA production by *P. marinus* at various parasite densities declined at high densities (Saunders et al. 1993). Also, a decrease in hemolymph protein in oysters has been noted during summer months when *P. marinus* infection intensity is high (Chintala and Fisher 1991) and as a result of MSX infection (Ford 1986). Using the measurements from Saunders et al. (1993), an

TABLE 4.

Coefficient definitions and values for the *P. marinus* population model.

Coefficient	Definition	Value	Units
$r_d(T)$	Specific rate of parasite division	Calculated	d^{-1}
r_{d0}	Base specific parasite division rate	0.555	d^{-1}
α	Q_{10} conversion	0.06931	$°C^{-1}$
T_0	Base temperature for parasite division rate	20	°C
S_0	Base salinity for parasite division rate	20	ppt
r_{d1}	Base specific parasite division rate	0.555	d^{-1}
β	Parasite density scaling factor	2.454×10^{8}	g AFDW $cell^{-1}$
C_k	Parasite number	Calculated	Number of cells
W_j	Oyster weight	Table 1	g AFDW
γ	Parasite density scaling factor	−1.5	No units
$r_m(T,S)$	Specific parasite loss rate	Calculated	d^{-1}
r_{m0}	Base specific parasite loss rate	0.555	d^{-1}
δ	Q_{10} conversion	0.08153	$°C^{-1}$
Ec	Total *P. marinus* energy demand	Calculated	cal d^{-1}
Eg	Energy for *P. marinus* population increase	Calculated	cal d^{-1}
Er	Energy for *P. marinus* respiration demand	Calculated	cal d^{-1}
El	*P. marinus* mortality	Calculated	cal d^{-1}
ϵ	Conversion	1.16×10^{4}	hr cal d^{-1} nl^{-1}
D	Average parasite cell diameter	8	μm
ζ	Conversion	9.57×10^{-10}	cal μm^{-3}
ω	Respiration scaling factor	−4.09	ml hr^{-1} μm^{-3}
θ	Respiration scaling factor	0.75	No units
κ	Filtration scaling factor	0.58	No units
λ	Filtration scaling factor	579	No units
μ	Conversion	-2.287×10^{-4}	g AFDW $cell^{-1}$
$FR_{D_j,k}$	Filtration rate, infected oyster	Calculated	ml $oyster^{-1}$ min^{-1}
C_{L_j}	Lethal parasite density	Calculated	Cells $oyster^{-1}$
ν	Mortality scaling factor	2.057	No units
z	Weight scaling factor	1.3258×10^{-7}	g AFDW
q	Weight scaling factor	0.2625	No units
σ	Mortality scaling factor	3.2	No units
υ	Weight conversion factor	5	g wet wt (g dry wt)$^{-1}$
τ	Infection level scaling factor	1409.9	Cells (g wet wt)$^{-1}$
ϕ	Infection level scaling factor	0.64296	No units
r_t	Specific rate of transmission	Calculated	d^{-1}
r_{tb}	Base specific interpopulation transmission rate	0.2	y^{-1}
r_{t0}	Base specific intrapopulation transmisson rate	12	y^{-1}

empirical relationship that modifies the specific parasite division rate at high parasite density, $r_d(\rho)_{j,k}$, was derived as:

$$r_d(\rho)_{j,k} = \beta r_d(T,S)\left(\frac{C_k}{W_j}\right)^{\gamma} \quad (13)$$

where $r_d(T,S)$ is determined from equation (12). Coefficient definitions and values are given in Table 4. In the model, the parasite division rate that is used is the minimum of that determined from equations (12) and (13).

Perkinsus marinus *Mortality*

Mortality of *P. marinus* is presumably a result of the oyster defense system response. Thus, parasite mortality was parameterized using data obtained for hemocytes, which are an important component of the oyster's defense mechanism (Fisher 1988). These data show that parasite mortality is temperature and salinity dependent. Moreover, field (Soniat 1985, Burrell et al. 1984) and laboratory (Fisher et al. 1992) observations show that the effect of salinity on parasite mortality is discernible only at high temperatures. One explanation for this is that hemocytes are already maximally active at low temperature so that salinity changes have little effect. However, at higher temperatures where hemocyte activity is reduced, some capability is recovered when the oyster is exposed to low salinity. Thus, a temperature- and salinity-dependent relationship for the specific parasite mortality rate, $r_m(T,S)$, was obtained using measurements of hemocyte activity reported in Fisher and Newell (1986), Fisher and Tamplin (1988), Fisher et al. (1989, 1992), and Chintala and Fisher (1991) as:

$$r_m(T,S) = r_{m0}\, e^{-\delta\left(\frac{S(t)}{S_0}\epsilon\right)}\, e^{-\delta\left(\frac{T_0-10}{S_0}\right)(S(t)-S_0)}. \quad (14)$$

where ϵ is the larger of the temperatures at 10°C or the difference between the ambient temperature and the base temperature of 20°C, i.e., $\max(10°C, T(t) - T_0)$. The definitions and values for the coefficients in the above equation are given in Table 4. The value used for δ was obtained by applying a Q_{10} of 2.26 to the base mortality rate. The specific parasite mortality rate assumes no reduction in hemocyte activity at extreme low salinity. Ford and Haskin (1988) found active hemocytes down to 6 ppt, and oyster mortality from low salinity begins at lower salinities.

As with parasite division, mortality should also be dependent on parasite density. As parasite density increases, the effectiveness of the defense system should decrease. Anderson et al. (1992) showed that the number of hemocytes in heavily infected oysters is only about double that in lightly infected oysters, whereas the number of *P. marinus* cells is a factor of 1000 or more higher. Thus, the relative activity of the hemocytes must decline at high parasite density. Measurements sufficient to exactly describe the relationship between parasite concentration and mortality are not available. Hence, the parasite density effect on mortality rate was assumed to follow the same relationship as was used for the parasite density effect (equation 13) on parasite division rate:

$$r_m(\rho)_{j,k} = \beta r_m(T,S)\left(\frac{C_k}{W_j}\right)^{\gamma}. \quad (15)$$

Coefficient definitions and values are given in Table 4. The specific parasite mortality rate was taken to be the minimum of the rate calculated using equations (14) and (15). It is assumed that no *P. marinus* mortality occurs as a direct result of extremes in temperature and salinity. Available data suggest that *P. marinus* is as resistant to environmental extremes as its oyster host (Goggin et al. 1990) and calibrating simulations of *P. marinus* growth against existing data sets did not require an additional mortality source beyond that provided by the host's defense system (Hofmann et al. 1995).

Perkinsus marinus *Energy Demand*

The *P. marinus* population depends on the oyster host to provide sufficient energy to support parasite respiration and growth. Thus, the energy requirement of the parasite population, Ec, can be expressed as:

$$Ec = Eg + Er - El \quad (16)$$

where Eg is the energy required to increase the population biomass through parasite division and Er is the energy requirement for population respiration. The last term on the right of equation (16), El, represents the return of energy to the host from the parasite which occurs through parasite mortality. Although hemocyte exomigration (Cheng 1983) might limit the importance of El, exomigration is not included in the model. The terms in the above equation are formulated as described below.

The energy requirement for population growth is defined by $Eg - El$ and is determined by the net change in parasite number ($C_{j,k}$) in the *P. marinus* population in a specific time interval. This is calculated from the difference in the specific parasite division and mortality rates as:

$$\frac{dC_{j,k}}{dt} = (r_d(\rho)_{j,k} - r_m(\rho)_{j,k})\, C_{j,k}. \quad (17)$$

The change in parasite number in a time interval, $\Delta C_{j,k}$, obtained from the above equation is converted to calories exchanged between the parasite and its host by:

$$Eg_{j,k} - El_{j,k} = \epsilon\, V\, \Delta C_{j,k} \quad (18)$$

where ϵ is a conversion factor obtained by assuming that 5 g wet weight is equivalent to 1 g dry weight and that 20 joules is equivalent to 1 mg dry weight (Laybourn-Parry 1987). Parasite cell volume, V, is calculated as:

$$V = \frac{4}{3}\pi\left(\frac{D}{2}\right)^3. \quad (19)$$

The average cell diameter, D, is from Ray (1954). Coefficient values and definitions are given in Table 4.

The respiratory energy required by the *P. marinus* population is obtained from:

$$Er_{j,k} = \zeta\, e^{(\alpha(T(t) - T_0))}\, 10^{\omega} V^{\theta} C_{j,k} \quad (20)$$

where the conversion factor, ζ, assumes 4.83 ml O_2 per calorie (Powell and Stanton 1985). The exponents ω and θ, which scale respiration rate to parasite cell volume, are from measurements made for protozoa (Fenchel and Finlay 1983). The value for α assumes a Q_{10} of 2 (Laybourn-Parry 1987). The effect of salinity on *P. marinus* respiration rate is unknown and therefore is not included. Coefficient values and definitions are given in Table 4.

Effects of Perkinsus marinus *on Oyster Physiology*

The primary effects of *P. marinus* infection on oysters are to reduce oyster filtration rate (Lund 1957) and eventually cause host mortality. Although increased predation is frequently described as a product of parasitism (Jakobsen et al. 1988, Hadeler and Freed-

man 1989, Schmid-Hempel and Schmid-Hempel 1988), no evidence exists for selective predation of *P. marinus*–infected individuals. Thus, selective predation is not included in the model. Also, the possible loss of *P. marinus* during spawning (Dungan and Roberson 1993) is not included.

Mackin and Ray (1955) provide measurements of *P. marinus* that can be used to derive a relationship that describes the reduction in oyster filtration rate with infection intensity. These measurements show an exponential decrease in oyster filtration rate that depends on the ratio of the number of cells of the parasite to the size (weight) of the host. This reduction in filtration can be expressed as:

$$Dred_{j,k} = \frac{\kappa}{\lambda e^{\mu \frac{C_k}{W_j}} + 1} \quad (21)$$

Coefficient definitions and values are given in Table 4. The expression given in equation (21), when applied to the oyster filtration rate, FR_{τ_j}, defined in Table 2, results in a fractional reduction in filtration rate as:

$$FR_{D_{j,k}} = FR_{\tau_j}(1 - Dred_{j,k}) \quad (22)$$

where $FR_{D_{j,k}}$ is the filtration rate that results when the oysters are infected with *P. marinus*.

The level of *P. marinus* infection in an oyster population is typically diagnosed in terms of a 0- to 5-point scale that was developed by Mackin (1962), with 5 being the heaviest infection level. Field and laboratory measurements show that oyster mortality generally occurs in individuals that have an infection intensity that corresponds to a 5 on this scale (Andrews 1988). Populations with mean infection intensities of 3 or more generally suffer 50 to 75% mortality per year (Ray and Chandler 1955, Mackin 1961, Mackin and Hopkins 1961). These observations provide a basis for determining the lethal *P. marinus* infection level in the simulated oyster populations.

A relationship was developed between host mortality, host size, and *P. marinus* number by assuming that host mortality occurs when the energy demand of the *P. marinus* population is some fraction of the host's net production. This relationship is based on net production values calculated for uninfected oysters as described by White et al. (1988) and is of the form:

$$\frac{2NP_{w_j}}{Ec} = C_{L_j} \quad (23)$$

where NP_{w_j} is net production, Ec is the caloric requirement of the *P. marinus* population as determined from equation (14), and C_{L_j} is the lethal parasite density (cells oyster^{-1}) for any oyster size class, j.

The above equation allows for a size dependency in lethal parasite density that is suggested by measurements given in Choi et al. (1989) and is consistent with a size-dependent scope for growth in oyster populations (Hofmann et al. 1992). The factor of 2 used in equation (21) was determined empirically by using yearly mortality rates of 90, 50, and 10% for the market-size population and comparing the resulting simulated populations with oyster populations reported in the field studies by Ogle and Flurry (1980), Soniat (1985), and Crosby and Roberts (1990).

The lethal parasite density from equation (23) can then be related to oyster size through a regression of the form:

$$C_{L_j} = 10^{\left(\upsilon \log_{10}\left(\left(\frac{W_j}{z}\right)^q + \sigma\right)\right)} \quad (24)$$

Note that because equation (24) is obtained from a regression, the units on the two sides of the equation are not equivalent. Coefficient definitions and values are given in Table 4.

Assuming that *P. marinus* infections are initiated by one cell, then depending on oyster size, 22 to 27 population doublings are needed to reach the lethal density. Smaller oysters require fewer population doublings to reach the lethal parasite level. As required by field observations, the above equation yields a value of 5 on Mackin's Scale when converted according to Choi et al. (1989) as:

$$C_{L_j} = \upsilon\tau(10^{\phi M})W_j \quad (25)$$

where M is the Mackin's Scale infection intensity as defined by Craig et al. (1989). Coefficient values and definitions are given in Table 4.

Equation (24) is consistent with the suggestion that oyster mortality could be at least partly explained by a negative energy budget produced when the energy demand of *P. marinus* exceeds the assimilation rate of the oyster (Choi et al. 1989). However, the exact mechanism by which *P. marinus* causes mortality of the oyster host is unknown, and some studies have reported significant effects on the host at lower infection levels (e.g., Paynter and Burreson 1991). The justification for using the approach given above comes from favorable comparisons between simulated and observed levels of *P. marinus* infection under equivalent environmental conditions (Hofmann et al. 1995).

Perkinsus marinus ***Transmission***

The available studies of the transmission of *P. marinus* indicate that oyster density and distance between infected host populations affect the rate of infection (Andrews and Ray 1988, Ford 1992, Mackin 1952). However, little information on the transmission of this disease from controlled experiments is available (Andrews 1965, 1988). Therefore, the transmission of *P. marinus* was modeled using general relationships for disease transmission. These formulations were then calibrated against field data.

The specific rate of infection of uninfected oyster individuals, r_t, was assumed to be the result of an interpopulation transmission rate, r_{tb}, and an intrapopulation specific transmission rate, r_{t0}, as:

$$r_t = r_{tb} + r_{t0}\left(\frac{P_1 + P_2 + P_3}{3}\right) \quad (26)$$

where P_1, P_2, and P_3 are factors that modify the intrapopulation transmission rate.

Insufficient data were available to include the expected relationship between oyster filtration rate and *P. marinus* transmission rate as occurs in other host-parasite systems (e.g., Gee and Davey, 1986). This effect could be important at higher latitudes where filtration ceases during the winter, thus limiting transmission rate. However, the decrease in *P. marinus* prevalence and infection intensity produced by the effects of low temperature on parasite growth and mortality, that occur during the winter, should minimize any error due to exclusion of this effect. Also, a suspected influence of salinity on *P. marinus* transmission rate (Paynter and Burreson 1991, Chu and La Peyre 1993) is not included in the model.

The interpopulation infection intensity was determined by using observations from San Antonio Bay, TX, obtained as part of the NOAA National Status and Trends program. A catastrophic

flood produced 100% mortality of oysters in this bay in 1988. As the bay recovered, the infection intensity and prevalence of *P. marinus* were monitored in the oyster population. These observations showed that *P. marinus* infection returned to regional norms in about 2 years. Simulations of this event required an interpopulation infection intensity (r_{tb}) of 0.2 y^{-1}. Field experiments by Paynter and Burreson (1991) yielded similar results.

Three variables—oyster density, *P. marinus* prevalence, and *P. marinus* infection intensity—were used to determine the intrapopulation transmission rate. Factors affecting the intrapopulation transmission rate were formulated as follows. The prevalence of infection in a population varies between 0 and 1, where 0 represents an uninfected population and 1 represents a population in which all individuals are infected. At each time step in the model the fraction of the total population that was infected with *P. marinus* was calculated and this value was used to specify P_1 as:

$$P_1 = \textit{fraction infected} \tag{27}$$

Mean population infection intensities of 3.5 and above on Mackin's Scale are associated with substantial oyster mortality. Mortality should maximize transmission rate by releasing infective elements into the water column where they are transmitted to other individuals. Thus, P_2 was specified by establishing a ratio between the total parasite density, *TCD*, in the simulated oyster population and the parasite density that corresponds to an infection level (*IL*) of 3.5. Limiting the maximum value of this ratio to 1 yields a maximum transmission rate at all population infection intensities $\geq$3.5 of:

$$P_2 = \min\left(1., \frac{TCD}{IL}\right) \tag{28}$$

where

$$TCD = \frac{\Sigma_{k=1}^{28} C_k}{\Sigma_{j=1}^{11} = O_j\nu} \tag{29}$$

and *IL* corresponds to 2.5×10^5 cells (g wet wt)$^{-1}$.

The proximity of oyster individuals to one another also affects the rate of disease transmission. This effect is included by comparing the total simulated oyster population density with a relatively high oyster population density and limiting this value to a maximum of 1 as:

$$P_3 = \min\left(1., \sum_{j=1}^{11}\sum_{k=1}^{28}\frac{O_{j,k}}{OD}\right) \tag{30}$$

where *OD* is 4000 oysters m^{-2} (May 1971, Dame 1976).

A dense, heavily infected population $[(P_1 + P_2 + P_3)/3 = 1]$ should produce an intrapopulation transmission rate that is capable of infecting all uninfected individuals within 6 months. To achieve this effect, the maximum intrapopulation transmission rate (r_{t0}) was set to 12 y^{-1}.

Model Implementation

The oyster and *P. marinus* model described above requires input of environmental measurements that describe ambient food supply, turbidity level, current flow velocity, salinity, and temperature conditions. For this study, time series of these data were constructed to illustrate specific environmental effects. In all cases, the structure of the environmental time series was based on measurements made in Galveston Bay, TX. The time series consist of monthly averaged values that extended for 1 year. The various environmental time series are given in Table 5. The specific combination of the environmental series for the different simulations is given in Table 6.

The oyster population–*P. marinus* model was solved numerically using an implicit (Crank-Nicolson) tridiagonal solution technique with a 1-day time step. All simulations began on January 1 (Julian day 1) and ran for 6 years. This amount of time was sufficient for the oyster and parasite population to adjust to the environmental forcing. Each simulation was initialized with an oyster size-frequency distribution obtained from a reef, South Deer Island, in the West Bay section of Galveston Bay, TX, in spring 1992 (Fig. 3). The initial density of the individuals in the oyster population was set at 20 individuals m^{-2}. The mussel size-frequency distribution used in the model is also from Galveston Bay, TX, and is given in Table 5. Initially, *P. marinus* was specified to be at 50% prevalence in each oyster size class. This allowed the simulated populations to more rapidly come into equilibrium with environmental conditions than would occur using 0 or 100% prevalence.

The simulated distribution of *P. marinus* in the oyster population depends on the rate of larval recruitment and juvenile mortality because new recruits, being uninfected, reduce prevalence and population infection intensity. In most of the simulations, obtaining *P. marinus* prevalence and infection intensities that were comparable to observed values required a larval survivorship of 1 individual in 10^8 larvae spawned and an independent (non–*P. marinus*) source of juvenile mortality yielding a 1% survivorship the first year after settlement. Both survivorship rates are typical of those reported for bivalves (Brousseau et al. 1982, Powell et al. 1984, Cummins et al. 1986). Other survivorship rates were used as indicated in Table 6.

MECHANISMS FOR STARTING AN EPIZOOTIC

A Growing, Parasitized Oyster Population

The first simulation with the oyster-parasite model was designed to provide a reference against which simulations considering factors that produce epizootics can be compared. The reference simulation was configured to represent conditions in Galveston Bay, TX. Galveston Bay supports a substantial oyster fishery in most years and is currently in a phase of significant oyster reef expansion (Powell et al. 1995b). Food supply throughout the bay is adequate to support the present oyster population; however, a 15% decrease in food supply would restrict population growth (Powell et al. 1995a). Other environmental factors, such as temperature and salinity, are usually within ranges that are conducive to oyster growth to market size (76 mm). The specific conditions used for the reference simulation are given in Tables 5 and 6.

P. marinus prevalence in Galveston Bay oyster populations normally exceeds 90% (Powell et al. 1992a), and significant yearly *P. marinus*–produced mortality, frequently in excess of 50% of the market-size portion of the population, can occur. However, epizootics rarely occur and oyster populations normally exist in quasiequilibrium with *P. marinus* such that prevalence remains high and mortality remains moderate. Hence, limitations on growth of the oyster population tend to be from *P. marinus*–induced disease, the vagaries of larval survival, and predators such

TABLE 5.

Environmental time series used as input to the oyster population–*P. marinus* model.

Food Supply Time Series (mg l^{-1})											
Summer bloom (SB)—after Hofmann et al. (1992)											
Jan	Feb	Mar	Apr	May	June	July	Aug	Sep	Oct	Nov	Dec
0.50	0.50	0.75	0.75	1.25	1.25	1.25	1.25	0.75	0.75	0.50	0.50
Summer bloom—reduced winter food (LW)											
Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
0.25	0.25	0.75	0.75	1.25	1.25	1.25	1.25	0.75	0.75	0.25	0.25
Summer bloom—reduced summer food (LS)											
Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
0.50	0.50	0.75	0.75	1.00	1.00	1.00	1.00	0.75	0.75	0.25	0.25
Summer bloom—increased summer food (HS)											
Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
0.50	0.50	0.75	0.75	2.00	2.00	2.00	2.00	0.75	0.75	0.50	0.50
Turbidity Time Series (g l^{-1})											
High-turbidity event (HT)											
Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.00	0.00	0.00	0.00
Current Speed Time Series (cm s^{-1})											
Low-flow event (LF)											
Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
1.0	1.0	1.0	1.0	0.001	0.001	0.001	0.001	1.0	1.0	1.0	1.0
Salinity Time Series (ppt)											
High-salinity event (Hsal)											
Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
20	20	20	35	35	35	35	35	35	20	20	20
Low-salinity event (Lsal)											
Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
20	20	20	10	10	10	10	10	10	20	20	20
Low-salinity event (Lrsal)											
Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
20	20	20	15	15	15	15	15	15	20	20	20
Temperature Time Series (°C)											
Galveston Bay, Texas (GB)											
Given in Dekshenieks et al. (1993)											
High winter temperature (Ht)											
Winter temperatures (October to March) are 2°C higher than those measured in Galveston Bay											
Low winter temperature (Lt)											
Winter temperatures (October to March) are 2°C lower than those measured in Galveston Bay											
Oyster Abundance—Confederate Reef											
Size (upper size limit in mm)											
25.	35.	50.	63.5	70.	76.	88.9	100.	110.	125.	150.	
Abundance (number m^{-2})											
1.8	2.0	4.8	4.6	2.4	1.6	2.2	0.8	0.4	0.2	0.0	
Mussel Abundance											
Size (upper size limit in mm)											
10.	20.	30.	40.	50.	60.	70.	80.	90.	100.		
Abundance (number m^{-2})											
50	50	50	50	0	0	0	0	0	0		

as crabs and oyster drills (Powell et al. 1995a), which represent top-down controls, as defined by Hunter and Price (1992).

Environmental conditions that are typical of high-salinity reefs in Galveston Bay, TX, result in the simulated oyster population shown in Figure 4. A relatively stable market-size population is maintained over 5 years (Fig. 4A) and the submarket-size component (not shown) of the population rises gradually during the first 4 years and more rapidly thereafter. A decline in year 6 is produced by high population densities exceeding the available food supply. The biomass-to-length conversion given in Table 1 was used to calculate the number of market-size individuals. This relationship is representative of Galveston Bay oyster reefs, although substantial variation exists within the bay (Powell unpublished data). Minor mortality events, due to *P. marinus*, occur during the summer of the second and fourth years (Fig. 4A). The large decrease in oyster abundance seen at the end of 6 years results from the effects of crowding caused by significant population expansion in years 5 and 6 (see Powell et al. 1994 for a discussion). The oyster population maintains reproductive capability throughout the simulation, with spawning occurring throughout much of the late spring and summer (Fig. 4B) in each year.

TABLE 6.

The combination of environmental time series given in Table 5 and additional parameter values used for the simulations. *The different environmental time series are defined as: summer bloom (SB), summer bloom with reduced winter food (LW), summer bloom with reduced summer food (LS), summer bloom with increased summer food (HS), high-salinity event (Hsal), low-salinity event (Lsal), low-salinity event with slightly higher summer values (Lrsal), high-turbidity event (HT), low-current-flow event (LF), high winter temperatures (Ht), and low winter temperatures (Lt). The *P. marinus* division time and juvenile oyster mortality used in each simulation are also shown. Except where indicated, juvenile survival was 1 in 10^8. Values indicate a 12-month continuous time series at that level.

Figure Number	Salinity (ppt)	Temperature (°C)	Food	Turbidity ($g\ l^{-1}$)	Flow ($cm\ s^{-}$)	Halving Time (hours)	Juvenile Mortality (d^{-1})	Comments
4	20	GB	SB	0	1.0	60	0.0064	
5	20	GB	SB/LW/SB	0	1.0	60	0.0064	Time series split 1/1/4
6	20	GB	SB/LS/SB	0	1.0	60	0.0064	Time series split 1/1/4
7	20	GB	SB	0/HT/0	1.0	60	0.0064	Time series split 1/1/4
8	20	GB	SB	0	1.0/LF/1.0	60	0.0064	Time series split 1/1/4
9	20	GB	SB	0	1.0	60	0.0064	Mussels present days 450–650
10	20/Hsal/20	GB	SB	0	1.0	60	0.0064	Time series split 1/1/4
11	20/Lsal/20	GB	SB	0	1.0	60	0.0064	Time series split 1/1/4
12	20/Hsal/20	GB/Ht/GB	SB	0	1.0	60	0.0064	Time series split 1/1/4
13	20	GB	SB	0	1.0	120	0.0064	
14	20	GB	SB/LS/HS/SB	0	1.0	60	0.0064	Time series split 1/1/1/3
15	20/Hsal/Lrsal/20	GB/Ht/LT/HB	SB	0	1.0	60	0.0064	Time series split 1/1/1/3
16	20	GB	SB	0	1.0	60	0.0064	Summer recruitment rate: year 2, 1 in 10^9; year 3, 6 in 10^9

One of the checks on the simulation is to ensure that the simulated seasonal progression of *P. marinus* infection intensity and prevalence corresponds to measured patterns. The observed pattern of *P. marinus* prevalence in oyster populations usually shows lows in late winter to early spring, an increase in late spring, and a peak in mid to late summer. The pattern of prevalence for the total simulated oyster population (Fig. 4C, solid line) shows lows in the summer and highs in the winter, which is exactly the opposite of field measurements. The lows in prevalence in the simulated populations occur during recruitment following major spawning events.

The standard approach for measuring *P. marinus* prevalence involves the collection of the largest individuals in the population, normally those of market size. If only this portion of the simulated oyster population is considered, prevalence exceeds 80% in most months of the year (Fig. 4C, dotted line). Lows occur in fall and winter as submarket-size adults grow to market size, but prevalence does not decline to the normally measured winter levels. Recent experimental studies (Choi et al. 1989) have shown that the thioglycollate technique typically used to assess *P. marinus* prevalence (Ray 1966) frequently misdiagnoses light infections as negative. Thus, if it is assumed that infections of $\leq 2^{12}$ cells ind^{-1} are normally misdiagnosed as negative, then the pattern of prevalence in the market-sized portion of the simulated oyster populations shows the observed seasonal cycle (Fig. 4C, dashed line). Low prevalences occur in February and March, when many false negatives are reported, and peak prevalences occur in the late summer.

A similar problem exists for the calculation of mean infection intensity for the population depicted in Figure 4B when using Mackin's (1962) Scale as modified by Craig et al. (1989). The simulated seasonal progression of infection intensity matches the observed pattern only when the market-sized portion of the population is considered (Fig. 4B, dotted line). The seasonal cycle of infection intensity in the entire population (Fig. 4B, solid line) can be discerned but summer highs are depressed as disease intensification in the adults is offset by recruitment of uninfected individuals. Thus, the seasonal cycle that is observed in *P. marinus* prevalence and infection intensity is dependent upon the size class structure of the sampled population. The routine sampling of only the largest individuals in the population normally does not accurately portray the disease status of the entire population. The implications of this are discussed more fully in Hofmann et al. (1995).

The level of *P. marinus* prevalence that occurs in the simulated oyster populations shown in Figure 4 in response to Galveston Bay environmental conditions remains above 60% throughout most of the year. Yearly highs exceed 90% in most years. Mean infection intensity of the market-size adults reaches 4 on Mackin's Scale in years when mortality occurs. Infection intensity in the entire oyster

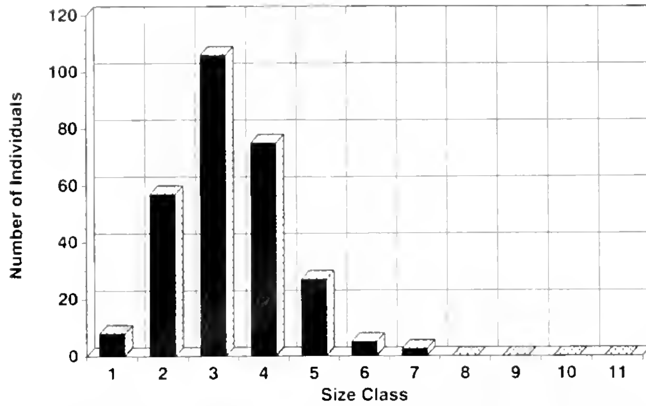
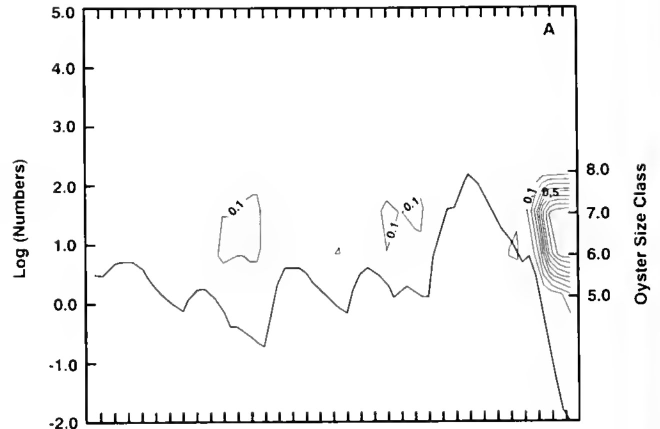
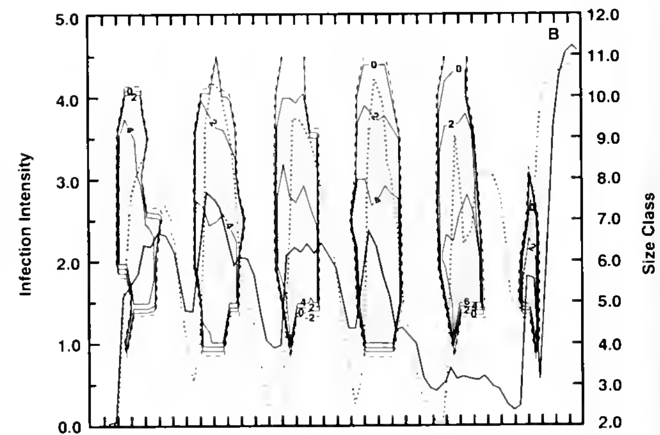
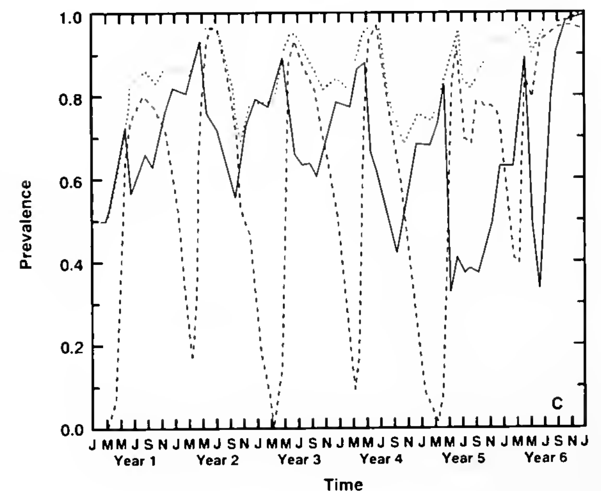


Figure 3. Size-frequency distribution of oysters that was used to initialize the oyster population model. Data are from observations made at South Deer Island in the West Bay section of Galveston Bay, TX, in spring 1992.







population averages 2 to 3, a light to moderate infection, during the summer and fall. This is lower than that for the market-size population due to the dilution effect of new recruits. The infection intensity for the entire oyster population drops to about 1 during the winter. This is higher than the infection level in the market-size fraction of the populations because the newly recruited smaller individuals, with the same number of *P. marinus* cells, have higher infection intensities on a cell per gram basis.

Infection intensity in the market-size individuals is about 3.5 on Mackin's Scale (moderate infections) in most years. However, in years in which there is significant *P. marinus* mortality, infection intensity nears 4 on Mackin's Scale. This small variation in infection intensity, which corresponds to about 1 to 2 population doublings (Hofmann et al. 1995), is all that is required to separate years of moderate-to-low mortality from years having significant mortality events.

TRIGGERING MECHANISMS FOR EPIZOOTICS

Rapid growth and high fecundity are the principal defenses against predation and disease for many host-prey/parasite-predator systems (e.g., Onstad and Maddox 1989, Warburton 1958). Oyster populations are no exception, with recruitment, growth, and fecundity usually exceeding, by some small amount, the combined rate of *P. marinus* transmission, intensification, and mortality. Therefore, mechanisms that can potentially trigger epizootics should be sought primarily among the variables that modify the oyster population potential for recruitment, growth, and fecundity. Obvious choices for potential triggering mechanisms are variations in environmental conditions, such as food supply, turbidity level, current flow, salinity, and temperature. Other factors that influence the ability of the oyster population to grow such as competition for food (i.e., mussels), variations in recruitment and juvenile mortality, and the ability of the oyster to resist disease also potentially affect the occurrence of epizootics.

Frequently, the factor(s) that triggers an epizootic occurs well before the detection of the event (Gill 1928), and once initiated, epizootics can persist during what would be considered normal or optimal conditions. Furthermore, only a small change in conditions may be needed to trigger an epizootic because populations often exist in quasiequilibrium with the disease (Anderson 1991, Lenski and May 1994). Thus, the simulations that were designed to investigate epizootic triggering mechanisms used food, temper-

ature, salinity, and turbidity conditions for Galveston Bay in which 1 year of the 6-year time series was modified to introduce a small change in conditions. For each simulation, year 1 represented normal environmental conditions (as used for the simulation shown in Fig. 4), year 2 included the modified condition, and years 3 to 6 returned to normal conditions. Thus, the oyster populations were exposed to 5 years of environmental conditions that are conducive to growth and expansion (Fig. 4) and 1 year that potentially was not.

Food Supply

Decreased food supply reduces oyster growth and fecundity (Soniat and Ray 1985, Robinson 1992) but does not affect the cell division rate of *P. marinus*. Low food supply, then, is potentially an epizootic-triggering mechanism. However, the time during which oysters experience low food supply can be important because the rate of *P. marinus* division is temperature dependent. Thus, the effect of low food supply might be expected to be less in the winter than in the summer. In the following simulations, food supply in the second year was reduced by 0.25 mg l^{-1} for 4 months in either the winter or the summer, which gives a 10% decrease in food over the year.

A reduction in food supply during the winter has little impact on the oyster population (Fig. 5). Oyster ingestion rates are primarily a function of filtration rate which is temperature controlled. Decreased temperatures in the winter result in reduced filtration rates so that the impact of low food supply during this time is minimal. Moreover, the division rate of *P. marinus* is at its yearly low. Thus, low winter food supplies do not substantially alter the pattern of *P. marinus* prevalence and infection intensity from that seen in the reference simulation. *P. marinus* mortality is increased somewhat, but the oyster population continues to grow and expand.

By contrast, a reduction in food supply during the summer triggers an epizootic (Fig. 6). This epizootic contains all of the basic characteristics of most epizootics (e.g., Gill 1928, Plowright 1982, Shields and Kuris 1988). Although the reduced food supply occurs in the summer of the second year, the response of the oyster population is not immediately obvious and no dramatic mortality event occurs in the following year (year 3, Fig. 6A). In the next 2 years (4 and 5), however, the population declines to extinction as the primary phase of mortality begins 18 months after the triggering event (Fig. 6A).

Spawning continues during the entire epizootic phase and rates do not decline substantially until significant mortality begins in the adult population in year 5 (Fig. 6B). Fecundity through year 5 would be adequate for population recovery were *P. marinus* infection intensity to decline. Infection intensity rises persistently from about 3 in the summer and 1.5 in the winter of year 2 to above 4 in the summer and 2 in the winter in year 6 (Fig. 6B). The rise in infection intensity is most noticeable in the entire popula-

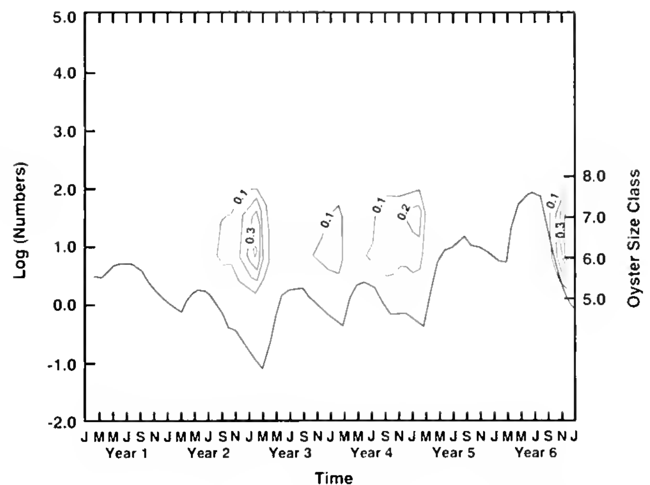


Figure 5. The number of market-size individuals (solid line) in the population expressed as $\log_{10}$(number m^{-2}), from a simulation that used environmental conditions that are characteristic of a high-salinity reef in Galveston Bay, TX (Table 5), that experienced a decline in food supply during the winter of year 2 (Table 6). Mortality events, calculated as the fraction of the population in a given size class that dies during a 1-month period, are indicated by the shaded contours, with an interval of 0.1.

tion rather than in the normally sampled market-size component because mortality in the latter size classes continually removes the most heavily infected individuals from the population.

During the epizootic, disease prevalence in the population gradually increases from about 60 to 80% to near 100% (Fig. 6C). However, prevalence, as usually measured (dotted line in Fig. 6C), changes little during the epizootic. Thus, false-negatives and inadequate sampling of the entire oyster size-frequency distribution can inhibit observation of this phase of disease intensification.

In this simulation, the population crash is not produced by a dramatic decrease in fecundity or recruitment. These are products of the crash. The population crash occurs because the rate of *P. marinus* growth and transmission exceeded the rate of expansion of the oyster population by just a small amount in year 2. A reduction in food supply during the summer months produces a subtle change in the balance between oyster population expansion and disease intensification which permits the disease to nudge ahead and gradually exert control over the host population. The initial food conditions for this simulation, which are typical of Galveston Bay, TX, allow population expansion but are near the threshold that can trigger an epizootic. For higher food supplies, a 10% decrease would have had a lesser effect. Thus, this simulation shows that a small change in environmental conditions may be all that is needed to generate an epizootic once the population nears the carrying capacity of the environment, and once the epizootic is triggered, simply returning to pretrigger environmental conditions

Figure 4. (A) The number of market-size individuals (solid line) in the population expressed as $\log_{10}$(number m^{-2}), from a simulation that used environmental conditions that are characteristic of a high-salinity reef in Galveston Bay, TX (Table 5). Mortality events, calculated as the fraction of the population in a given size class that dies during a 1-month period, are indicated by the shaded contours, with an interval of 0.1. (B) Simulated oyster population reproductive effort (shading) expressed as $\log_{10}$(total joules spawned per month) in each size class. Contour interval is 2 log units. *P. marinus* infection intensity expressed in terms of Mackin's (1962) 0- to 5-point scale is shown for the entire population (solid line) and the market-size (≥3-inch) portion of the population (dotted line). (C) *P. marinus* prevalence expressed as the fraction of the total population that is infected. Prevalences in the entire oyster population, the market-size (≥3-inch) portion of the oyster population, and the market-size population, assuming that all infections $\leq 2^{12}$ cells ind^{-1} are judged negative using the method described by Ray (1966), are represented by the solid, dotted, and dashed lines, respectively.

is insufficient to prevent disaster. Moreover, the simulation shows that an epizootic generated by a small but significant decline in food supply can be characterized by a delay of about 18 months between the trigger and an observed increase in mortality and that a decline in fecundity may not become obvious until significant mortality begins in the adult population. An increase in prevalence and infection intensity in the population may serve as an early warning sign of an impending epizootic. However, this rise may only be noticeable in that fraction of the population smaller than market size, a fraction normally not sampled by field surveys.

Turbidity

Increased turbidity decreases feeding efficiency and therefore should also restrict food supply. Increasing turbidity during the summer of year 2 to 10 mg l^{-1} (Table 5) initiates an epizootic that produces significant mortality about 12 months after the high-turbidity event and results in a crash of the oyster population in year 4 (Fig. 7). The simulated disease prevalence and intensity associated with this event are essentially identical to those obtained for the reduced food scenario (Fig. 6).

Current Flow

Certain combinations of food content, population density, and current flow may significantly affect the flux of food over the oyster reef (Muschenheim 1987, Wilson-Ormond et al. in press). A reduction in current flow during the four summer months of year 2 gives results that are similar to those shown for reduced food conditions. Significant oyster mortality begins about 18 months after the low-flow event and the population eventually crashes in years 5 and 6 (Fig. 8). The pattern of intensification of *P. marinus* infection in this and the reduced food simulation is similar, but the epizootic that results from low current flow develops more slowly.

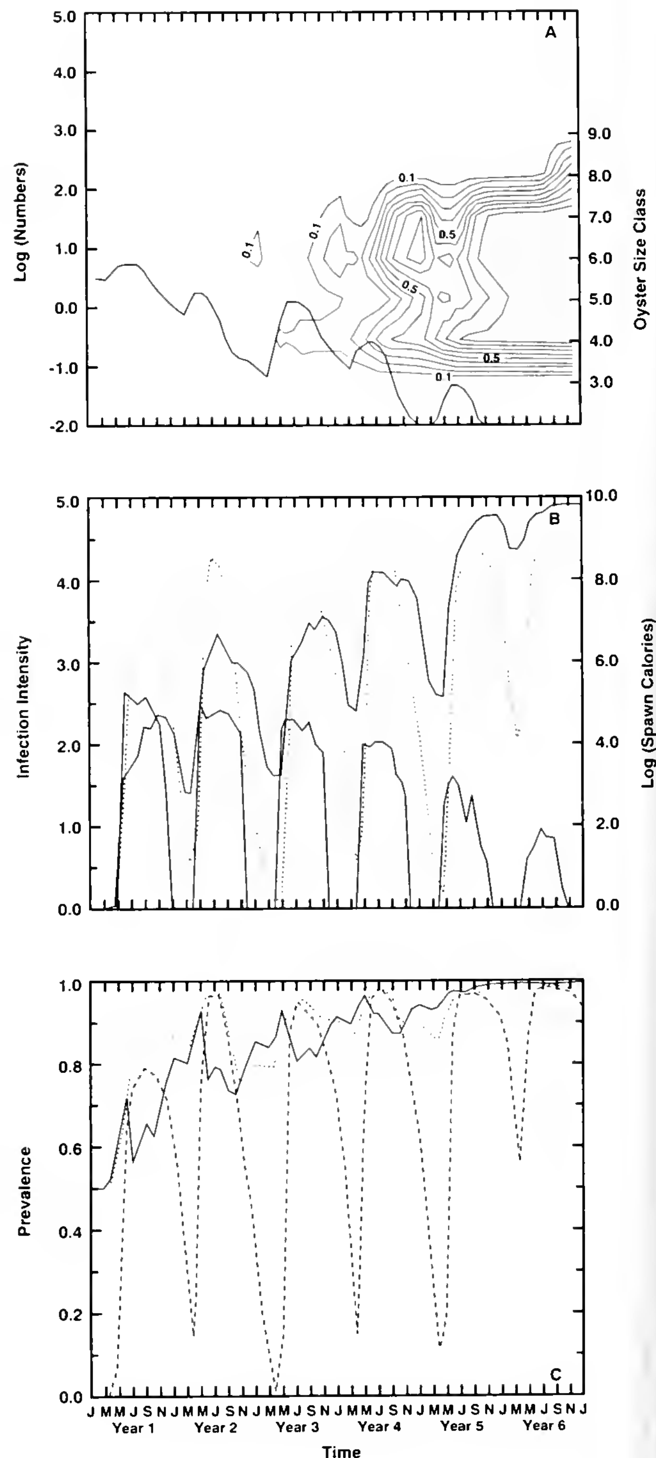
Mussels

Competition from other filter feeders may reduce food supply and thus adversely impact the oyster population. In Texas bays, mussels of the genus *Brachidontes* are abundant. To provide a simulation comparable to the low-food, increased turbidity, and low-flow simulations, the mussels were allowed to impact food supply for only the summer months in year 2. The competing effect of the mussels acts to decrease the food supply to the oysters. The time-dependent evolution of the oyster population (Fig. 9) is similar to that shown in Figure 6 and the pattern of disease intensification and intensity is essentially identical to that seen in Figure 6B and C. An epizootic triggered in year 2 results in significant mortality about 18 months later and the population begins to decline in years 4 and 5 (Fig. 9).

Salinity

Small changes in climate have been shown to significantly modify recruitment to marine populations (Turrell et al. 1992) and disease (Jarosz and Burden 1992). *P. marinus* responds to temperature and salinity variations and even small perturbations in these environmental conditions arising from changes in climate can significantly modify *P. marinus* disease intensity over large geographic areas (Powell et al. 1992a). High-salinity conditions have a greater impact during the warmer half of the year. Temperature has a major effect in winter through its influence on parasite division rate and mortality.

The effect of salinity was investigated by raising salinity by 15



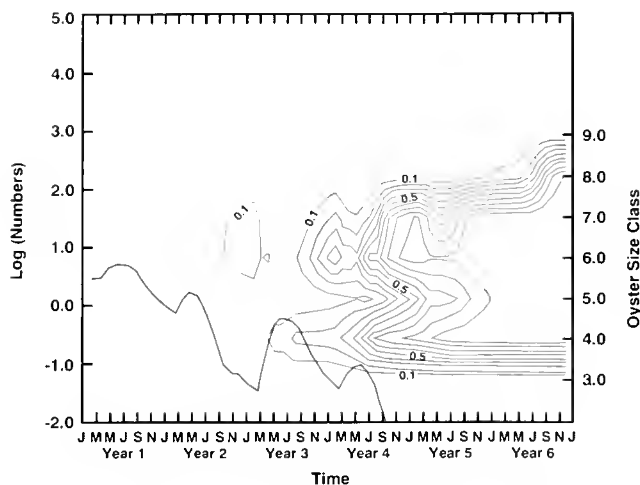


Figure 7. The number of market-size individuals (solid line) in the population expressed as $\log_{10}$(number m^{-2}), from a simulation that used environmental conditions that are characteristic of a high-salinity reef in Galveston Bay, TX (Table 5), that experienced an increase in turbidity during the summer of year 2 (Table 6). Mortality events, calculated as the fraction of the population in a given size class that dies during a 1-month period, are indicated by the shaded contours, with an interval of 0.1.

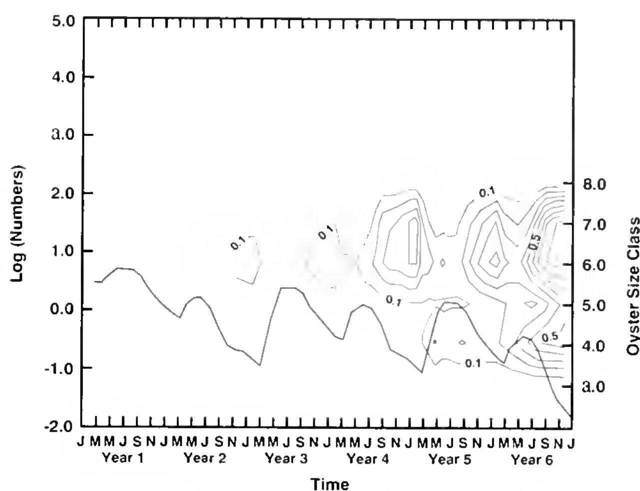


Figure 8. The number of market-size individuals (solid line) in the population expressed as $\log_{10}$(number m^{-2}), from a simulation that used environmental conditions that are characteristic of a high-salinity reef in Galveston Bay, TX (Table 5), that experienced a decrease in current flow during the summer of year 2 (Table 6). Mortality events, calculated as the fraction of the population in a given size class that dies during a 1-month period, are indicated by the shaded contours, with an interval of 0.1.

ppt for 6 months, April to September, in year 2. Increased salinity during the warmer months reduces the rate of population growth in comparison to the simulation that used normal conditions (cf. Fig. 4) by increasing the mortality of market-size oysters from *P. marinus*. However, an epizootic does not occur (Fig. 10). High-salinity is usually associated with epizootics (e.g., Crosby and Roberts 1990, Mann et al. 1991). However, many of the oyster populations in the Gulf of Mexico exist at salinities above 20 ppt for much or all of the year and maintain productive and expanding populations. High salinity may facilitate the development of an epizootic, but high salinity alone is unlikely to trigger one.

Exposing an oyster population to a 10-ppt decrease in salinity for 6 months, however, produces an immediate mortality event, which continues for an indefinite time (Fig. 11A). During the low-salinity event, infection intensity (Fig. 11B) and prevalence (Fig. 11C), as usually measured, decline as expected. However, the population prevalence and infection intensity rise as individuals of market-size decline, which is counterintuitive. Low salinity restricts scope for growth, and in the absence of a balancing effect such as increased food supply, this decrease restricts oyster growth, particularly in individuals already growth restricted by high *P. marinus* infection intensity (e.g., Menzel and Hopkins 1955). Such a growth restriction would be just enough, in heavily infected oysters, to produce a lethal infection. It is well known that oysters are more sensitive to low-salinity mortality during the summer months (e.g., Gunter 1953, Ray 1987, E. Powell unpublished data). This simulation suggests that *P. marinus* infection may be one important reason for this sensitivity, although no observations are available to support this speculation. Furthermore, the dramatic decrease in prevalence and infection intensity noted during and after low-salinity events in the summer (e.g., Soniat 1985) may well be due as much to the removal of heavily infected individuals from the population as to inhibition of *P. marinus* intensification. No evidence from field observations is available to support or refute this suggestion.

Temperature

Water temperature variation in Gulf of Mexico bays and estuaries between warm and cold years is rarely more than 2°C from the long-term mean (Sittel 1994). A change in temperature of this magnitude failed to initiate an epizootic. Frequently, however, extremely warm years co-occur with extremely dry years (about 10% of all years) in Galveston Bay and these years may be characterized by relatively warm winters or relatively warm summers. To test these effects, the summer temperature for the Galveston Bay time series was increased by 2°C (Table 5) and used with the summer salinity conditions that produced the simulated oyster population shown in Figure 10. The resultant extremely warm and dry summer produced a simulated oyster population distribution that was not significantly different from that shown in Figure 10. One reason is that summer conditions in the Gulf of Mexico are

Figure 6. (A) The number of market-size individuals (solid line) in the population expressed as $\log_{10}$(number m^{-2}), from a simulation that used environmental conditions that are characteristic of a high-salinity reef in Galveston Bay, TX (Table 5), that experienced a decline in food supply during the summer of year 2 (Table 6). Mortality events, calculated as the fraction of the population in a given size class that dies during a 1-month period, are indicated by the shaded contours, with an interval of 0.1. (B) Simulated oyster population reproductive effort (shading) expressed as $\log_{10}$(total calories spawned per month). *P. marinus* infection intensity expressed in terms of Mackin's (1962) 0- to 5-point scale is shown for the entire population (solid line) and the market-size (≥3-inch) portion of the population (dotted line). (C) *P. marinus* prevalence expressed as the fraction of the total population that is infected. Prevalences in the entire oyster population, the market-size (≥3-inch) portion of the oyster population, and the market-size population, assuming that all infections $\leq 2^{12}$ cells ind^{-1} are judged negative using the method described by Ray (1966), are represented by the solid, dotted, and dashed lines, respectively.

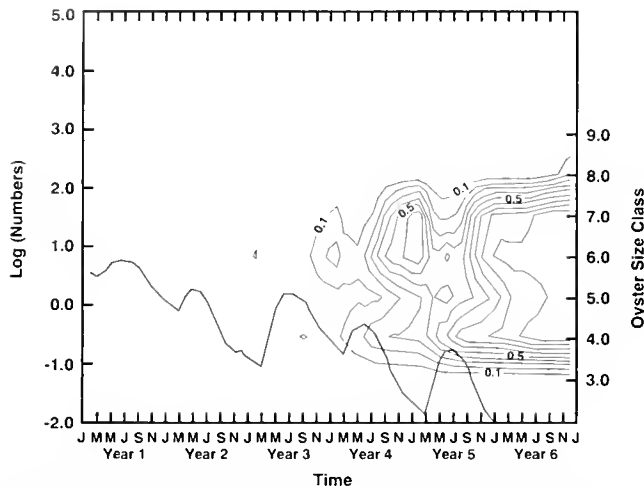


Figure 9. The number of market-size individuals (solid line) in the population expressed as $\log_{10}$(number m^{-2}), from a simulation that used environmental conditions that are characteristic of a high-salinity reef in Galveston Bay, TX (Table 5), that experienced a competitive interaction with mussels during Julian Days 450 to 630 (Table 6). Mortality events, calculated as the fraction of the population in a given size class that dies during a 1-month period, are indicated by the shaded contours, with an interval of 0.1.

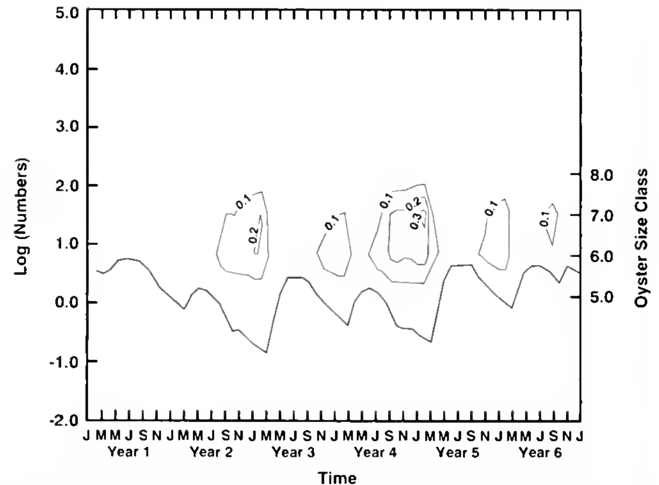


Figure 10. The number of market-size individuals (solid line) in the population expressed as $\log_{10}$(number m^{-2}), from a simulation that used environmental conditions that are characteristic of a high-salinity reef in Galveston Bay, TX (Table 5), that experienced a high-salinity event during the summer of year 2 (Table 6). Mortality events, calculated as the fraction of the population in a given size class that dies during a 1-month period, are indicated by the shaded contours, with an interval of 0.1.

already so conducive to *P. marinus* intensification that slightly warmer conditions have very little additional impact. Oyster populations must routinely withstand warm, dry summers to maintain the population abundances normally observed.

The same is not the case for a warmer winter. For this simulation, the winter water temperatures from Galveston Bay were increased by 2°C (Table 5) and used with the higher summer salinity conditions (Fig. 10) to produce a dry summer following a warm winter. These conditions produce a classic *P. marinus* epizootic (Fig. 12) which is similar in all respects to those described in previous simulations (e.g., Figs. 6–8). A warm winter increases the ratio of parasite division rate to parasite mortality rate so that winter infection intensities remain relatively high. This simulation suggests that the coincidences of appropriate summer salinities and winter temperatures are the environmental factors that contribute the most to the generation of an epizootic in Gulf of Mexico bays and estuaries.

Recruitment and Juvenile Mortality

Factors that affect population fecundity, recruitment, or juvenile mortality may destabilize host/parasite populations in quasiequilibrium (Dobson 1988). Decreasing recruitment success by 50% in the summer of year 2 (Julian days 450 to 630) or increasing juvenile mortality by 50% in the same time frame produced an epizootic qualitatively identical to the one depicted in Figure 6. The intensification of *P. marinus* infection closely followed the pattern shown in Figure 6B and C. An epizootic began to produce significant mortality about 12 months after the event, in each case, and the population crashed in years 4 and 5.

Changing Disease Resistance or Virulence

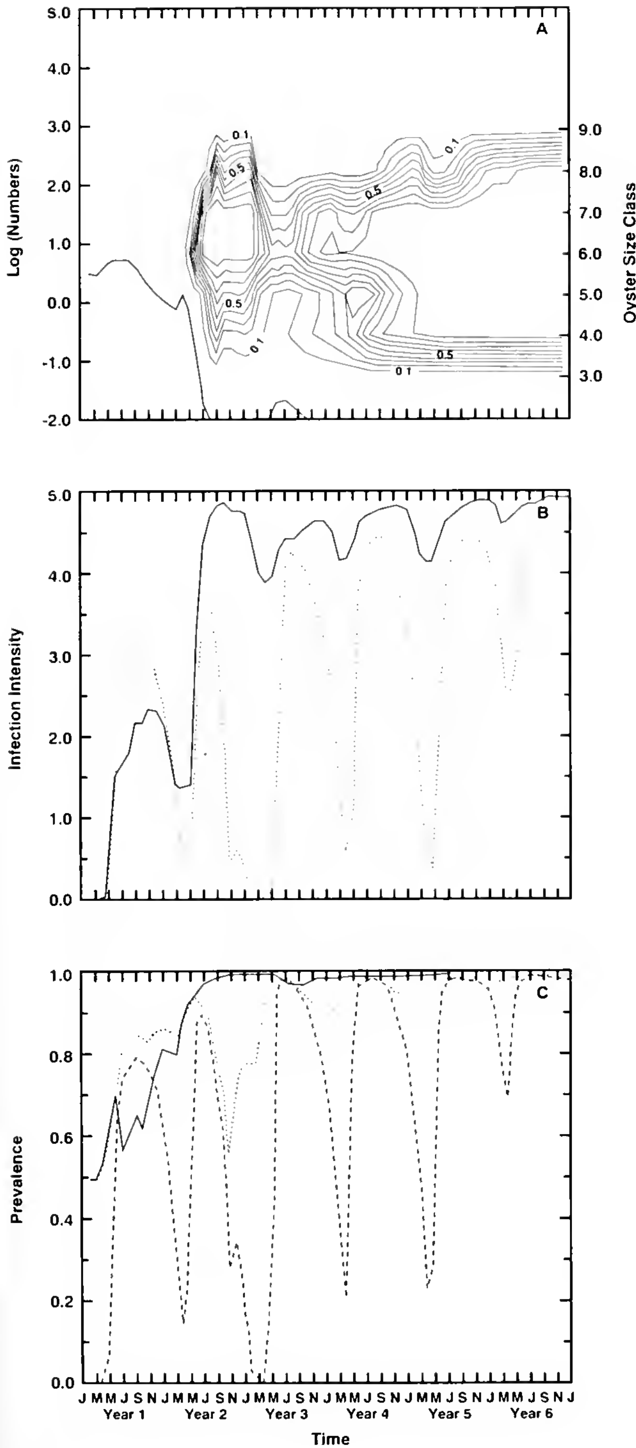
Resistance to disease is often important in initiating or stopping an epizootic (Ross 1982, Kent et al. 1989, McCallum 1990, Möller 1990). Although the development of resistance to *P. marinus* has been questioned (e.g., Lewis et al. 1992), Hofmann et al. (1995) suggested that some regional variation in oyster resistance or *P. marinus* virulence is probably required to explain regional variations in *P. marinus* prevalence and infection intensity. This effect was simulated by reducing the rate of parasite mortality $[r_m(T,S)]$ by changing the population halving time at 20°C–20 ppt from 60 to 120 hours. Note that a reduction in population doubling time (increased virulence) would yield similar results. The resultant simulated oyster population undergoes an epizootic with mass mortality starting in year 3 (Fig. 13). The reduction in parasite mortality (or increase in parasite division time) primarily affects the winter drop in infection intensity and produces conditions similar to those produced by a warm winter. Variations in the rate of parasite division or mortality have little effect in the summer when parasite density effects exert a major control on the growth rate of the *P. marinus* population.

MECHANISMS FOR STOPPING AN EPIZOOTIC

General Considerations

Once started, an epizootic is difficult to stop. In most cases, epizootics cease when the host population's density drops to a

Figure 11. (A) The number of market-size individuals (solid line) in the population expressed as $\log_{10}$(number m^{-2}), from a simulation that used environmental conditions that are characteristic of a high-salinity reef in Galveston Bay, TX (Table 5), that experienced a low-salinity event during the summer of year 2 (Table 6). Mortality events, calculated as the fraction of the population in a given size class that dies during a 1-month period, are indicated by the shaded contours, with an interval of 0.1. (B) *P. marinus* infection intensity expressed in terms of Mackin's (1962) 0- to 5-point scale is shown for the entire population (solid line) and the market-size (≥3-inch) portion of the population (dotted line). (C) *P. marinus* prevalence expressed as the fraction of the total population that is infected. Prevalences in the entire oyster population, the market-size (≥3-inch) portion of the oyster population, and the market-size population, assuming that all infections $\leq 2^{12}$ cells ind^{-1} are judged negative using the method described by Ray (1966), are represented by the solid, dotted, and dashed lines, respectively.



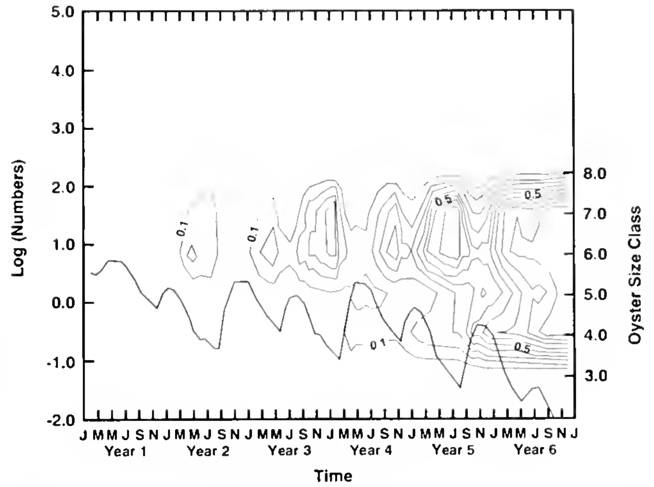


Figure 12. The number of market-size individuals (solid line) in the population expressed as $\log_{10}$(number m^{-2}), from a simulation that used environmental conditions that are characteristic of a high-salinity reef in Galveston Bay, TX (Table 5), that experienced a high-salinity event during the summer and a warm temperature event during the winter of year 2 (Table 6). Mortality events, calculated as the fraction of the population in a given size class that dies during a 1-month period, are indicated by the shaded contours, with an interval of 0.1.

critical level which is sufficiently low to inhibit transmission of the disease (Kermack and McKendrick 1991a,b, Anderson 1991). In most cases, this level is near local extinction, in comparison to densities normally found (e.g., Bartlett 1960, Plowright 1982, Ross 1982); this is especially true for *P. marinus* which has an efficient transmission mechanism even at low host densities. Thus, can epizootics be stopped by mechanisms other than local extinction?

Since triggering mechanisms normally are defined by small changes in some environmental or biological variable, small

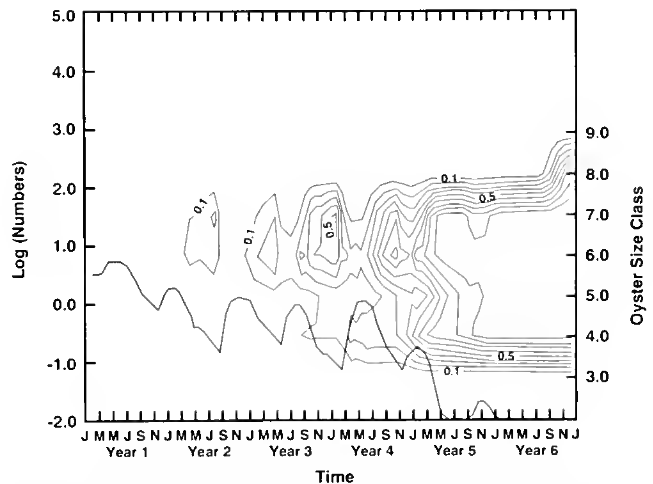


Figure 13. The number of market-size individuals (solid line) in the population expressed as $\log_{10}$(number m^{-2}), from a simulation that used environmental conditions that are characteristic of a high-salinity reef in Galveston Bay, TX (Table 5), that experienced a decrease in the rate of *P. marinus* cell mortality during the winter of year 2 (Table 6). Mortality events, calculated as the fraction of the population in a given size class that dies during a 1-month period, are indicated by the shaded contours, with an interval of 0.1.

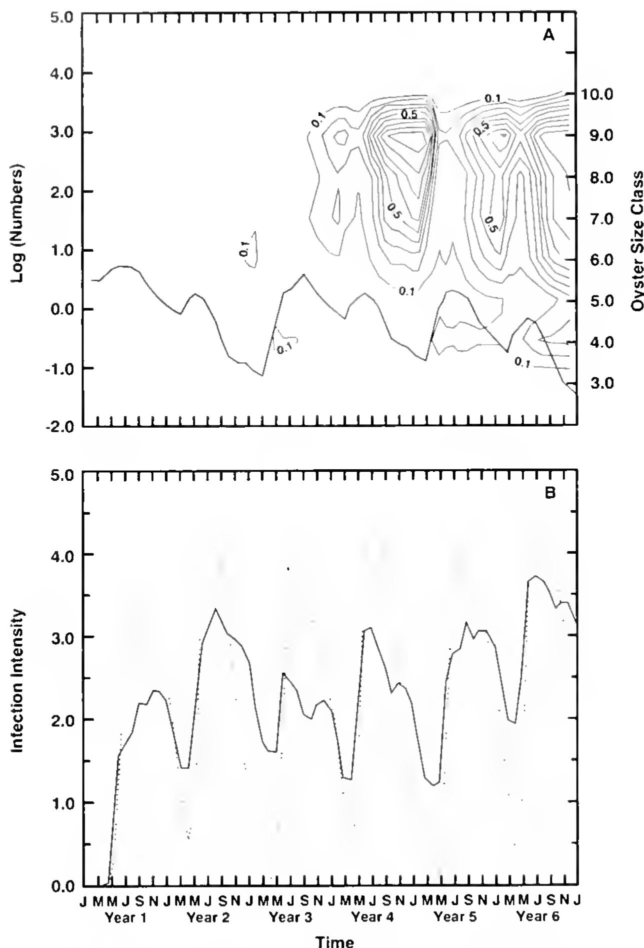


Figure 14. (A) The number of market-size individuals (solid line) in the population expressed as $\log_{10}$(number m^{-2}), from a simulation that used environmental conditions that are characteristic of a high-salinity reef in Galveston Bay, TX (Table 5), that experienced a decline in food supply during the summer of year 2 followed by an increase in food supply during the summer of year 3 (Table 6). Mortality events, calculated as the fraction of the population in a given size class that dies during a 1-month period, are indicated by the shaded contours, with an interval of 0.1. (B) *P. marinus* infection intensity expressed in terms of Mackin's (1962) 0- to 5-point scale is shown for the entire population (solid line) and the market-size (≥3-inch) portion of the population (dotted line).

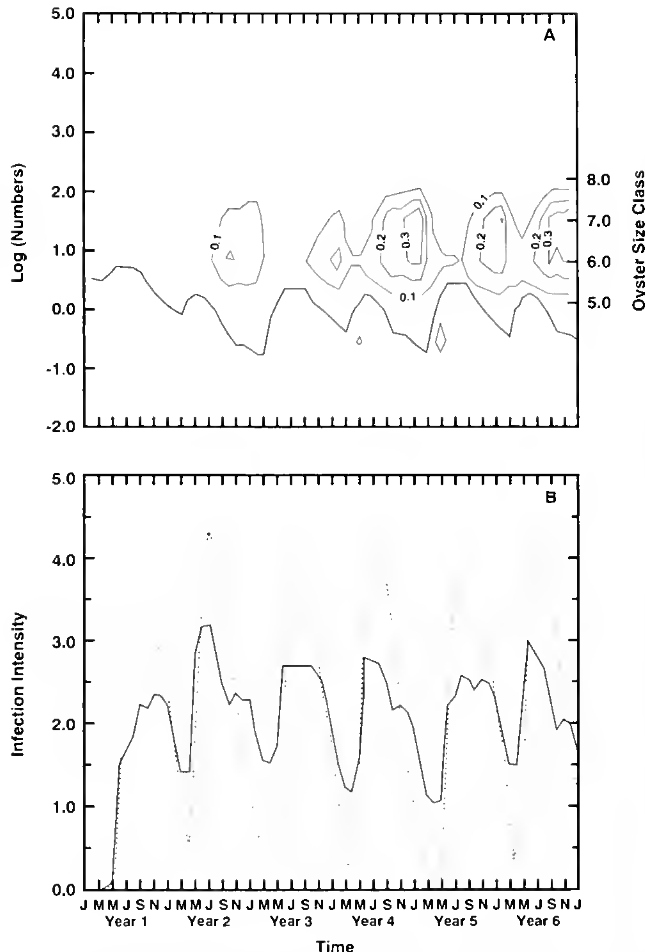


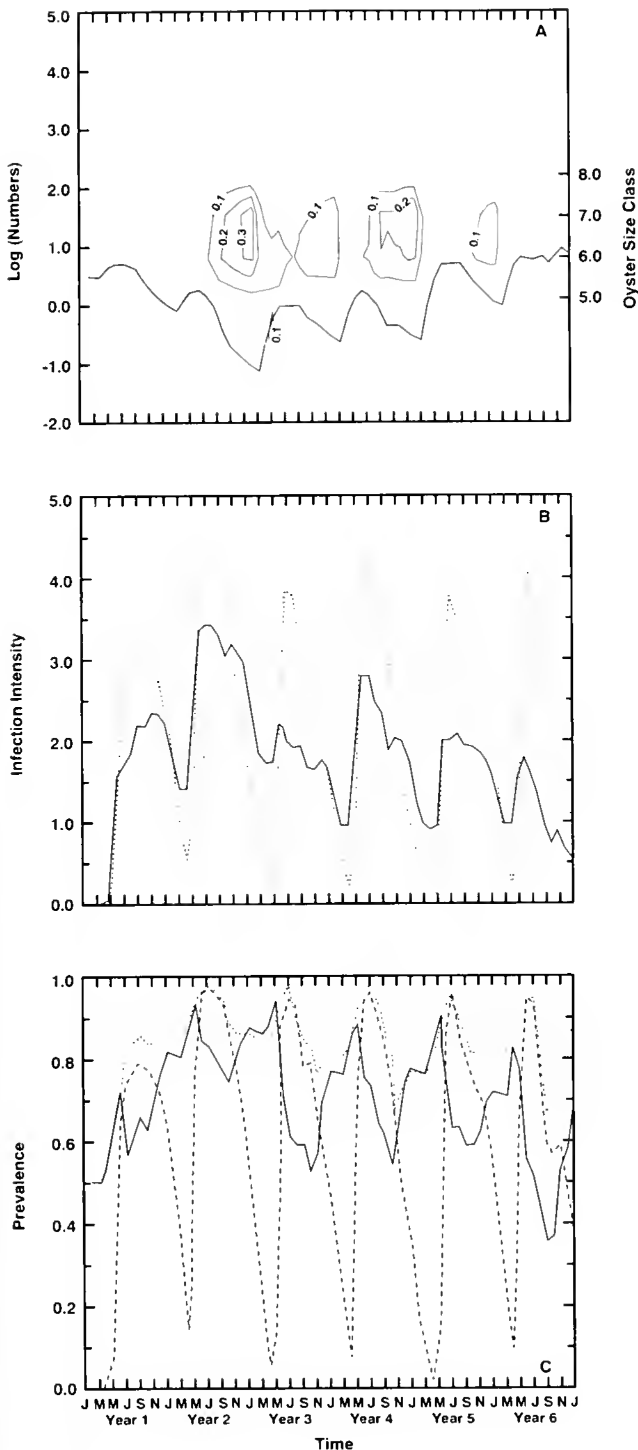
Figure 15. The number of market-size individuals (solid line) in the population expressed as $\log_{10}$(number m^{-2}), from a simulation that used environmental conditions that are characteristic of a high-salinity reef in Galveston Bay, TX (Table 5), that experienced warm, dry conditions in year 2 followed by cool, wet conditions in year 3 (Table 6). Mortality events, calculated as the fraction of the population in a given size class that dies during a 1-month period, are indicated by the shaded contours, with an interval of 0.1. (B) *P. marinus* infection intensity expressed in terms of Mackin's (1962) 0- to 5-point scale is shown for the entire population (solid line) and the market-size (≥3-inch) portion of the population (dotted line).

changes in these variables may also be able to stop an epizootic. A series of simulations was designed to test this possibility. For each, an epizootic was triggered in year 2 as described previously. Year 3 environmental conditions were then modified in the opposite direction to produce a favorable (better than normal) year. Years 1 and 4 through 6 were unchanged and normal conditions prevailed.

Food Supply

The simulation results given in Figure 6 show an epizootic triggered by a decrease in food supply of about 25% during the summer months of year 2. A 75% increase in food supply during the summer months of year 3 is used to offset the decrease in year 2. This increase in summer food supply failed to prevent the epizootic (Fig. 14A), although its onset was delayed. Infection in-

Figure 16. (A) The number of market-size individuals (solid line) in the population expressed as $\log_{10}$(number m^{-2}), from a simulation that used environmental conditions that are characteristic of a high-salinity reef in Galveston Bay, TX (Table 5), that experienced a decline in recruitment rate during the summer of year 2 followed by an increase in recruitment rate during the summer of year 3 (Table 6). Mortality events, calculated as the fraction of the population in a given size class that dies during a 1-month period, are indicated by the shaded contours, with an interval of 0.1. (B) *P. marinus* infection intensity expressed in terms of Mackin's (1962) 0- to 5-point scale is shown for the entire population (solid line) and the market-size (≥3-inch) portion of the population (dotted line). (C) *P. marinus* prevalence expressed as the fraction of the total population that is infected. Prevalences in the entire oyster population, the market-size (≥3-inch) portion of the oyster population, and the market-size population, assuming that all infections $\leq 2^{12}$ cells ind^{-1} are judged negative using the method described by Ray (1966), are represented by the solid, dotted, and dashed lines, respectively.



tensity is reduced in year 3 but then rises back to epizootic levels in subsequent years (Fig. 14B). A lesser increase of food (25 to 50%) failed to delay the epizootic, so that stopping an epizootic requires a much higher proportional increase in food supply than the decrease that triggered it. This occurs because of the inhibiting effect of high infection intensity on oyster filtration rate. Heavily infected oysters are food limited by their disease.

Temperature and Salinity

For the simulation shown in Figure 15, a warm dry year (year 2) was followed by a cool wet year in year 3. The cool wet year contained winter temperatures 2°C cooler than the standard Galveston Bay conditions and summer salinities that were 5 ppt fresher (Table 6). With these conditions, an epizootic fails to develop. Population abundances remain more or less stable, as does *P. marinus* mortality (Fig. 15A), prevalence, and infection intensity (Fig. 15B). Thus, a cool wet year following a warm dry year is sufficient to terminate an epizootic.

Recruitment and Juvenile Mortality

The decrease in recruitment in year 2 used for the simulation shown in Figure 6 was a 50% reduction from 2 in 10^9 to 1 in 10^9 between March and August. Offsetting this decrease required a recruitment success of 6 in 10^9 during the same period in year 3. The decrease in recruitment produces a mortality event, which is the beginning of an epizootic, in year 2 (Fig. 16A). The start of the epizootic is also identified by an increase in infection intensity (Fig. 16B) and prevalence (Fig. 16C). These trends are offset by increased recruitment success in year 3 as evidenced by decreases in prevalence and infection intensity that continue for the remaining 3 years of the simulation. As seen in other simulations, the primary record of the epizootic is found in the prevalence and infection intensity for the entire population rather than in the market-size individuals (Fig. 16B and C). Population abundance and mortality from *P. marinus* infection return to normal by year 4 and retain the characteristics of an expanding population (cf. Fig. 4) for the remainder of the simulation (Fig. 16A).

DISCUSSION

Thresholds exist which trigger *P. marinus* epizootics. These thresholds are defined by a combination of oyster fecundity, recruitment, and growth which determines the population dynamics of the species relative to the capabilities of its parasite. In oyster populations near the threshold, subtle changes in the environment are sufficient to trigger an epizootic. How frequently populations approach threshold conditions is unclear; however, the infrequency of epizootics in light of the small environmental perturbations required to trigger an epizootic in a susceptible population suggests that the population dynamics of most populations allows them to reside some distance from the epizootic threshold.

Epizootics are triggered by three general classes of environmental and biological perturbations: factors affecting food supply, factors affecting environmental characteristics, and factors affecting the supply of juveniles in the population. Factors affecting food supply include food supply itself, turbidity, competition with other filter-feeding species, and current flow. Environmental characteristics are principally temperature and salinity. Factors affecting the supply of juveniles include settlement success and juvenile mortality. Each of these interferes in one way or another with the rate at which populations recruit adult individuals.

In the common case where prevalence exceeds 60% and infection intensity rises to 3 or more during the summer months, most oyster populations will suffer adult mortality due to *P. marinus*. Stability is maintained by an adequate rate of adult replacement to minimize the effect of these losses on adult density and population fecundity. These recruits not only eventually maintain population fecundity, but also they replace heavily infected individuals with those with lighter infections. Epizootics are triggered when adult recruitment fails to replace those adults that die with adults of lower infection intensity at a rate adequate enough to dilute the adult infection intensity below about 3.5. The simulations show that one of the key changes in the population is the increase in infection intensity of the subadult and submarket-size adult portions of the population.

Accordingly, the simulations suggest that the key to triggering an epizootic is to raise the infection intensity in the subadult and submarket-size adult portions of the population, and indeed, most of the triggering mechanisms do just that. Infection intensity of market-size individuals is maintained at a relatively stable level by the death of heavily infected individuals. Consequently, an increase in the infection intensity of market-size individuals is normally neither obvious nor important. If the simulations are correct, it is ironic that the standard methods used to assess field infection intensities in *P. marinus* select for that fraction of the population least likely to provide information on the health of the population and least likely to provide early warning signals of an impending epizootic.

Ranking the triggering mechanisms by their ability to generate an epizootic is relatively difficult. Some of the variables, like temperature, do not vary over a wide range. Others, like recruitment, vary over orders of magnitude. The simulated conditions have been chosen to fit within the range of the variable as usually observed in the field. Based on these simulations, a rough ranking would suggest that factors affecting food supply are more likely to trigger epizootics than changes in temperature and salinity. The duration of the trigger is also important, however, and conditions conducive to the triggering of an epizootic may remain present longer for temperature and salinity than for food supply. One of the problems in assessing the importance of these variables in oyster populations is the limited information available on triggers of observed epizootics.

The characteristics of a developing epizootic were identical for all triggering mechanisms except low salinity. A drop in salinity resulted in an immediate mortality event suggestive of mortality due simply to low-salinity conditions as normally described for the warm months of the year. Such events may be intensified by disease rather than being due simply to the low salinity present.

Excepting this unusual case, all other epizootics followed a typical time course observed for epizootics of most other invertebrate and vertebrate species. The conditions triggering the epizootic occurred and disappeared well before, and as much as 18 months before, the initiation of mortality in the population. Unfortunately, identifying triggering mechanisms for observed epizootics is likely to be difficult unless, by serendipity, a long-term time series of data is available for the affected population. Once started, most epizootics progressed more or less rapidly toward population extinction. No internal mechanism was available to limit their time course. In the intermediate period between the trigger and the first major mortality event, prevalence and infection intensity rose in the population; the majority of this rise was concentrated in the subadult and submarket-size adult fractions of the population.

One of the interesting outcomes of this set of simulations was the difficulty in generating an epizootic simply with changes in temperature and salinity. Temperature and salinity share the blame for most epizootics. However, many populations, particularly in the Gulf of Mexico, exist under conditions of high temperature and salinity without initiating an epizootic. Although evidence is meager, most epizootics may occur in populations stressed by one of the other mechanisms prior to the high-temperature and high-salinity conditions that facilitate the mortality event. One of the most likely is recruitment failure. Timely failure to introduce uninfected individuals into the population is likely a principal mechanism increasing population infection intensity and initiating an epizootic. However, in cases where temperature and salinity are the cause, it is the winter conditions that seem to be most important, at least for the Gulf of Mexico.

Stopping an epizootic may be hard to do. The simulations generally required conditions substantially more extreme to stop an epizootic than to start one. Accordingly, local extinction is likely the most common outcome and the most common mechanism of terminating the epizootic. Stopping an epizootic otherwise requires reducing the infection intensity in the submarket-size adults and subadults in the population. The simulations suggest that a principal mechanism is a large recruitment event which dilutes *P. marinus* in the population, although it does not affect the infection intensity of the market-size adults. One crucial message from these simulations is that the infection intensity of the market-size adults does not need to be reduced to stop an epizootic nor does it need to be raised to start one. It is the infection intensity of juveniles recruited to the adult population and of adults recruited to market size that is important.

Overall, the most important message from this series of simulations may be the implications for management of oyster populations. Clearly, apparently healthy populations may reside very near the threshold for an epizootic. Also, an epizootic may be triggered 1 to 2 years prior to the major mortality event defining it, depending upon the environmental conditions and the external supply of recruits to the population. The simulations suggest that identifying populations nearing epizootic mortality levels may be as easy as obtaining an adequate time course record of prevalence and infection intensity across the entire size-frequency spectrum of the population. Identifying populations residing near the epizootic threshold is likely to be extremely difficult. Whether such populations have specific population dynamics attributes or specific disease characteristics is not clear based on the simulations presented here. One possibility is that such populations cannot be identified without simulation modeling of the host and its disease.

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MANAGEMENT ALTERNATIVES FOR PROTECTING *CRASSOSTREA VIRGINICA* FISHERIES IN *PERKINSUS MARINUS* ENZOOTIC AND EPIZOOTIC AREAS

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ABSTRACT We review management of oyster stocks infected by *Perkinsus marinus*, comparing previously published recommendations with current practices, with emphasis on the public fishery in the Maryland portion of Chesapeake Bay. The epizootiology of perkinsiasis is described, particularly the spread of the disease into low salinity areas. We also describe recent attempts to develop a policy and management framework for restoration of oyster populations that have been depleted by *P. marinus* and *Haplosporidium nelsoni*. It is apparent from experiences in Gulf of Mexico estuaries, Long Island Sound, and Maryland that strong recruitment can, to some extent, offset the impacts of *P. marinus* on oyster fisheries. Although improved management practices so far have had very limited success in maintaining harvestable stocks in the Chesapeake, it is clear that the recruitment potential of oyster populations has not been diminished to a critical point. Strategies designed to enhance and supplement natural recruitment, along with maintaining growing areas as free from *P. marinus* infections as possible, currently offer the most promise for maintaining harvestable stocks. In combination, new developments in research, management, monitoring, and policy are cause for guarded optimism, both for larger, sustainable harvests and for restoration of some of the ecological functions of healthy oyster populations.

KEY WORDS: *Crassostrea virginica, Perkinsus marinus*, oyster management, fisheries management

INTRODUCTION

The protozoan *Perkinsus marinus*, phylum Apicomplexa, parasitizes eastern oysters, *Crassostrea virginica*, causing extensive mortality throughout its ecological range. The present known range for the parasite extends from the southern Gulf of Mexico through southern New England. Basic aspects of the taxonomy, life history, and ecology of the parasite have been described (Andrews 1954, 1965, 1988; Mackin 1962; Ray 1966; Perkins 1988). Many field and laboratory infection studies have provided sufficient information to predict the impacts of enzootic and epizootic episodes of perkinsiasis ("dermo disease") in oyster populations (e.g., Paynter and Burreson 1991, Fisher et al. 1992, Smith and Jordan 1992). Two of the more prolific investigators of this disease, Andrews and Ray (1988) delineated several management strategies to control the impacts of *P. marinus* in oysters. Their suggested management practices focused on manipulation of privately owned or leased beds where oyster populations were traditionally completely harvested and replaced by a new crop of seed oysters. However, several important attributes of the host-parasite interaction need to be reconsidered and some of the basic management concepts modified to reflect recent research findings (Saunders et al. 1993, Bushek et al. 1994, Roberson et al. 1993). In this paper, we first review the interactions of the host-parasite relationship with 1) environmental factors, 2) oyster recruitment, and 3) traditional fishery and management practices. Second, we reiterate long-established concepts of how oyster populations can be managed to minimize the impacts of perkinsiasis. Third, we present the current status of management and some future directions, with emphasis on the experience in Maryland and the Chesapeake Bay.

INFLUENCE OF ENVIRONMENTAL FACTORS ON *P. MARINUS* EPIZOOTIOLOGY

Throughout its range, *P. marinus* is most active in producing pathology in oysters during the warmer months of the year. Perkinsiasis exhibits a gradient of infection with high prevalence and intensity in high salinity areas and lower infection levels in populations at low salinity in the same estuaries. North of Cape Hatteras, the parasite becomes dormant during winter and early spring and frequently is undetectable by histology or by thioglycollate culture of rectal and gill tissue. Subpatent infections are known to exist in many individuals within an overwintering population. These animals initiate new generations of intense infections in the population from May through October. The process of re-establishment of detectable infections in individual oysters is compromised by water temperatures below 20°C and by salinity below 8–10 ppt. As a result, a gradient of severe to light infections is re-established annually in populations along the salinity gradient in all estuaries. Upon re-establishment of detectable infections in spring, a range in diagnosable infections may be found even in populations that had 100% high intensity infections in the fall of the previous year. The gradient of *P. marinus* disease prevalence and intensity in estuaries was first reported by Mackin (1962) in his classic study where he found heavy infections of *P. marinus* in oyster populations living in environments where salinity frequently dropped to 2 ppt. Surveys along the coast of the Gulf of Mexico conducted by later investigators (Craig et al. 1989, Soniat 1985, Turner 1985) described variations in disease pressure among oyster populations and confirmed the existence of active *P. marinus* infections in oyster populations living in salinities of 1–2 ppt.

Since 1988, perkinsiasis has become more active in Chesapeake Bay and has spread into the low salinity environments of Maryland and Virginia estuaries. The disease is now found throughout oyster-producing areas of the Chesapeake. Populations that have high prevalence and intensity of infection during the summer months have high annual mortality regardless of their geographic location (Andrews and Ray 1988, Otto and Krantz 1980, Krantz, 1991, 1993, 1995).

Infections by *P. marinus* in Chesapeake Bay oysters occur primarily in the warm summer months; previous investigators hypothesized that direct transmission of *P. marinus* occurred from

overwintered infected oysters to uninfected oysters and that proximity of uninfected to infected oysters was a significant variable subject to management manipulation. Careful monitoring of the water column using optical sorting of filtered particles labeled by fluorescent antibodies has suggested that *P. marinus* may be widely spread throughout the Chesapeake Bay by waterborne stages of the parasite (Roberson et al. 1993). Oyster exposure studies in trays deployed in Chesapeake Bay demonstrated that site-specific infection rates had more influence on seasonal levels of *P. marinus* than initial infection levels in the experimental oysters (Meritt 1993). In an unpublished study, G. F. Smith (Cooperative Oxford Laboratory) found evidence that large doses of apparently waterborne particles could initiate epizootic levels of perkinsiasis during late summer in a deployment of experimental oysters. Any successful management strategy must consider that epizootics can be initiated by two mechanisms: 1) rapid proliferation and spread of enzootic infections within a local subpopulation, or 2) advection of waterborne infective stages of the parasite from other infected subpopulations of oysters. The spatial scale on which the latter mechanism operates is unknown.

The invasion of *P. marinus* into low salinity seed oyster areas was a turning point in the host-parasite relationship within the Chesapeake Bay system (Andrews and Ray 1988). Once heavy levels of *P. marinus* infection occurred in seed oysters, the pathogen was spread rapidly throughout the Chesapeake Bay system by the practice of planting private and public seed beds. Based on recent surveys (Burreson 1989, Krantz 1993) it appears that *P. marinus* is established throughout Chesapeake Bay, with the highest intensity of infections on some of the best oyster-growing areas. Annual, seasonal, and spatial variations in salinity within the Bay suppress the impact of the disease on some host populations. However, once a specific population becomes infected with *P. marinus* it may be expected to experience epizootic losses whenever environmental conditions favor the parasite. Therefore, because *P. marinus* never can be eradicated from enzootic areas, management should focus on activities that will reduce the losses of oysters in the expectation of epizootic levels of perkinsiasis (Andrews and Ray 1988).

Temperature apparently is a regulating factor for *P. marinus* prevalence and infection intensity. Warmer water temperatures in the Gulf of Mexico increased the intensity and duration of perkinsiasis even at lower salinities (Andrews and Ray 1988). Recent experience in Maryland has supported the idea that cold winters and high rainfall are mitigating factors. The prevalence and intensity of *P. marinus*, which had reached epizootic levels throughout Chesapeake Bay by 1992, decreased considerably in 1994 (at least in Maryland waters) after two successive wet, cold winters. Improved growth and survival during 1994 led to geographic expansion of the commercial harvest during the 1994–1995 season, along with increased landings (Krantz 1995).

Alternate molluscan hosts in the natural environment can be important as reservoirs for *P. marinus* (Perkins 1988, Andrews and Ray 1988). Andrews (1954) found putative *P. marinus* cells in a majority of the potential molluscan host species that he sampled in the Chesapeake Bay system. Many of these species are natural inhabitants of Chesapeake Bay oyster bars, and some develop dense populations in the muddy bottom immediately adjacent to natural oyster bars. When an oyster-planting site has been depopulated (e.g., by disease), the remaining shell base rapidly becomes heavily colonized by other molluscan species. Mollusks parasitized by *P. marinus*–like organisms appear to shed cells from their intestinal tract throughout the summer and could be a significant source of infectious material in the immediate vicinity of any managed oyster bar.

The question remains whether all of these molluscan parasites are in fact *P. marinus* or closely related species that do not parasitize oysters. White et al. (1987) found a gastropod, *Boonea impressa*, that was capable of transmitting *P. marinus* from one oyster to another or from populations of the ectoparasitic snail, a natural inhabitant of oyster bars. Antisera against *P. marinus* cells cultured *in vitro* recently were used to assess the serological similarity of the *P. marinus* parasites found in other molluscan species; several Chesapeake Bay mollusk species contained organisms which cross-reacted strongly with antiserum prepared with *P. marinus* cultured from oysters (Dungan and Roberson 1993).

MANAGEMENT CONCEPTS

In a discussion of management strategies to control levels of mortality caused by *P. marinus*, Andrews and Ray (1988) focused their recommendations on the importance of planting disease-free oysters on cultivated barren bottom or on natural oyster bars from which all previous generations of diseased oysters had been removed. At the time of their recommendations, a few sanctuaries of seed oysters free of *P. marinus* infections existed in Chesapeake Bay. By the early 1990s, however, virtually all natural oyster populations contained some oysters with detectable levels of *P. marinus* disease. These authors concluded that ". . . control [of perkinsiasis epizootics] depends upon the return of normal rainfall and low estuarine salinities to suppress the disease which remains active during summer at levels of 12 ppt . . ."

Andrews and Ray (1988) outlined management procedures for controlling *P. marinus*, with the following recommendations:

> "1. Transplant only disease-free stocks. Even low prevalence of *P. marinus* will accelerate mortality.
> "2. Select growing beds isolated at least 0.4 kilometers from any other bed with infected oysters.
> "3. Early harvest (2–3 inches) followed by fallowing of beds limits mortality and distribution of the disease.
> "4 Monitor beds of growing eastern oysters for *P. marinus*. All planted beds should be examined . . . each late summer, or early fall for dead animals to determine if harvesting or another year of culture is desirable or necessary."

These recommendations were focused on controlled cultivation of private, leased beds where the removal of diseased oysters and the planting of only disease-free oysters could limit mortalities caused by *P. marinus*. Public oyster bars, however, are never completely harvested because of regulatory minimum size limits and gear inefficiencies, and because natural recruitment maintains a population of small oysters that serves as a reservoir for the parasite. Thus, control of *P. marinus* is more difficult on public beds than on private beds.

APPLIED MANAGEMENT PRACTICES AND THEIR OUTCOMES

Natural oyster bars in the Maryland portion of the Chesapeake Bay are the mainstay of a public fishery, with legally defined areas where only specific types of gear (hand tongs, patent tongs, oyster dredges, or hand collection by divers) can be used to harvest oysters. Hand tongs are extremely inefficient, and even under high harvest pressure, a significant percentage of adult oysters remains on the bottom following the prescribed harvest season. Patent

tongs are slightly more efficient at collecting oysters, but still a large percentage of the population remains. Power dredging and scuba divers are much more efficient on specific bottom types. However, any legal harvest operation will leave pockets of infected oysters on the natural bars. Smith and Jordan (1992) estimated that 53% of harvestable oysters (≥76 mm) were taken from harvested oyster bars (all gears combined) in Maryland during 1990–1991.

For many years, oyster managers have depended on movement of oysters from established seed areas to growing areas. In earlier decades, the main purposes of this practice were 1) to relieve overcrowding in areas of very high recruitment, where oysters did not grow well because of competition for food and space, and 2) to distribute the harvest geographically according to the locations of private growers or public fisheries. When *P. marinus* and, to some extent, *Haplosporidium nelsoni* (MSX) became epizootic in the Chesapeake, this practice was adapted to increase survival of stocks to harvestable size by concentrating growing areas in lower salinity reaches of the Bay and tributaries. Maryland instituted a new practice of producing seed oysters for the public fishery by planting dredged fossil shell on hard bottom with few if any existing natural oysters. The seed plantings now are made several miles from heavily infected natural oyster populations. Several of the most recent plantings have been made on the western side of Kedges Straits (Fig. 1); production of seed oysters has been very successful. Movement of these seed oysters, even though lightly infected with *P. marinus* and *H. nelsoni*, to areas of low salinity in the upper reaches of the mainstem of the Bay and other subestuaries has been successful in producing harvestable oysters (Krantz 1995). In the Chester River this strategy has maintained a public fishery even during a series of summer drought conditions that induced epizootic levels of *P. marinus* over all of the growing grounds in Maryland (Fig. 2).

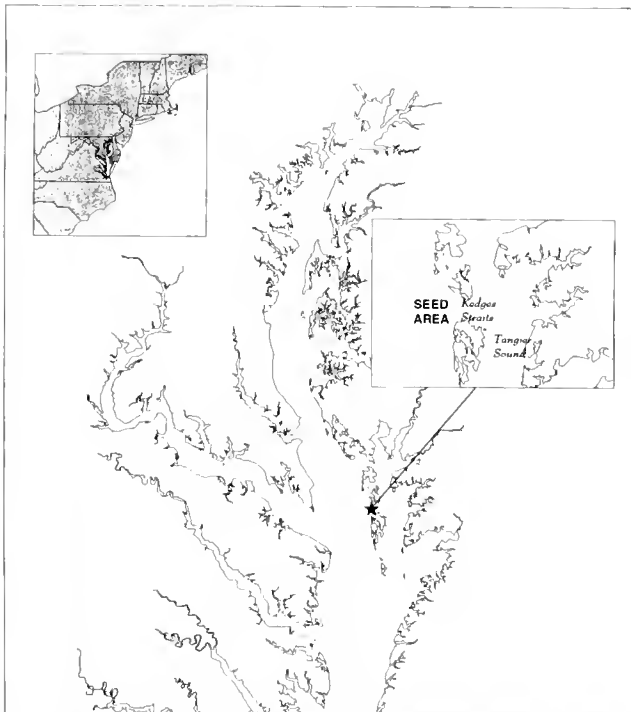

Figure 1. Northern Chesapeake Bay showing the location of the principal current (1995) Maryland seed oyster production area.

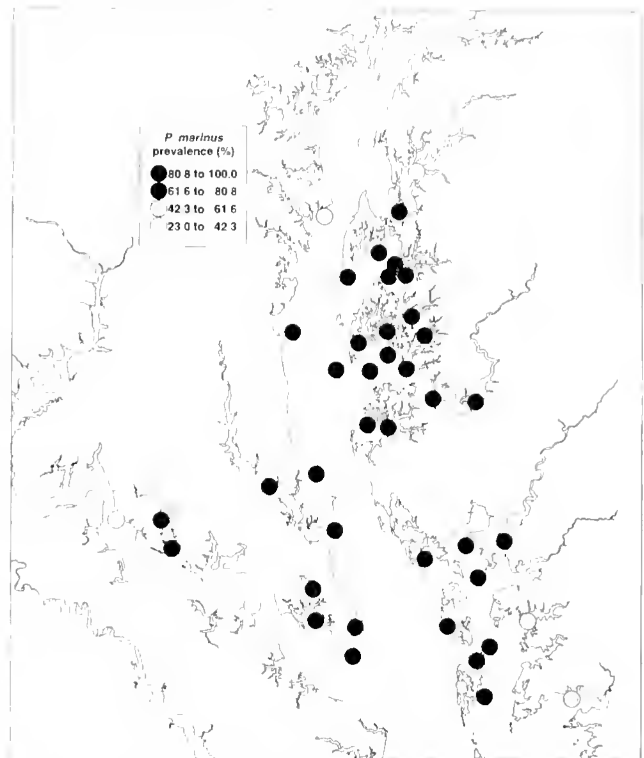

Figure 2. *P. marinus* prevalence (%) in Maryland oyster populations in the fall of 1992, based on thioglycollate incubations of hemolymph samples from 30 oysters at each of 43 monitoring sites.

Concerns about spreading pathogens through movement of infected seed stocks, and strong interest in relieving growing areas from the pressure of *P. marinus*, led the Maryland Oyster Roundtable (Maryland DNR 1993) to establish six Oyster Recovery Areas in Chesapeake tributaries (Fig. 3). Zones were designated in each of these tributaries where no diseased seed oysters (defined as oysters with zero prevalence of *P. marinus* and *H. nelsoni* as determined on a random sample of seed by standard diagnostic methods) were to be planted. Because production of disease-free seed oysters in the natural waters of Chesapeake Bay cannot be assured, experimental quantities of seed oysters are being produced in hatcheries, with a long-range goal of supporting seed requirements for the disease-free zones, as well as for private growers. Ambient water, however, is used to grow the newly set spat in all of Maryland's oyster hatcheries. Therefore, contamination of hatchery-produced seed by waterborne *P. marinus* could occur during the postsettlement and early growth periods. Little is known about *P. marinus* infections in very young oysters or larvae, but if hatchery seed is grown in water where *P. marinus* is enzootic, it is likely that they will become infected within the first year (Krantz 1993).

Selecting oyster-growing beds that are isolated from other beds is difficult where natural oyster bars are the basis of a public fishery, as in Maryland. Although oyster "bars" in parts of the Atlantic and Gulf coasts are discrete features of the estuarine seascape, in large areas of Chesapeake Bay there are nearly continuous populations of oysters in much of the habitat, albeit with large variations in the density of oysters. Recent studies on waterborne particles in the Maryland portion of the Chesapeake Bay indicate that putative *P. marinus* particles are present in the water column from April through October, with peak concentrations in

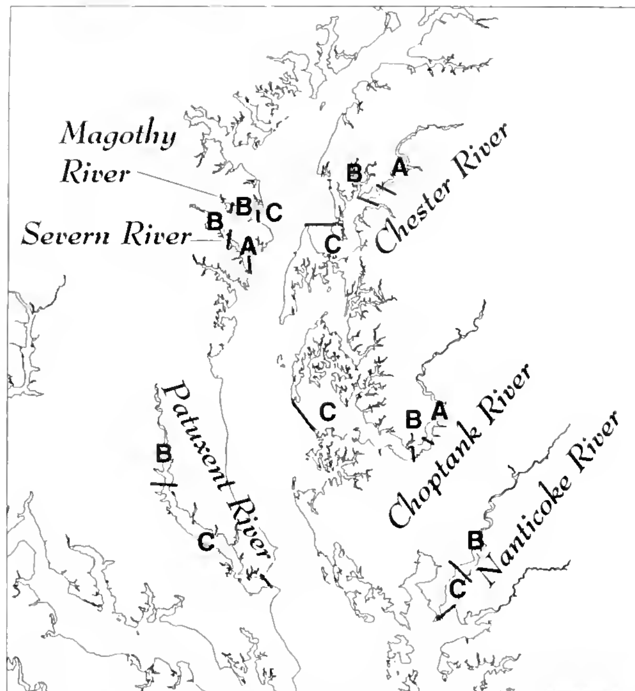

Figure 3. Maryland Oyster Recovery Area tributaries with approximate zone boundaries. See text for zone definitions.

June (Roberson et al. 1993). Burreson and Andrews (1988) found that spatial isolation of groups of oysters did not protect them from *P. marinus* during drought conditions in 1985–1987. This was a period of geographical expansion of *P. marinus* into uninfected populations in the low salinity sanctuaries in Maryland. Evidence, albeit indirect, has accumulated that waterborne concentrations of *P. marinus* may be more important in establishing epizootic levels of the disease than proximity of infected natural oysters to the newly planted seed.

Early, exhaustive harvest of planted beds and allowing the beds to remain fallow between plantings could not be an option for a public fishery without major changes in law and management practice. For example, the Maryland 3″ (76-mm) cull law has been in place since the early part of the 20th Century and has been thought to protect the spawning potential of the stock. The problem is that perkinsiasis, alone or in concert with MSX, crops a significant percentage of a given year class before it reaches 76 mm. Lowering the legal size to, say, 2½″ (64 mm) would allow increased exploitation of younger year classes while increasing the abundance, if not the biomass, of the harvest. Exploitation of the smaller oysters, however, could decrease the recruitment potential of the population. Further, it has been suggested that minimum size limits cause selective pressure for smaller size at maturity (Allen and Bushek 1994); if so, then decreasing the cull size could exacerbate this effect. A decrease in the cull size also could interact with the host-parasite relationship by providing fewer opportunities for oysters to survive *P. marinus* infections, reproduce, and thereby expand any genetic resistance, should it exist, in the population. A "slot limit" (e.g., 2½–4″ or 64–102 mm), as proposed for evaluation in the Maryland Oyster Recovery Action Plan (Maryland DNR 1993), would protect some larger, more fecund, and perhaps resistant oysters. It is questionable, however, whether there are enough oysters >102 mm remaining in Chesapeake populations to mitigate the negative effects of a smaller harvest size.

A smaller size limit also could lower the retail value of shucked oysters to a point where it would make the fishery economically unattractive to both processors and watermen. Virginia implemented a reduction in size limit to 2½″ for the James River seed area in 1991. This action resulted in the taking of increased amounts of James River seed. In the following years, harvests and recruitment were greatly depressed, and Virginia now lacks natural seed oysters to implement improved management on any significant scale.

MONITORING OF *P. MARINUS*

Andrews and Ray (1988) suggested monitoring oysters 2 years of age or older by the thioglycollate culture method in late summer and early fall. They suggested that the beds be concurrently examined for mortality. Population mortality rates and disease prevalence then could predict whether planted oysters could survive and grow another season or had developed high intensity infections of *P. marinus* that would predispose them to high mortality during the coming months. If oysters were found to be heavily infected, then they should be harvested regardless of their size or meat quality. If prevalences and mortality were low, indicating an enzootic *P. marinus* infestation, the grower could risk leaving the oysters in place until the spring and early summer of the following year. After 2 years of exposure to *P. marinus*, however, summer mortalities can be significant, and any population found to be enzootic for *P. marinus* in the fall should be monitored early in the following summer.

Maryland implemented a comprehensive disease-monitoring program for natural oyster bars in 1987. It was designed to document and follow recently established *P. marinus* infections, as well as the impacts of epizootic *P. marinus* in populations where the parasite had been established for several years. This monitoring program was instrumental in determining the geographic expansion of perkinsiasis in Maryland waters and has delineated areas within subestuaries where *P. marinus* remained at low enzootic levels and induced only minimal levels of mortality in natural oyster populations. The monitoring program has continued to follow seed oysters planted in both enzootic and epizootic portions of Maryland waters and documented site-specific changes in prevalence and intensity over an 8-year period (Krantz 1989a, 1990, 1991, 1993, 1995). The monitoring program was modified in 1990 to produce consistent annual observations on selected oyster bars regardless of their disease status. Information from the fall field surveys on population structure, size frequency, mortality, and spatfall is now collected from 64 stations distributed throughout the Maryland portion of the Bay. A subset of 43 of these stations is monitored for *P. marinus* and *H. nelsoni* prevalence and intensity (Fig. 4). These stations were originally part of a long term monitoring effort to document oyster recruitment dynamics on natural bars in the Maryland portion of the Bay. The monitoring program is linked to a geographical information system (Smith and Jordan 1992) that includes several attributes of oyster habitat such as water quality, bathymetry, and substrate type, in addition to the population and disease data. Analysis and modeling studies are in progress to better understand the dynamics of *P. marinus* in oyster populations and to evaluate the effectiveness of management strategies, both tried and proposed.

In areas known or suspected to harbor *P. marinus* (virtually the entire Atlantic and Gulf coasts), seed oysters to be transplanted should be monitored for diseases before they are moved. The seed

Figure 4. Maryland Fall Oyster Survey population and disease-monitoring sites. Circles: population samples (size, mortality, spatfall, etc.) only; crosses: population and disease samples.

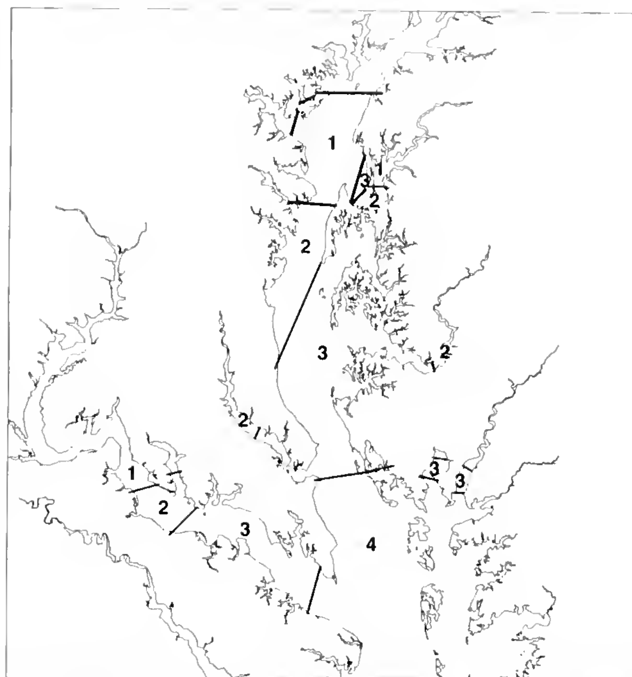

Figure 5. Maryland Chesapeake Bay oyster management areas (1992) based on potential disease impacts (adapted from Krantz 1993). The areas are numbered according to potential disease impacts, with Area 1 representing the safest growing areas and Area 4 representing areas most likely to be affected by both *P. marinus* and *H. nelsoni*. Areas 2 and 3 represent increasing risks of parasite-induced mortality, primarily from *P. marinus*, although *H. nelsoni* can affect these areas during drought periods.

area should be evaluated during the fall prior to seed movement for prevalence and intensity of diseases. After planting, disease status and mortality should be monitored in the fall of the second and third growing seasons.

NEW DIRECTIONS IN MANAGEMENT

Oyster management in Chesapeake Bay has taken on a posture of avoidance of areas where the disease is epizootic and has focused plantings of seed oysters in areas of low *P. marinus* pressure, the equivalent of low salinity sanctuaries described by Andrews and Ray (1988). The collective experience of previous surveys for disease prevalence, oyster mortality, and response of planted seed oysters to documented levels of fall disease prevalence and intensity permitted the delineation of management areas in Maryland (Fig. 5). The four areas were based on the combined effects of *P. marinus* and *H. nelsoni* during the past two decades. Each year's management recommendation for the areas is tempered by the results of the previous Fall Survey, which may detect diminution or increases in prevalence and intensity of the diseases in the areas. The recent report of the 1993 and 1994 Maryland surveys (Krantz 1995) is an example of how monitoring technology can assist the management agency in selecting areas that will optimize expenditure of funds for the placement of seed oysters, shell for the collection of natural spat and oyster bar rehabilitation, and the development of seed-producing areas.

Although oyster population and disease monitoring in Maryland is reasonably comprehensive, information on salinity, temperature, and water quality patterns throughout the year could help management to anticipate disease and recruitment conditions. A geographical information system (developed for managing oyster-monitoring data) is being used to develop spatial interpolations of the extensive Chesapeake Bay water quality monitoring database (Heasly et al. 1989), in combination with modeling studies of population and disease dynamics. Eventually, these efforts should provide some ability to predict oyster infection prevalence and intensity, mortality, and population structure from knowledge of seasonal and interannual environmental conditions (Hoffman et al. 1995).

Maryland, like most mid-Atlantic coastal areas, relies on naturally set seed for planting oyster bars that received light or no spat set, yet are in very productive growing areas. These areas have been identified by annual fall surveys in Maryland waters that began in the mid-1930s. Collective knowledge and memory of the performance of plantings at specific sites are passed from one generation of oyster management biologists to another through practical field exposure. Seed production areas in the state require a comprehensive knowledge of the past history of the natural oyster bars in that area, changes in the bottom type, the potential impact of storm events during the winter and early spring, environmental water quality conditions during the summer setting area, and most important, the past history of natural spat set. The investigation of natural spat set is one of the pursuits of the Fall Survey, and historical information has been used to locate areas that have the most probable chance of acquiring a natural spat set. Detailed information on natural spat set on the oyster bars has been described by Krantz and Meritt (1976) and is currently being updated by the senior author for publication. During the past three decades, seed areas in the Maryland portion of the Bay that have produced a cost-effective quantity of spat on planted shell have

changed dramatically. Historically, the management agency had the choice of producing seed in St. Marys River, upper Tangier Sound, Honga River, Little Choptank River, Harris Creek, Broad Creek, and Eastern Bay (Fig. 6). Naturally set seed oysters first showed a decrease in numbers and frequency of set in St. Marys River, Eastern Bay, and portions of Tangier Sound. During the past decade, seed production has shown a radical decline in Harris Creek and Broad Creek and a diminution in the Little Choptank and Honga Rivers. As this decrease in spat set occurred, a concurrent increase in spat set on sparsely populated bottom along the Chesapeake Bay side of St. Marys County and in lower Tangier Sound was noted. As a result, state-managed seed areas now have been moved to areas where the highest spat set can be obtained.

Substrate for seed production areas is critical to continuous production of seed to be transported. Shell is collected from all Maryland processing and shucking plants, to be planted back in the Bay. However, as harvest has decreased, the quantity of freshly shucked shell has become inadequate for seed production. Unfortunately, fresh shell is dispersed throughout the entire Chesapeake Bay, and the cost of hauling all of the shells to the seed beds is excessive. Therefore, fresh shell from packing houses is now used to rehabilitate oyster bars located near packing houses, or to add to the planting of dredged fossil shell on seed beds.

Dredged fossil shell is obtained in the upper Chesapeake Bay through a contract with a large dredging company that has the capability of digging 35–40 feet below the Bay surface and escalating oyster shell to the surface. The shell is then graded, washed, and transported by barges and tug boats to areas selected by the management agency to be planted. Annual planting of dredged shell on the best seed areas usually produces 500–2,500 spat per bushel of dredged shell material at a given location. Spat set on natural oyster bars adjacent to these areas would range between 50 and 250 spat per bushel of shell during the same season. The

Figure 6. Areas used historically for natural seed oyster production in Maryland's Chesapeake Bay.

seasonal periodicity of planting the dredged shell has been a point of great concern. Maryland has accumulated records of periodicity of spat fall on natural oyster bars, planted shell, and experimental spat collectors for over three decades. These data show 90% of the annual recruitment in any given year occurs between the second week of June and the end of July. Shells planted during this time will usually receive an adequate concentration of spat to be used as seed the following spring.

By law, seed areas may not be located on productive natural bars in Maryland waters. Therefore, considerable effort must be expended to find firm bottom capable of supporting dredged shell. Frequently, natural oyster bars that have been heavily harvested or devastated by disease become covered by a layer of sediment 5–10 cm in depth, but they can be used as seed areas. The planting of dredged shells to a depth of 10–15 cm on top of this substrate provides a base for oyster attachment, but at the expense of losing some of the shells in the reconditioned substrate. Records of shell placement to establish new seed areas revealed that only ⅓ to ½ of the planted shell can be recovered with spat on it. A high percentage of shell settles into the soft surface and becomes anaerobic and unsuitable for the attachment of spat.

Two decades ago, there were areas in the Chesapeake Bay in Maryland and Virginia that had relatively high spat fall and no disease in the natural oyster populations. Since 1980, this situation has changed. Andrews and Ray (1988) pointed out that the ability to manage around perkinsiasis was greatly altered when all of the seed area became infected. Even the low salinity sanctuaries in the Chesapeake Bay now have occasional and temporary excursions of *P. marinus* in the natural populations that establish reservoirs to infect seed oysters.

Maryland management agencies have found by trial and error that new seed areas can be established annually if the areas are carefully chosen. Spat settlement in Maryland waters is between June and the end of July. By fall of the first year, some of these seed areas have been demonstrated to be free of detectable levels of *P. marinus*. In certain years, some seed areas will acquire low prevalences of very light infections (up to 30%). These seed can be moved the following spring, prior to the biologically active season for *P. marinus* development, and placed on oyster bars for growth. Current policy, however, does not permit planting of oysters with any detectable level of *P. marinus* infection in Zones A or B of Oyster Recovery Areas (Fig. 3).

Occasionally, spat set on a selected seed area is light and the number of seed is below the level that is economically attractive for movement (350–500 spat per bushel). Such seed beds will be left for another biological season in which more spat will attach to the shell. In these situations, it has been the experience in Maryland waters that *P. marinus* will increase in prevalence and intensity during the second biological season. Often, seed that remained on the seed areas for two seasons had a prevalence of 100%, with infections at lethal severities in 30% of the animals. In other cases, seed can remain on the dredged shell for two biological seasons without contracting detectable levels of *P. marinus*. It is rare, however, to find a seed area that is free of disease, because all oyster bars surrounding productive seed areas in Maryland have high prevalence and intensity of *P. marinus* infection. In summary, the management agency has encountered four distinct classes of seed beds, which vary both spatially and from year to year:

1. Seed areas with high prevalence and high intensity of *P. marinus* infections.

2. Seed areas with high prevalence, but low intensity of *P. marinus*.
3. Seed areas with low prevalence of *P. marinus*, but infected animals have severe infections.
4. Occasionally seed areas will have the most desirable disease status—low prevalence of *P. marinus* with low severity of infection.

The general outcome of moving the above four categories of seed can be predicted based on general observations of cumulative mortality of oysters caused by epizootic levels of *P. marinus* in Maryland waters (Table 1). Table 1 summarizes field observations, along with experimental exposure of oysters in bags and trays at various sites throughout Maryland during the last decade. In some cases, all of the oysters would be destroyed by a combination of *H. nelsoni* and *P. marinus* in two seasons, leaving only a few trays free of MSX, from which the data on *P. marinus* were taken. However, a voluminous amount of data on the relationship between prevalence and intensity of perkinsiasis and resultant mortality have been accumulated by the annual Fall Surveys (Krantz 1991, 1993, 1995; Smith and Jordan 1992).

The outcome of transplanting seed oysters with a high prevalence and intensity of *P. marinus* infections will be moderated by both the environment and the disease pressure of the area in which they are planted. Seed oysters with a high prevalence and high disease intensity routinely develop a prevalence in excess of 65% during the first year of life. Population mortality during the first and second biological seasons will reach levels shown in Table 1. By the third biological growing season, cumulative mortality of seed oysters with these characteristics may reach 90–95% and very few of the animals will enter the annual harvest.

Transplantation of seed oysters with a high prevalence and low severity of *P. marinus* usually requires 2 years at the new site before the population reaches epizootic levels with 20–30% severe infections. By the time the third biological season is completed, population mortality may range from 50 to 75%. Planting seed oysters with a low prevalence but with a high severity of infection usually follows a similar track. By the end of the second year, the population reaches epizootic status and begins experiencing high mortality. During the third biological season cumulative mortality may exceed 50–60%. Planting of seed oysters with low prevalence and infection in low salinity environments will permit the population to grow at normal rates for the area until the third biological season, in which time the population may reach epizootic levels, with losses of 10–30% of the animals by the time they reach harvest size.

Seed oysters with low prevalence and severity of *P. marinus* infection may show no significant mortality when transplanted into a low salinity area typical of the sanctuary zones described by Andrews and Ray (1988). This concept is the basis for Maryland's present oyster management program, in which seed oysters are taken from setting areas that traditionally have high prevalence of *P. marinus*. This seed is planted in low salinity sanctuaries. The Chester River (Fig. 3) is one of the areas with chronic *P. marinus* infections, but prevalence and intensity are low enough so that oysters will grow to market size in a 3- to 4-year period with a loss of approximately 30% of the transplanted seed. Maryland has been able to maintain a "put-and-take" fishery in the Chester River and the upper Chesapeake Bay mainstem through the movement of lightly infected seed oysters. Table 2 shows the results of maintaining a "put-and-take" fishery while the harvest from natural bars in Maryland's waters dropped from 1.5 million bushels in the 1985–1986 season to 65,000 bushels in the 1993–1994 season. Less than 25,000 bushels of seed oysters were planted in the Chester River in 1986, with a harvest about equal to this input. At this point, the decision was made to stop planting oysters in the lower Bay, where high disease prevalence was killing the oysters before they reached market size. The seed that would have been planted in these areas was then relocated to the Chester River where a fishery resulted that by 1993 accounted for over 50% of Maryland's harvest. In earlier years, the Chester River could hardly be considered an area to support significant sustainable harvests because of very low annual recruitment.

From 1985 to 1995, the cost of collecting and hauling the seed oysters from the lower Bay seed areas has risen from $1.00 to $1.30 per bushel for the contracted labor and vessel. In addition, there is a highly variable cost for the amount of shell that is placed on the seed area from which the seed is taken. In some cases, as much as 50% of the shell can be recovered and moved, but in most cases only 30% of the planted shell will have seed on it. Dredged shell costs have been fairly stable during the past decade, ranging from 18.5 cents per bushel to the present cost of 26 cents per bushel. The "put-and-take" fishery being maintained in the Chester River has been characterized as a subsidized fishery, since the Department of Natural Resources has expended more than the severance tax (45 cents per bushel) gained from the packer who bought the oysters. For instance, in 1986, 24,695 bushels planted at a cost of $29,140 produced 50,679 bushels of harvested oysters (Table 2). The planting cost of $0.57 per bushel harvested was not recovered from the $0.45 per bushel severance tax, although other indirect benefits such as employment, income taxes, and economic

TABLE 1.

Expected cumulative mortality (%) caused by oyster diseases at epizootic levels in Maryland waters, based on a synthesis of several years of observations from natural populations and experimental deployments by the senior author.

	Year			
Parasite	**1**	**2**	**3**	**4**
P. marinus	27	58	81	92
H. nelsoni	46	77	89	94

TABLE 2.

Oyster production in the Chester River (Maryland) in response to planting natural seed oysters. The oysters were harvested 2–3 years after planting. Units are Maryland bushels.

Year	Seed Oysters Planted	Bushels Harvested
1986	24,695*	24,738
1987	55,465	20,597
1988	155,530	50,679
1989	114,675	54,029
1990	37,770	60,468
1991	83,590*	55,123
1992	161,684	53,803
1993	62,089	51,271
1994	74,431	Not available

* Supplemented by natural spat fall

multipliers would have to be considered in a realistic economic analysis.

If a state agency is considering management of oysters under conditions influenced by epizootic perkinsiasis, it must be prepared to subsidize the immediate costs of maintaining the fishery. One of the most important aspects of management of an oyster fishery during a *P. marinus* epizootic is the establishment of new seed areas each year, to produce a single year class of seed that will have a low level of infection. If dredged shell are planted on top of a previous year class, a percentage of the older year class with higher prevalence and more intense infections would be mixed with the new year class being produced by the seed area. However, the re-establishment of the new seed areas each year utilizes a greater percentage of shell resources as base material and contributes to the increased cost of the seed. The selection of the geographical location is an empirical and qualitative type of decision. All of the past experiences with planting and production of seed oysters, as well as the most recent monitoring data on the disease prevalence for seed and oysters on natural bars adjacent to the seed area, must be considered. Spat fall is highly erratic and virtually unpredictable from season to season in the Maryland portion of the Chesapeake Bay (Krantz and Meritt 1976, Krantz 1992, Dekshenieks et al. 1993). At present, the Maryland management agency selects two or three new seed production sites each year, in an attempt to produce the greatest concentrations of seed with the lowest prevalence and intensity of *P. marinus*.

In addition to the use of a disease-monitoring program and production of uninfected or lightly infected seed, some states use a quarantine zone (*P. marinus*-free) for producing natural seed oysters. No diseased oysters are introduced into the seed area and seed could remain in that area and not be transferred into the zone where *P. marinus* was present. This situation exists in Delaware Bay and some portions of Long Island Sound. Unfortunately, many areas in Delaware Bay that are free of *P. marinus* have low spat set, at concentrations that are not economically attractive to be moved. A quarantine concept could also be used to protect New England stocks from contamination by seed oysters or brood stocks from areas where *P. marinus* is epizootic or enzootic. The Atlantic States Marine Fisheries Commission initiated a shellfish transport management plan in 1989 that could be implemented to assist in establishing quarantine procedures to protect oyster-growing areas from importation of disease (Krantz 1989b). At the present time, the plan has been approved by Atlantic States Marine Fisheries Commission, but funding has not been appropriated to implement the committee to oversee and coordinate management among the New England states. This program would have a great impact on prevention of spreading *P. marinus* into new areas.

Low salinity culture of oysters is a possible alternative. Essentially, a program such as described above for Maryland could be implemented by the private sector. State and private sector organizations, working cooperatively, could establish hatcheries and seed areas in low salinity environments where the impact of *P. marinus* is minimal. The biological cost of this approach is usually a slower growth rate and poor, or at least unpredictable, meat quality. Low salinity culture sites must be very carefully selected and observations on small quantities of oyster seed and adults should be made over a period of years prior to the investment of large sums of capital. This strategy would allow management and investors in the low salinity mariculture system a chance to experience the natural diversity of responses of growth, disease fluctuations, spat set, and survival in the low salinity areas. Low salinity areas that inhibit *P. marinus* are usually those which are not optimal for the growth of oysters. Two to three additional years of growth are required in low salinity environments in Maryland waters for planted oysters to reach market size, compared to oysters grown in areas that are now heavily infected with *P. marinus*.

Technology for use of disease-free brood stock and oyster production in hatcheries is well known, but this approach has not been cost effective in the past (Krantz 1982). Closed-system hatcheries have the capability of maintaining brood stock and seed oysters that have not been exposed to disease. The costs of operating closed-system seed production has not been evaluated at this time. The greatest shortcoming to the hatchery approach is still the environmental setting in which the disease-free seed could be grown at their optimum. The low salinity environments with characteristics of the sanctuaries proposed by Andrews and Ray (1988), or the present growing grounds used in the Maryland portion of the Chesapeake Bay, are areas where oyster growth rate and meat production are not very good. Therefore, the economic return from an expensive hatchery seed oyster may not be realized when placed in the natural environment. Attempts to grow seed oysters to market size in hatcheries have been conducted in the past, and none of these have been shown to be cost effective.

Oyster geneticists continually express interest in developing oyster strains that are resistant to perkinsiasis. Andrews and Ray (1988) speculated on the possibility of finding strains of oysters that were resistant to perkinsiasis. In their studies, they found there were always oysters that survived epizootics. However, after studying 40 years of natural selection in the Gulf of Mexico, Ray responded to a question at the 1993 National Shellfisheries Association Annual Meeting in Charleston, SC, that he has never found a population of oysters in the Gulf of Mexico to become resistant and show a diminution in the prevalence of *P. marinus* and subsequent mortality to perkinsiasis. Andrews reported on numerous occasions to have obtained stocks of oysters from Virginia waters that acquired heavy infections of *P. marinus*, but that did not have much resulting mortality (e.g., Andrews 1965, 1967). Offspring from these strains appeared to have better survival than uninfected seed oysters that were placed in trays for comparison. However, Andrews' experiments always were compromised by the interaction of *H. nelsoni* that cropped a large percentage of his test animals. Interest in development of *P. marinus*-resistant strains has recently intensified. For example, Bushek et al. (1994) evaluated the host-parasite interaction for strains from a range of East Coast and Gulf Coast locations and determined that oysters and parasites, respectively, had heritable variations in resistance and virulence. Recently, a subpopulation of oysters from the Nanticoke River in Maryland was observed to have survived to large size (>102 mm) in an area that had experienced *P. marinus* epizootics for several years. Selective breeding and evaluation of F1 and F2 generations for *P. marinus* resistance are in progress, in a Maryland Department of Natural Resources and National Marine Fisheries Service cooperative investigation.

Despite the hopes, frustrations, and in some cases, moderate successes of attempting to manage around perkinsiasis, nature still has the upper hand. For example, after several successive years of record low harvests in Maryland in the late 1980s and early 1990s, the harvest more than doubled between the 1993–1994 (~70,000 bushels) and 1994–1995 season (~150,000 bushels by preliminary

estimates). This reversal followed two consecutive years (1993 and 1994) of high spring rainfall and runoff. Salinities in the northern Chesapeake Bay and tributaries dropped to the point where the Maryland Bay-wide average of *P. marinus* prevalence decreased from 84 to 53%, and *H. nelsoni* virtually disappeared after causing a record epizootic in 1992 (Krantz 1995). Harvests not only increased, but several areas produced harvests that had not been productive for a decade or more.

CONCLUSIONS

Clearly, there remains great potential for natural recruitment and recovery of oyster populations in the northern Chesapeake Bay, despite the depredations of the parasites and the often-voiced concerns about overharvesting a depleted resource. This potential probably exists in other oyster-growing areas, whether assisted by rational management strategies or not. Harvesters, packers, markets, and managers, however, cannot thrive (or survive?) in the face of the uncertainty of waiting for the next freshet. The problem, then, is to establish stable harvests that are minimally subject to natural variations in climate, recruitment, and parasitic infections.

Maryland's dominant oyster management strategy of recent years—establishing new seed beds and transplanting to low salinity growing areas—has resulted only in slowing the decline in harvests. The cost effectiveness of this technique has been marginal, at best. It remains to be seen whether the state's new oyster recovery strategy of establishing quarantined areas and sanctuaries, and encouraging and enhancing hatchery seed production, research and monitoring, will succeed. Experiences in the Chesapeake, the Gulf of Mexico, Long Island Sound, and probably other East Coast oyster-growing areas indicate that management of oyster populations infected with *P. marinus* should include the following elements:

1. Optimizing natural recruitment by developing and maintaining clean seed beds free of infected older oysters;
2. Preventing movement of infected stocks into growing areas—in some cases growing areas may need to be depopulated and left fallow for a time prior to planting;
3. Enhancing the capability of local hatcheries to supplement natural recruitment and to provide uninfected seed oysters and larvae for mariculture operations;
4. Careful monitoring of oyster populations and seed stocks, along with important environmental information such as temperature, salinity, dissolved oxygen, and turbidity;
5. Directed research, particularly in the areas of genetic resistance to *P. marinus* and improved brood stocks, more efficient diagnostic methods, and the ecological dynamics of host-parasite-environmental interactions.

Several major contributions have been made very recently to our knowledge of *P. marinus* and our ability to detect and experiment with the parasite. Genetic studies and selective oyster breeding are promising to produce a more resistant oyster, or at least to generate a better understanding of the problem. Improved monitoring programs are yielding excellent information on oyster population and disease dynamics on both system-wide and local scales. Modeling studies are applying this new knowledge to begin to predict outcomes of management strategies in the face of natural variability. New tools, new knowledge, and new skills are making *P. marinus* a more tractable problem and may soon result in practical approaches to better management of oyster stocks.

What is perhaps most hopeful in Maryland is that the Oyster Recovery Action Plan has laid the groundwork for an unprecedented partnership between the public fishery, private growers, managers, scientists, and environmentalists, with the goal of restoring both the ecological and economic benefits of oyster populations. Long term cooperation between these groups could ensure that scientific advances are applied responsibly and that concerns about overharvesting, habitat loss, and conflicts between the public and private fisheries are addressed jointly, rather than competitively. These groups are sharing information and working together to plan and implement moderate-scale oyster enhancement projects. New developments in research, monitoring, management, and policy, however promising, certainly will not make *P. marinus* (or *H. nelsoni*) go away, but they may, given enough time, result in larger and healthier oyster populations.

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THE ECOLOGICAL ROLE OF THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA*, WITH REMARKS ON DISEASE

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ABSTRACT Historical writings and the presence of pre-Colonial shell middens provide evidence that individual eastern oysters *Crassostrea virginica* (Gmelin, 1791) grew larger and formed more extensive reefs than they do under present-day conditions of harvesting, habitat destruction, and disease. Their diminished abundance has reduced their roles in providing hard substrate, in filtering the estuarine water column, and in affecting energy flow and nutrient flux. The poorly understood interactions among the abundant inhabitants of oyster assemblages have undoubtedly also been affected, although it is not clear how or by how much. The one important difference between the results of mortality from harvesting compared with mortality from disease may be that the shells of disease-stricken oysters remain on the oyster bar to continue to serve as substrate. There may be a connection between stress imposed by overfishing or habitat alteration and susceptibility of eastern oysters to disease.

KEY WORDS: Eastern oyster, ecological role, disease, *C. virginica*

> Every oyster-bed is thus, to a certain degree, a community of living beings, a collection of species, and a massing of individuals, which find here everything necessary for their growth and continuance. . . . I propose the word Biocoenosis for such a community. Any change in any of the relative factors of a biocoenose produces changes in other factors . . . (Mobius 1877).

INTRODUCTION

Historians of ecology credit Karl Mobius (1877) with being perhaps the first scientist to recognize (at least in print) the existence of assemblages of organisms that interact with one another and with their abiotic environment. He coined the term "biocoenose" to categorize this "social community" and used as his example the beds formed by the European flat oyster *Ostrea edulis*, reporting that (macrofaunal) species diversity was greater on the beds than on the adjacent soft sediments. Presciently he commented:

> In North America the oysters are so fine and cheap that they are eaten daily by all classes. Hence they are now, and have been for a long time, a real means of subsistence for the people . . . but as the number of consumers increases in America the price will also surely advance and then there will arise a desire to fish the banks more severely than hitherto, and if they do not accept in time the unfortunate experience of the oyster culturists of Europe, they will surely find their oyster beds impoverished for having defied the biocoenotic laws (quoted by Winslow 1881 and Sweet 1941).

Unfortunately, Mobius' pessimism was justified. Populations of the eastern oyster *Crassostrea virginica* in North America became overfished (e.g., Ingersoll 1881, Oemler 1894, Haven et al. 1978, Kennedy and Breisch 1983), just as happened to populations of other oyster species elsewhere (see Mobius 1877, Gross and Smyth 1946). In addition, habitat degradation and disease hastened the decline of some eastern oyster populations. In this review I will consider the former abundances of the eastern oyster (also referred to herein as "the oyster" or "oysters") in North America, its ecological role, and some hypotheses as to how that role might have diminished as a result of population depletion due to human activities and disease.

POPULATION CHANGES OVER TIME

The ability of unexploited, or relatively unexploited, eastern oysters to flourish over time is demonstrated by two pieces of evidence. The first is the fact that the body size of oysters and the spatial extent of their beds on the New World's East Coast astounded the earliest colonial observers. For example, Ingersoll (1881) cited two accounts of oyster size—one by Wood in 1634 that Charles River, MA, oysters had to be cut in two in order to be eaten, and an undated report by Josselyn that Gulf of Maine oysters had to be trisected for the same reason. Similarly, Wharton (1957) quoted the Swiss writer Michel's comment in 1701 that Chesapeake Bay oysters made two mouthfuls. Ingersoll (1881) also repeated Wood's report of 1634 that oysters in the Charles and Mystic Rivers in New England formed reefs that were navigational hazards. Similarly, Michel stated in 1701 that the incredible abundances of Chesapeake Bay oysters resulted in reefs so large that ships had to avoid them and that his sloop was grounded on one for 2 hours until the tide returned (Wharton 1957). Finally, early explorers of west Florida reported that oysters formed reefs that broke the water surface (Ingersoll 1881).

The second piece of evidence concerning oyster abundances is the presence of coastal shell middens that point to long-term pre-Colonial use of oysters by American Indians. Ingersoll (1881) described such a midden in Damariscotta, ME, that was built by Quoddy Indians. It contained an estimated 226,000 m^3 of mostly single shells of eastern oysters, many of them over 30 cm long (supporting the reports quoted above about the size of the edible tissue). Ingersoll (1881) also reported that shells from middens in the St. John's River region of Florida were generally larger than

those of living oysters on nearby oyster-producing beds. Similarly, Lunz (1938) found that shells in a South Carolina midden were 61% longer (hinge to bill) and 43% wider than shells of oysters living on nearby commercial beds (the percentages I report are those corrected by Gunter 1938).

Commercial fishing initially depleted the oyster "capital" that so astounded the colonists. For example, in Canada's Maritime Provinces the early fishery grew as improved transportation inland led to expanded markets and increased demand (Stafford 1913). Prices rose, oyster abundances fell, and eventually beds yielded few or no oysters. In some Maritime regions, most of the season's catch was taken in the first day of fishing, with boats massed over beds awaiting the opening hour of the season (Stafford 1913). This description of the rise and fall of Canadian oyster fisheries is generic for the industry on the North American East Coast.

By the early 20th Century then, oyster populations had declined greatly along the Atlantic Coast of North America. Few oysters remained on the southern shore of Nova Scotia or along the southwest coast of the Gulf of Maine (Stafford 1913, Churchill 1920); the remaining commercial beds in the species' northern range were in warmer Gulf of St. Lawrence waters. Ackerman (1941) reported that there were no recorded landings from Maine or New Hampshire, with the only fished beds in the Gulf of Maine being tiny remnants on the northern shore of Cape Cod. The once plentiful public beds in southern New England had become of minor importance to the fishery, and most oysters were produced by cultivation. Matthiessen (1970) reported that production from North Carolina to east Florida had declined since the turn of the century and that the only region with stable production since about 1900 was that of the Gulf States (west Florida to Texas). In addition to overfishing, destruction of habitat (e.g., Kennedy and Breisch 1981, Hargis and Haven 1988) and mortalities due to disease (e.g., Farley 1992) were implicated in these population declines. The diminished abundances of eastern oysters undoubtedly had a negative effect on their important ecological role in estuaries.

THE ECOLOGICAL ROLE OF OYSTERS

Oysters as Substrate

Oysters play a functional role in providing hard substrate (shell) that is used by members of the oyster biocoenose in the sediment-dominated environment of an estuary. Dauer et al. (1982) demonstrated in lower Chesapeake Bay that clumps of scrubbed oyster shells on experimental plots became associated with significantly higher densities of invertebrate macrofauna, both on the shells themselves and in the soft sediments between clumps, than occurred in untreated control plots. The shells provided attachment space for tubiculous suspension feeders (primarily polychaetes) whose tubes in turn became attachment space for additional epifauna. Shells of barnacles and byssate mussels that attach to oyster shell also serve as sources of hard substrate for epifaunal foulers (personal observations).

The role of substrate provider is enhanced by the irregularity of oyster shell surfaces. The shell veneer of an oyster bed displays a diversity of configurations; that is, there can be single shells or shell pieces, articulated shell pairs, and clumps or clusters of attached shells scattered on the bed, producing a complex, three-dimensional structure. Bahr (1974) estimated that 1 m^2 of intertidal oyster reef in Georgia provided 50+ m^2 of available surface for epifauna to exploit.

Community Structure and Dynamics

The ecological role of oyster communities in the economy of estuaries is influenced by the structure (physical, biological) and function of oyster beds and their components. As to physical structure, Kennedy and Sanford (unpublished observation) have examined early descriptions of beds of eastern oysters in North America, their morphology, and the influence of environmental factors, especially water circulation, on physical structure. Here I examine biological structure briefly and then consider the topic of function. Note that in general on the East Coast of North America, oyster beds from Chesapeake Bay north are subtidal, with beds from South Carolina south being mainly intertidal and North Carolina representing a transition zone of intertidal and subtidal habitat (Kennedy and Sanford, unpublished observation). In the Gulf of Mexico, most oyster beds are subtidal.

The spatial distribution of oysters on a bed is not well understood, nor are the factors influencing such distributions. In perhaps the only study of its kind, Powell et al. (1987) examined the small-scale spatial distribution of eastern oysters on 11 beds on the Texas coast. The size categories used were: small, <2 cm; medium, 2.1–5.0 cm; large, >5 cm. Small oysters were patchily distributed, presumably as a result of contagious settlement or, perhaps, differential mortality. Patch size decreased with increasing oyster size as oysters became less contagiously distributed. Clumps with relatively few large oysters tended to be found immediately adjacent (<12 cm apart) to clumps with relatively many large oysters. Powell et al. (1987) attributed this latter finding to possible superior competition for food by the more abundant and larger clumped oysters, with such competition also enhancing the negative effects of predators and disease on the adjacent clump of smaller and fewer oysters.

Another poorly understood aspect of community dynamics is that of interactions among the numerous associates on oyster beds. To begin with, only a few studies (Table 1) have examined the faunal composition of oyster assemblages in eastern North America. The most southerly investigations identified from 31 to 155 invertebrate taxa (not all identified to species) associated with oyster beds. Studies in Chesapeake Bay (Larsen 1985) and Delaware Bay (Maurer and Watling 1973) yielded from 129 to 138 invertebrate taxa; Frey's (1946) cursory collection of invertebrates produced about 41 species in the Potomac River, a tributary of Chesapeake Bay. Wells' (1961) extensive examination of oyster beds in North Carolina yielded 284 taxa, with 55–65 taxa collected from predominantly intertidal beds (he did not provide details on intertidal versus subtidal diversities). The macrofaunal invertebrates in all studies represented a mixture of filter and deposit feeders, as well as mobile predators and a variety of tunicates and benthic fish. The four studies that measured faunal density produced estimates of thousands of individuals per square meter (Table 1), with Larsen (1985) recording a high of 125,573 individuals m^{-2} in the James River, VA.

The cited studies are only comparable qualitatively because of differences in sampling methods (sampling gear, screen sizes) and sampling intensity (spatially, temporally) and because community structure can change with season and along the salinity gradient (Dame 1979, Dauer et al. 1982, Larsen 1985). Nevertheless, the impression is one of relatively high species diversity and (especially) high faunal abundances among invertebrate macrofauna on oyster beds. For example, Larsen (1985) found that species richness on James River oyster beds was generally similar to values

TABLE 1.

Number of invertebrate taxa (not all identified to species), species' biomass, and faunal density (individuals m $^{-2}$) associated with beds of *C. virginica* in USA (S = subtidal; I = intertidal; — = not measured. Location abbreviations refer to states in USA).

Geographic Location	Location of Beds	Number of Taxa	Biomass (g m $^{-2}$)	Faunal Density	Reference
Northern Gulf of Mexico	S/I	155	—	—	Kilgen & Dugas 1989
Redfish Bay, TX	S/I	—	479	—	Copeland & Hoese 1966
Apalachicola Bay, FL	S	90	—	—	Pearse & Wharton 1938[a]
Crystal River, FL	I	31	253	6,200	Lehman 1974
Georgia	I	42	705	24,747	Bahr 1974
North Inlet, SC	I	37	214	2,476–4,077	Dame 1979
Newport River, NC	S/I	284	—	—	Wells 1961
James River, VA	S	138	—	5,757–57,857[b]	Larsen 1985
Potomac River, MD	S	41[c]	—	—	Frey 1946
Delaware Bay, DE	S	129	—	—	Maurer & Watling 1973

[a] My estimate of benthic species, excluding protozoa and parasites.
[b] Mean faunal density.
[c] My estimate, excluding internal parasites and pelagic species.

reported for mesohaline soft-bottom assemblages but that mean densities of macrofauna were much higher on the beds.

There is scope for much more research on the influence of oyster aggregations on biodiversity. Increased faunal biodiversity has been associated with clumps of mytilid species on both soft-sediment shores (e.g., Dittmann 1990, Thiel and Dernedde 1994) and hard-sediment shores (e.g., Lintas and Seed 1994 and references therein). Breitburg (unpublished observation) reported that recruitment rates of the naked goby, *Gobiosoma bosc*, are 10–100 times higher than recruitment rates recorded for coral reef fish or other temperate reef fish species. The modification of water flow by oyster shell on the beds enhances aggregation of naked goby larvae (Breitburg et al., 1995). Juvenile striped bass (*Morone saxatilis*) are abundant over oyster beds in Chesapeake Bay, perhaps preying on naked goby larvae (Breitburg, unpublished observation).

Interactions among the various species on oyster beds are not well understood because research has been limited. In one instance, Ortega (1981) examined intertidal oyster assemblages in North Carolina. At a wave-exposed coastal site, *C. virginica* was uncommon and the scorched mussel *Brachiodontes* (= *Brachidontes*) *exustus* was dominant in the lower and middle intertidal. Ortega (1981) attributed this pattern to low colonization rates by the oyster, its sensitivity to wave shock, and overgrowth by the mussel. At a protected site, she found that the oyster dominated the low and middle intertidal and attributed this pattern to higher colonization rates than those of barnacles, greater tolerance of heat and desiccation (thought to be deleterious to the mussel), and rapid growth. She proposed that the absence of predatory gastropods in the protected intertidal region also helped the oyster dominate the habitat.

The partitioning of oyster bed habitat by various mud crabs (family Xanthidae) has received limited attention. Their distribution on oyster beds was examined in Delaware Bay by McDermott and Flower (1952) and in Chesapeake Bay by Ryan (1956), and Day and Lawton (1988) demonstrated that three species of mud crabs preferred broken oyster shell over four other types of substrate. However, we barely understand how these small crustaceans make use of the three-dimensional shell structures on oyster beds. In South Carolina, *Panopeus herbstii* (an oyster predator) and *Eurypanopeus depressus* co-occur on intertidal beds, with the smaller *E. depressus* generally restricted to the narrower spaces among shells (McDonald 1982). Meyer (1994) confirmed this finding for these same two species on intertidal beds in North Carolina. He also reported that *P. herbstii* (especially, large individuals) is more common under shell on and within the oyster bed compared with *E. depressus*, which is common in oyster-shell clusters that project above the surface of the bed. Winter temperatures are associated with a shift in distribution by *E. depressus* and small *P. herbstii* from shell clusters to subsurface shell, presumably away from the more extreme air temperatures (Meyer 1994). In Florida, adult and juvenile specimens of another species of mud crab, *Panopeus obesus*, are concentrated in the high intertidal zone on oyster reefs, whereas adult *Panopeus simpsoni* occupy the lower intertidal, with juvenile *P. simpsoni* found homogenously over the reef (Menendez 1987). Finally, in Georgia, ribbed mussels, *Geukensia demissa*, that attach to the exterior of oyster clumps experience nearly four times the mortality from attack by *P. herbstii* than do mussels within the clumps' interstices (Lee and Kneib 1994).

The partitioning of habitat by mud crabs on subtidal reefs is unknown, save for the report that in Delaware Bay the abundant *Dyspanopeus sayi* (= *Neopanope texana sayi*) is common in the bodies of red-beard sponge (*Microciona prolifera*) that attaches to oyster shell (McDermott and Flower 1952). In terms of other oyster-associated invertebrates, habitat partitioning probably occurs among epifauna like hydroids, bryozoans, and barnacles. Such ecologically interesting problems can be investigated readily on oyster beds.

At a scale smaller than that of the oyster bed, the landscape microecology of eastern oyster shell is an area awaiting detailed study. Korringa (1951) described the assemblage of plants and invertebrate animals that used the shell of *O. edulis* as a habitat. No such detailed study has been performed on the shell of eastern oysters. However, Osman et al. (1989) examined the interplay among fouling organisms and settling or settled eastern oysters. They found that growth of newly settled spat was inhibited and survival was reduced in association with an assortment of sessile

invertebrates that co-occurred on the cultch, with competition for food apparently being an important limiting factor (Zajac et al. 1989). Additional experiments (Osman et al. 1990) showed that ontogenetic changes in trophic relationships led to complex interactions among spat and associated predators and competitors. In addition to the influence of such biological factors, physical factors such as depth in the water column may influence community structure, as was found by Hirata (1987) for a Japanese species of oyster, *Crassostrea nippona*, and some of its associates.

Oyster beds not only provide a refuge from extreme environmental conditions as noted for mud crabs above, but also a refuge from predation, and even as a waiting place for predators. For example, Posey et al. (1995) reported that oyster beds serve as a refuge for grass shrimp, *Paleomonetes pugio*, and other decapods from predation by predatory fish. In addition, some species of predators remain in moist habitat on the reef during low water, moving off the reef in high water to forage on the adjacent soft-sediment benthos.

The depletion of oyster beds has potentially major effects on species in addition to those using the shell as a substrate or a refuge from extreme temperatures or predation. Pea crabs, *Pinnotheres ostreum*, live within the shell cavity of oysters (e.g., Christensen and McDermott 1958). There appear to be no long-term data on abundances of pea crabs in relation to oyster abundances, so I cannot speculate on the effects of depleted oyster abundances. It is possible that pea crabs have sufficient alternative bivalve hosts (e.g., Sandifer and Van Engel 1970) to allow their populations to persist even as oysters have become less common. A similar lack of data hinders attempts to correlate abundances of two fish that feed on oysters (among other prey), the oyster toadfish (*Opsanus tau*) and the sheepshead (*Archosargus probatocephalus*), with changes in oyster abundances.

At the microscale level, oyster gametes (and, presumably, pelagic gametes of associated organisms) serve as food for microheterotrophs and metazoan suspension feeders, with oyster sperm rapidly ingested by microprotozoans (Galvao et al. 1989). These investigators estimated that over 50% of oyster sperm released in a salt marsh could be ingested by the resident population of microbial grazers. In the absence of understanding of energy budgets of microbial food webs, it is difficult to say how significant is the loss of gametes from residents of oyster beds when populations of those residents have been depleted by human activities or disease.

Energy Flow and Nutrient Flux

The difficult task of modelling the energetics of oyster beds has rarely been undertaken, with the bulk of such research on *C. virginica* occurring in South Carolina (Dame 1972, 1976, Dame and Patten 1981) and Georgia (Bahr 1976). Dame (1976) found that intertidal oysters in South Carolina had the greatest population production (P), assimilation (A; energy flow), and net growth efficiency (P/A * 100) of nine intertidal molluscs studied to that date. Dame and Patten (1981) reported that an intertidal reef in South Carolina consumed about 15,000 Kcal m^{-2} y^{-1}. Bahr (1976) and Bahr and Lanier (1981) estimated that the total community respiration of an intertidal reef in Georgia was 27,000 Kcal m^{-2} y^{-1}. These values are very high for natural heterotrophic systems (Dame and Patten 1981). Similar studies need to be performed for subtidal beds in more northerly climes and in the Gulf of Mexico to provide a comparison of energy flow under constant submergence.

In terms of nutrient flux, oyster beds are thought to play significant roles in habitat in which they abound. For example, on intertidal beds in South Carolina, Dame et al. (1984) measured release rates of ammonia that were comparatively higher than rates measured in other coastal and estuarine habitats and proposed that *C. virginica* was important in material cycling. Jordan (1987) reported that production of feces and pseudofeces by subtidal *C. virginica* in Chesapeake Bay played an important role in sedimentation and remineralization. It may be instructive that an intertidal bed of the Pacific oyster *Crassostrea gigas* in France yielded evidence of complex chemical interactions (oxygen consumption, fluxes of nutrients) depending upon the available biomass of oysters (Boucher-Rodoni and Boucher 1990). Much research is underway into the role of beds of other bivalves in nutrient exchanges (e.g., Asmus et al. 1995) and there is scope for similar research into the role played by intertidal and subtidal oyster beds.

Oysters and Food Webs

Limited attention was paid to the ecological role of oysters and associated macrofauna in food webs until Newell (1988) emphasized the extent to which suspension-feeding oysters link pelagic and benthic food webs. He proposed that Chesapeake Bay's extensive oyster populations before 1870 had the potential in the summer to filter the Bay's water column in less than a week, whereas the currently depleted populations may take over 46 weeks. Unless some other suspension-feeding group(s) made up the difference (and there is no evidence that they did), this decline in filtering capacity would lessen the grazing pressure on phytoplankton populations. Newell (1988) proposed that this change would shift Bay food webs from a benthic-dominated to a pelagic-dominated mode, with the sea nettle *Chrysaora quinquecirrha* assuming a major role in controlling energy flow.

There is evidence to support Newell's (1988) claims. For example, Ulanowicz and Tuttle (1992) used a simple network-analysis model of mesohaline Chesapeake Bay to ask "what if oyster populations became more abundant?" By decreasing oyster harvest per unit biomass by 23% in their model, they were able to predict an increase of 150% in oyster biomass and an 89% decrease in gelatinous zooplankton (which includes sea nettles). In addition, the model predicted a 29% increase in benthic diatoms. This prediction is important because Cooper and Brush (1993) have since demonstrated that the ratio of centric diatoms (usually planktonic and associated with eutrophic environments) to pennate diatoms (usually benthic and from clear waters) has increased many-fold over the past half-century in the Bay.

It is always difficult to perform hindcasting experiments on complex ecosystems. It can be argued that the model of Ulanowicz and Tuttle (1992) is simplistic and subject to making unrealistic predictions. Further, a decline in benthic diatoms can result from light limitation caused by increasing turbidity from sediment runoff in the wake of human land-clearing activities since Colonial times. The lack of available data on population changes over time in the various benthic and pelagic components of the Bay's ecosystems can make the exercises of Newell (1988) and Ulanowicz and Tuttle (1992) seem too speculative.

Serendipitously, some natural experiments involving invasions of aquatic ecosystems by exotic bivalves have demonstrated that bivalves can indeed have major effects when their populations expand. For example, Cohen et al. (1984) found that high densities of the introduced Asiatic clam *Corbicula fluminea* in the fresh-

water Potomac River, MD, coincided with a region in which phytoplankton abundances were 40–60% below abundances immediately upstream or downstream of the clams. Similarly, Alpine and Cloern (1992) documented declines in phytoplankton abundance as populations of the exotic Asian clam *Potamocorbula amurensis* increased in the estuary of San Francisco Bay. The invasion of North America's Great Lakes by the zebra mussel *Dreissena polymorpha* has led to many reports of depleted phytoplankton populations and increased water transparency in the lakes (e.g., Holland 1993). Further, Stewart and Haynes (1994) provide evidence that the zebra mussels have modified energy pathways in Lake Ontario by deposition of feces and pseudofeces. The mussel colonies also appear to enhance substrate complexity, and abundance and diversity of benthic macroinvertebrates in the vicinity of the colonies are higher than in areas without colonies.

As noted above, it is not clear that other suspension-feeding organisms have replaced oysters where the latter once thrived (Newell 1988). Mobius (1877) reported that populations of flat oysters that declined in western European waters were often replaced by blue mussels, *Mytilus edulis*, and cockles, *Cardium* sp. The Atlantic rangia clam *Rangia cuneata* has increased in abundance in soft sediments in oligohaline and upper mesohaline Chesapeake Bay (Hopkins and Andrews 1969), but there are no data on how its abundances and filtering capacities resemble those of the eastern oyster. In addition, its populations fluctuate in the upper Bay over time (pers. obs.). Interactions among aquatic herbivores and primary producers are complex and of widespread interest (e.g., Prins et al. 1995). The role played by bivalves other than the oyster, as well as other suspension feeders, in the economy of the Bay and elsewhere is a topic that requires detailed examination.

THE ECOLOGY OF DISEASE IN OYSTERS

In addition to overfishing and habitat degradation, disease has also taken its toll on populations of eastern oysters (Ford and Tripp 1996), beginning in recorded memory in Malpeque Bay, Prince Edward Island. Needler (1931) stated that oyster stocks in Malpeque Bay had been greatly overfished by the turn of the 20th Century, with landings in 1914 being 10% of those in 1882, the historical peak harvest. Subsequently, the onset of Malpeque Bay Disease (thought to have been introduced with oysters imported to bolster the local fishery) caused high mortalities in 1915 and 1916, leading to no measurable landings in 1918. In a similar manner, overfishing of Delaware Bay and Chesapeake Bay stocks led to diminished returns in the first half of the 20th Century, with subsequent depletion being made worse by MSX and dermo diseases (Andrews 1968, Haven et al. 1978, Haskin and Ford 1982).

Disease can be debilitating to oysters by inhibiting growth, lowering condition, and disrupting filtering activities, depending upon the particular etiological agent (see Ford and Tripp 1996 and Paynter 1996 for reviews). Negative but nonlethal effects on physiological activities can indirectly influence the oysters' roles in energy flow and nutrient flux. However, our knowledge of these matters is not sufficient to allow for predictions to be made about the extent of such nonlethal effects.

In most ways, depletion of oyster populations by the lethal effects of disease is similar in effect to depletion by human activities. That is, the extent of the biological activity of oysters (shell and gamete production, particle filtration and deposition, nutrient flux) on the bed is reduced as oyster abundances decline. Again, it is not yet possible to quantify such changes with any accuracy.

Beyond the physiological effects noted above, perhaps the major difference in possible ecological effects between disease and anthropogenic factors is that the shells of oysters killed by disease remain on the bed (in contrast to the removal of shell from beds by harvesting). This allows them to continue to function as substrate or as a refuge for small invertebrates and fish as well as their eggs and larvae.

Finally, although Farley (1992) associated most mass mortalities in oysters with transfers of infected animals, the occurrence of disease in the wake of overfishing as noted above is provocative. Is it possible that susceptibility to disease in oysters can be enhanced in some instances by heavy fishing activity? Is there a deleterious level of stress in oysters on physically disturbed beds that have been scraped to just above the estuarine bottom?

Gross and Smyth (1946) evaluated the history of declining oyster populations, especially with regard to *O. edulis*, and proposed that overfishing leads to loss of genetic variability and reduced adaptability to long-term environmental changes. Laird (1961) hypothesized that when physical factors (e.g., temperature, salinity, tidal factors) led to a physiologically unfavorable environment for eastern oysters, they became susceptible to disease (he was considering hexamitosis caused by *Hexamita inflata*). He proposed that the proximity of the sediment-water interface led to stress and depressed resistance in eastern oysters. Lee et al. (1995) suggest that a fungus associated with a species of yew tree may have been responsible for the precipitous decline in abundance of the yew. They hypothesize that the fungus was a relatively benign associate of the yew as an endophyte until environmental changes caused by unregulated forestry practices stressed the yew and rendered it vulnerable to attack by the fungus.

There is evidence that stressors can influence prevalence and intensity of *P. marinus* disease in eastern oysters. Laboratory studies have shown that oysters are increasingly infected by *P. marinus* in the presence of pollutants of various kinds (Wilson et al. 1990, Chu and Hale 1994, see also Paynter 1996). Whether this is due to increased susceptibility on the part of the host oyster (e.g., from physiological stress or suppressed immune systems) or to enhanced virulence of the parasite is not clear. In contrast to these observations and speculations, dermo disease has been observed in oysters held in trays in the upper water column (R. Newell, Horn Point Environmental Laboratory, pers. comm.). Presumably these oysters were less subject to hypoxia and sedimentation, with any attendant deleterious effects, yet they became infected.

Clearly, the subject of environmental influences on susceptibility to disease and any role that stress from harvesting and habitat alteration might play in susceptibility require additional research. I propose that, because disease is a natural component of biological systems, it is unlikely to depress populations of the eastern oyster for long (time measured in decades or centuries), except in concert with the stressors imposed by humans. Meanwhile, until oysters develop a natural resistance to disease or perhaps until advances in biotechnology produce such resistance, managers of eastern oyster fisheries will need to implement recommendations such as those of Andrews and Ray (1988) in order to manage around the effects of *P. marinus*.

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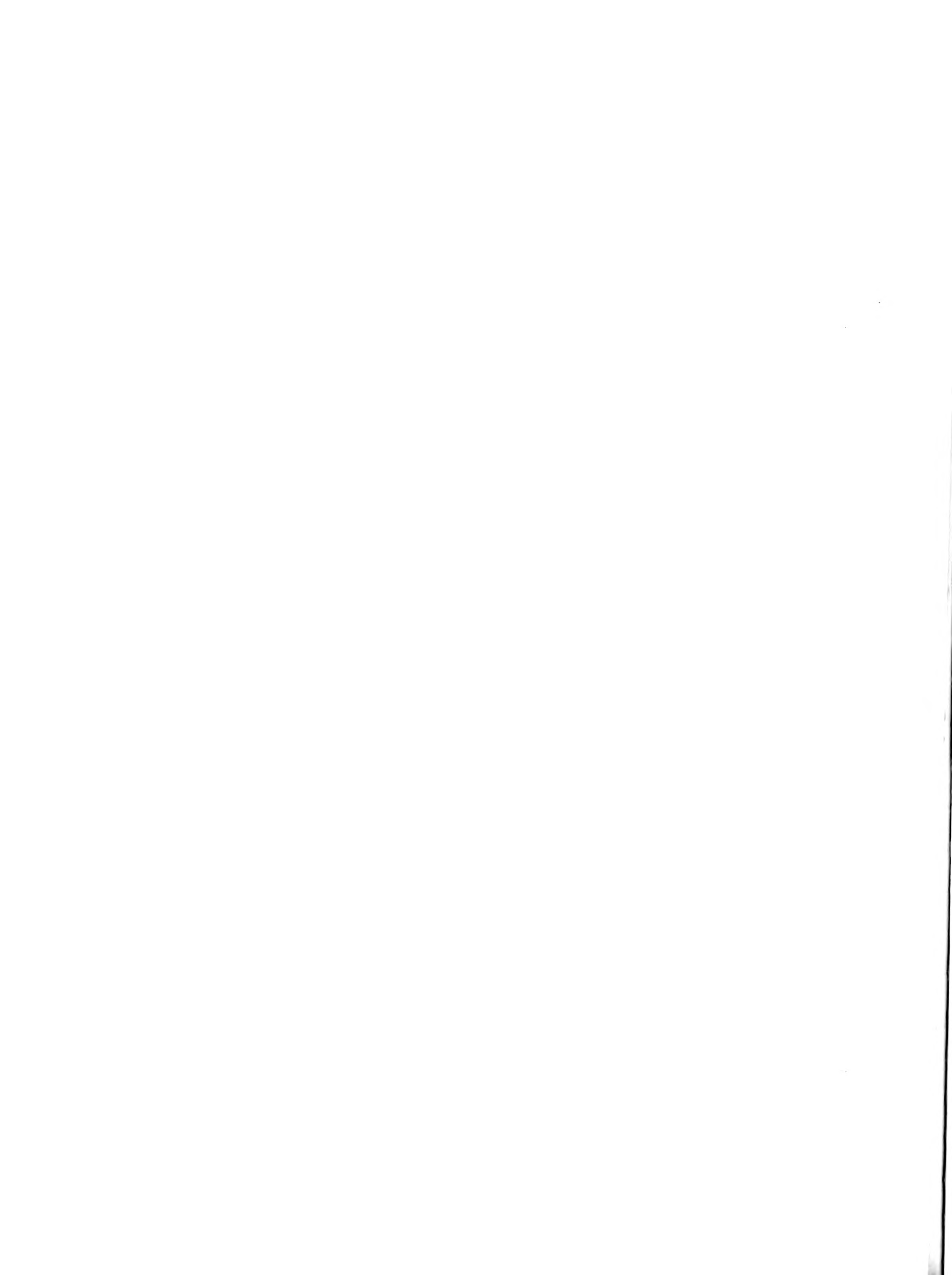

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IN MEMORIAM
R. TUCKER ABBOTT
1919–1995

Figure 1. Photograph of R. Tucker Abbott, circa the early 1970s, measuring gastropod shells at the Delaware Museum of Natural History.

Picture a likable, enthusiastic biologist surrounded by an admiring group of graduate marine biology students, on the sand flats at low tide near Indian River, DE. Repeatedly plunging his arm deep into siphonal holes in the loose sand, the fit biologist repeatedly comes up with a squirming, squirting razor clam—to the delight of the students who have been digging unsuccessfully for them with shovels and forks, the impact of each thrust merely stimulating the reactive burrowers to bury more deeply!

This was Dr. R. Tucker Abbott, a friendly, energetic, scholarly, highly productive systematic malacologist. He was as much at home in the field collecting and studying mollusks as curating them in museum collections, writing scientific papers and monographs, editing equisitely illustrated books and popular manuals about them, and associating with students and amateur and professional malacologists alike.

Tucker died of pulmonary fibrosis illness on November 3, 1995, at the age of 76, at his home on Sanibel Island, FL. He was Founding-Director of the new Bailey-Matthews Shell Museum. Tucker was survived by his charming wife, Cecelia White, who for many years enthusiastically supported him in his professional malacological activities.

Tucker was born September 28, 1919, in Watertown, MA. Little could he have anticipated the full, fruitful, satisfying professional life that would unfold in the years ahead. These are some of the highlights of his career:

1938–42. Research Assistant in the Museum of Comparative Zoology, Harvard University, MA.

1942. Received a B.S. degree at Harvard College.

1942–44. Was a United States Naval Aviator (Lt., USNR) working as a dive bomber pilot.

1944–46. Malacologist with the United States Naval Medical Research Unit 2 (Lt., USNR); the first medical malacologist in history to attempt to control schistosomiasis, the fatal blood fluke parasite. Studies took him to Guam, Marianas, and finally to China's Yangtze Valley, where he discovered the life cycle of the schistosome in a small freshwater snail.

1946–49. Assistant Curator in the Division of Mollusks, U.S. National Museum, Smithsonian Institution, Washington, D.C.

1949. Received an M.S. degree from nearby George Washington University, Washington, D.C.

1949–54. Associate Curator in the Division of Mollusks, U.S. National Museum. While there, he prepared the first edition of his *American Seashells*.

1955. Obtained the Ph.D. degree from George Washington University.

1954–69. Held the Pilsbry Chair of Malacology and the Chairmanship of the Department of Mollusks, Academy of Natural Sciences, Philadelphia. During this period, he was actively writing, editing, and publishing (see Partial Bibliography). Many of his works have been translated into several other languages.

1969–76. Held the du Pont Chair of Malacology and Chairmanship of the Department of Mollusks, as well as the Assistant Directorship of the Delaware Museum of Natural History, Wilmington. There he continued writing actively and increased the number of speaking engagements, especially to shell collectors and shell clubs, in many regions of the United States.

1972. Became president of his company, American Malacologists, Inc. Publishers of *Distinctive Books on Mollusks*.

1973. Accepted the active honorary position of Adjunct Professor in the College of Marine Studies, University of Delaware.

1979. Resigned as Adjunct Professor in the College of Marine Studies when he moved to Florida to continue writing, publishing books, and consulting in malacology. There his literary pursuits continued actively, and in addition, he began publishing the systematic malacological works of other scholars. At the time, he was also involved with the Conchologists of America, but his overriding interest became the development of the Bailey-Matthews Shell Museum on Sanibel Island. Subsequently he was honored as the Founding Director of the Museum, a facility of the Shell Museum and Education Foundation, Inc. Tucker remained active in this capacity until his death.

Tucker's research emphasis was distinctly systematic malacology, and in this field, he had few peers. This attention was strongly complemented by his intense and rewarding interest in collecting for and building museum collections. His work often took him far afield. The museums that benefitted most from these missions were the Philadelphia Academy of Natural Sciences, the Delaware Museum of Natural History, and the Bailey-Matthews Shell Museum. Tucker listed his principal expeditions as follows (Abbott 1987):

1934–76. Many trips to Bermuda.

1939–40. Harvard-Archbold Expedition to Melanesia and Polynesia.

1939, 1944, 1958. Philippines.

1939, 1945. China.

1942, 1944, 1946. Cuba.

1944–45. Marianas.

1952. National Research Council Expedition to East Africa.

1963. Anton Bruun Cruise to Bay of Bengal.

1970. Grand Cayman.

1972. Solomons.

1983. Bahamas, Senegal, Seychelles, Sri Lanka, Thailand, Indonesia, Australia, Tasmania, New Zealand, Tahiti.

1984. New Guinea and Admiralty Islands.

Tucker's outstanding international reputation is well deserved. In addition to his many other accomplishments, he published over 200 major and lesser works, of which 14 were major books (see Partial Bibliography); wrote dozens of books reviews; edited and published many books by other writers; and described one new family, 10 new genera or subgenera, and 70 new species of mollusks, many of these probably resulting from new discoveries during oceanic expeditions.

Tucker was widely recognized as an eminent malacological systematist. He received five major awards for his literary efforts between 1953 and 1978, was listed in some 15 . . . Who's Who . . . directories, was associated with some 17 shell clubs, and was a member of malacological societies as far away as Australia and Uruguay. But of the latter, he was probably most committed to the American Malacological Union, serving in several offices and finally as president in 1959.

My long friendship with Tucker began in 1954 when we corresponded on the taxonomy of the large ecologic form of oyster drills (*Urosalpinx cinerea follyensis*) from the Eastern Shore of Maryland and Virginia. He was then with the United States National Museum. During his subsequent tenures at the Academy of Natural Sciences, at the Delaware Museum of Natural History, and finally, in Melbourne and Sanibel Island, we continued to discuss systematic problems most frequently at meetings of the American Malacological Union. Ours was a long and extremely cordial association, especially during Tucker's stint at the Delaware Museum of Natural History, 1969–1977. Understandably, I was delighted by the presence of a close malacological colleague so near at hand.

In the fall of 1973, shortly after I joined the College of Marine Studies, University of Delaware, in Lewes, and on my recommendation, Dean William Gaither of the College of Marine Studies invited Tucker to join the College of Marine Studies as an Adjunct Professor. He was to co-teach with me a graduate course in malacology, instruct a course of his choosing, and serve on graduate committees. Tucker graciously accepted our invitation, and the faculty welcomed him cordially, proud to have him on our faculty.

Tucker co-taught a course in malacology with me in the spring of 1974 and the falls of 1975, 1976, 1977, and 1978. Among the graduate students who assisted us in the malacology course were M. G. (Jerry) Harasewych and Robert Prezant. Enrollment in the different classes ranged from a half dozen to 20 students.

Classes in the systematic aspects of the malacology course were held in the Mollusk Department of the Delaware Museum of Natural History, about 100 miles north of Lewes. Tucker illustrated his lectures with molluscan specimens from the impressively large collections of the museum (in excess of one million) and his professional color transparencies of living mollusks and their habitats. Classes were informal, attentatively relaxed, and often punctuated by animated discussions. During lunch we gathered around a large table in free space surrounded by some 500 museum shell cabinets, munched sandwiches, related experiences, and enjoyed hearing about Tucker's lively shell-collecting adventures around the world. Students were outspokenly impressed by the diversity of the specimens displayed, the wide range of habitats and geographic regions represented, and the intricacy of the nomenclature and classification of some of the taxa.

The remainder of the malacology course was held at the College of Marine Studies on the Delaware coast in Lewes. Ecologic field trips were taken by car to representative sand and mud flats, ocean beaches, and salt marshes and by boat on Delaware Bay. These trips, taken early during each course, not only "broke the ice" between students and professors, but they also provided experiences in the earthy exercise of slopping over mud flats and marshes, collecting live marsh snails, burrowing bivalves from sand, mud, or peat substrata, and dredging mollusks from the

bottom of the Delaware Bay. Even on cool, rainy days, students bantered among themselves as they pulled an occasional class member out of a marsh ditch or hole or prevented another from sliding overboard in a rough sea. Collected live animals were maintained in running seawater in the Lewes laboratory for later functional studies.

Using the systematic parts of the course as a foundation, we concentrated on the anatomical, behavioral, and functional biology of live mollusks in representative local taxa. Teachers, the teaching assistant, and students all participated in the lectures, discussions, and laboratory work. To all of this Tucker contributed substantially, not only by his fine reputation as a scientist, but also by his broad knowledge, experience, wit, warmth, and empathy for the students. Students still comment to me how much they appreciate the opportunity of having studied with Tucker. My gain likewise has been great. The favorite laboratory for students was a SEM study of a hard part of a mollusk of their choice.

For his own course, Tucker chose to teach a Winterim Course in Evolutionary Biology on the main campus of the University of Delaware in Newark. The course was taught during the years 1975, 1976, and 1977, with the same success that greeted Tucker during the instruction of our joint course in malacology.

Tucker was especially helpful to graduate students while serving on their graduate committees. He made available the very large molluscan collections of the Delaware Museum of Natural History and provided especially helpful advice on biosystematics and its application in thesis and dissertation research. Students on whose graduate committees Tucker served were Margaret Carter, Clement Counts II, G. M. (Jerry) Harasewych, Peter Kinner, and Robert Prezant. He was quick to discuss molluscan systematics with other students and faculty who sought his advice.

Reminiscing, Clem Counts once noted that Tucker was fond of autographing copies of his many books on mollusks. This practice was well known to his peers, and Tucker himself laughed about his "inability to refuse to sign" a cover page. At the 1990 meeting of the American Malacological Union in Woods Hole, MA, Clem was standing in the back of the lecture hall with Tucker during the book and shell auction. After a time, a copy of Tucker's classic *American Seashells* was brought out. The copy, coming directly from the publisher, was wrapped in plastic, which prompted Richard Petit, the auctioneer, to quip that the wrapper was significant because it indicated that the book was "untouched by the pen of R. Tucker Abbott." Chuckling, Tucker turned to Clem and counterquipped that "The book should fetch a very high price since there were fewer unsigned, than autographed copies!"

In the summer of 1979, Tucker resigned as Adjunct Professor in the College of Marine Studies in preparation for his permanent move to Florida. He had terminated his position with the Delaware Museum of Natural History late in 1977. On Tucker's departure, Dean William Gaither, College of Marine Studies, and I wrote Tucker expressing our deep regret for his departure, but gratitude for his many contributions to the University of Delaware during his tenure as Adjunct Professor.

Going to Florida permitted Tucker to concentrate on his writing and to continue building his successful publishing company, American Malacologists, Inc. Now his earlier wish to more seriously pursue writing could be fully realized. Tucker spent the remainder of his life collecting and studying mollusks, writing, and publishing, among other literature, a remarkable series of enthusiastically received books for amateur naturalists and collectors.

The formal opening of the Bailey-Matthews Shell Museum took place on November 18, 1995, just 2 weeks after Tucker's death. The museum was the materialization of his vision of a "monument to shells for people, not just a museum full of shells" (Scheu 1995). Although Tucker did not live to see the formal opening of the museum, he did take pleasure in seeing paying visitors pass through the halls of the museum earlier that year.

"On November 3, 1995, the world lost an extraordinarily respected man of science and a godfather to shellers everywhere!" (Hallstead 1995); and I would add, an extraordinarily gifted man of letters who had the natural talent of bridging between amateur and professional malacologists to the benefit of both as well as to the benefit of the field of malacology.

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RED-COLOURED DIGESTIVE GLANDS IN CULTURED MUSSELS AND SCALLOPS: THE IMPLICATION OF *MESODINIUM RUBRUM*

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ABSTRACT In April 1992, cultured mussels from Ship Harbour, a major estuary on the shore of Nova Scotia, Canada, were found to have red-coloured digestive glands. Examination of digestive gland tissue using spectrofluorometry and epifluorescence microscopy revealed the presence of the photosynthetic pigment phycoerythrin. A survey of the local phytoplankton suggested that the most likely source of the pigment was the ciliate *Mesodinium rubrum*. In general, the intensity of the red colouration in the digestive glands of the mussels varied consistently with the abundance of *M. rubrum*. Control (phycoerythrin-free) mussels transferred into Ship Harbour accumulated sufficient phycoerythrin to closely resemble the indigenous mussels within a week. Epifluorescent examination of the control mussels indicated the presence of phycoerythrin in the digestive tubules within 4 h of deployment. In depuration experiments, macroscopic evidence of red colouration disappeared within 48 h, but epifluorescence microscopy indicated that phycoerythrin persisted in the digestive gland for as long as 3 wk. Control scallops transferred into Ship Harbour accumulated phycoerythrin, but not to the extent of developing red-coloured digestive glands. The abundance of *M. rubrum* declined from 30,000 cells $\cdot l^{-1}$ in early May to <10,000 cells $\cdot l^{-1}$ in early June, coincident with an increase in water temperature and the disappearance of the red colouration in the mussels. During July and August, numbers remained low and the population was generally confined to the coldest, deepest (8–13 m) section of the estuary.

KEY WORDS: Mussels, scallops, *Mesodinium rubrum*, ingestion, phycoerythrin, depuration, aquaculture

INTRODUCTION

In the spring of 1991, cultured mussels from several sites along the shore of Nova Scotia, Canada, were found to have unusual red-coloured digestive glands. Local mussel growers expressed concern over the tissue discolouration and reported that the mussels tended to leak red fluid when harvested. Preliminary research efforts failed to identify the source of the red colour, but epifluorescence microscopy revealed that the digestive gland tissue had fluorescence characteristics similar to those of the accessory photosynthetic pigment phycoerythrin.

In April 1992, cultured mussels in one of the major Nova Scotian estuaries, Ship Harbour, again developed red digestive glands. Preserved phytoplankton samples obtained from local mussel growers as part of the Phytoplankton Monitoring Program (Carver et al. 1992) were found to contain high numbers of 30- to 60-μm particles. Although difficult to identify initially, they were found to exhibit the same phycoerythrin-like fluorescence as tissue samples from the digestive gland. Subsequent examination of unpreserved water samples revealed high concentrations of the photosynthetic ciliate *Mesodinium rubrum*. This delicate species varies in size from 20 to 70 μm and harbours an algal (cryptomonad) endosymbiont containing the accessory photosynthetic pigment phycoerythrin (Taylor et al. 1971, Hibberd 1977, Lindholm 1992). This pigment appears red under natural light and emits a characteristic orange-red fluorescence under green epifluorescent illumination.

Although *M. rubrum* occurs in coastal waters throughout the world and is frequently associated with "red tides" (McAlice 1968, Taylor et al. 1971, White et al. 1977), there are very few references linking this species with red digestive glands in shellfish. Clemens (1935) reported a bloom of *M. rubrum* at Nanaimo, B.C., in late April 1933 and noted that local oysters were observed to contain red-stained styles in their digestive glands. In another instance, Kat (1984) suggested that *M. rubrum* was responsible for the red discolouration of oyster digestive glands in Lake Grevelingen in the Netherlands.

The following study was undertaken to clarify the relationship between the occurrence of red digestive glands in cultured mussels and the abundance of *M. rubrum*. The specific objectives were (a) to demonstrate that the discolouration of the digestive gland was due to the presence of phycoerythrin, (b) to assess the rate of the uptake and depuration of phycoerythrin by the mussels; (c) to determine whether cultured sea scallops would also accumulate phycoerythrin; and (d) to document the temporal and spatial distribution of *M. rubrum* in Ship Harbour.

MATERIALS AND METHODS

Phycoerythrin Fluorescence

Extracts of digestive gland from cultured mussels grown in Ship Harbour (Fig. 1) and a control site near Lunenburg, N.S., were obtained by gently teasing the tissue in seawater. The resulting slurry was filtered onto 25-mm GF/F filters and examined by spectrofluorometry. Fluorescence (excitation and emission) spectra were acquired on a SPEX Fluorolog F111A spectrofluorometer interfaced with a SPEX DM3000 IBM-compatible computer. Excitation and emission wavelengths were scanned and recorded at 0.5-nm intervals.

Uptake of Phycoerythrin

Ship Harbour is a long, narrow estuary (8 × 1 km) with the deepest water located at the head of the system (Fig. 1). Three mussel longlines were selected as experimental sites: Location 1 (13-m deep) at the upper end, near the river mouth; Location 2 (11-m deep) roughly 2 km downstream; and Location 3 (6-m deep), another 2 km downstream. Preliminary sampling trips were undertaken in April 1992, and a weekly monitoring program was conducted from May to mid-August 1992.

To document the uptake of phycoerythrin, "control" mussels (i.e., with no evidence of phycoerythrin) were transferred from the Lunenburg site into Ship Harbour at weekly intervals (April 10 to June 5). Twenty mussels were placed in each of six Japanese pearl nets, which were then deployed at Location 1 (3 and 8 m), Location 2 (3 and 8 m), and Location 3 (3 and 5 m). After 1 wk, the digestive glands of the control mussels were examined for signs of red colouration or macroscopic evidence of phycoerythrin accumulation. This qualitative visual assessment was based on several criteria: (a) colour of the tissue; (b) size of the gland; (c) colour of the crystalline style; and (d) presence of red fluid in the style sac. A mussel with a red and swollen digestive gland, a pink-stained style, and red fluid in the stomach was assigned a rank of "4"; with no red fluid, a rank of "3"; with a clear style, a rank of "2"; with no swelling of the digestive gland, a rank of "1"; and with no red colouration (i.e., "normal"), a rank of "0". The rankings were then averaged to obtain an overall value or "red colouration index" for each location. Ship Harbour mussel sleeves, originally hanging from the longline at each location, were also sampled weekly and ranked on the basis of the same criteria.

On several occasions, the control mussels held for 1 wk in a pearl net at Location 1 (3 m) were sampled for histological assessment of phycoerythrin levels. These individuals were fixed in 1% glutaraldehyde: 4% formalin, embedded in paraffin, and sectioned (6 μm) through the digestive gland. Unstained tissue sections were examined under epifluorescent illumination (545 nm) with a Reichart-Jung Polyvar 1 microscope. An image analysis system equipped with a black-and-white camera was used to assess the intensity of the fluorescence, which was then used to derive a relative phycoerythrin ranking for each mussel. Values ranged from a maximum of "100%" down to "0%" for the control mussels before being transferred into Ship Harbour.

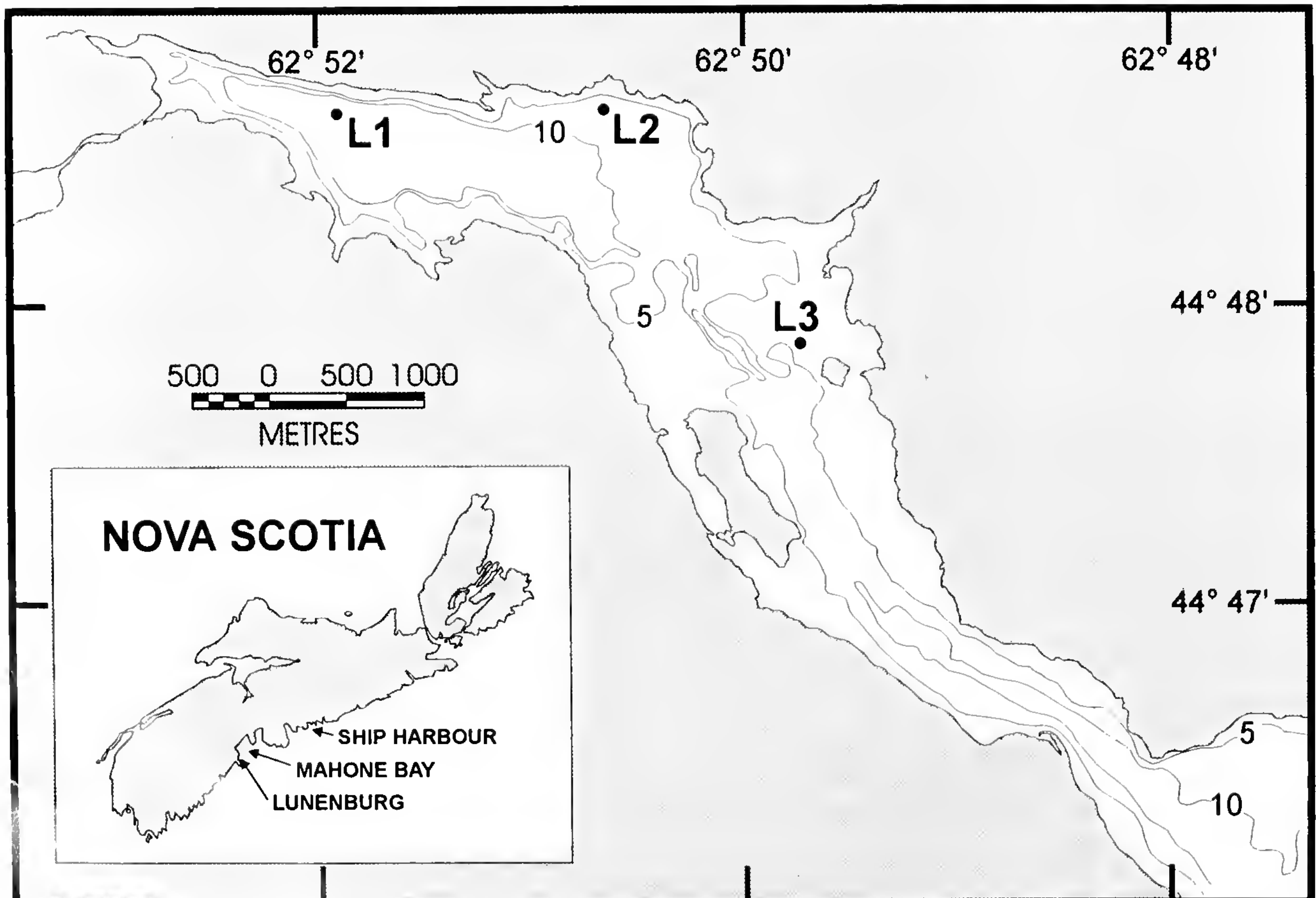

Figure 1. Map of the Ship Harbour estuary indicating the position of the three sampling locations (L1, L2, and L3). Note that the deepest section of the estuary (14 m) is located at the upper end.

A short-term field-grazing experiment was conducted on May 1. In this case, control mussels were deployed at Location 1, and samples for the histological assessment of phycoerythrin were taken hourly over the next 4 h.

To determine whether other species would also develop red digestive glands, "control" scallops were obtained on three occasions from a scallop culture site in Mahone Bay (Fig. 1). The scallops were deployed in pearl nets (six per net) adjacent to the control mussels at the three locations in Ship Harbour. After 1 wk, the scallops were examined for signs of red colouration and two were processed for histological assessment of phycoerythrin levels.

Depuration of Phycoerythrin

Depuration experiments were carried out on three occasions using mussels with a strong red colouration in their digestive glands. On April 10, 1992, 80 mussels from Ship Harbour were transferred to two tanks of filtered sea water (40 mussels/tank) at the Halifax Fisheries Laboratory (Department of Fisheries and Oceans, Canada), one set at ambient temperature (ca. 5°C) and the other at 15°C. A second set of tanks was stocked with 80 control mussels originally from the Lunenburg site. Ten mussels were sampled from each tank at 20, 53, and 77 h: five were visually assessed for red colouration, and five were prepared for histological examination. Two similar depuration trials were set up on April 20 and 24 with new groups of Ship Harbour and Lunenburg mussels.

A longer term depuration experiment was initiated on May 26; in this case, mussels from Ship Harbour were left in flowing sand-filtered seawater for 5 wk. Seven mussels were sampled initially and after 1, 2, 3, and 5 wk: three were examined for red colouration, and four were prepared for histological assessment of phycoerythrin levels.

Abundance and Distribution of M. rubrum

Preliminary phytoplankton samples collected during April 1992 indicated that *M. rubrum* was the most likely source of the phycoerythrin accumulated by the mussels. A weekly sampling program was therefore undertaken from May to mid-August to document the abundance and distribution of this species. On each sampling day, temperature and salinity profiles were obtained with a Conductivity-Temperature-Depth meter, and a vertically integrated phytoplankton sample was collected with a 20-μm-pore-size mesh net. Integrated water column samples (0–3, 0–8, and 0–13 m at Location 1, 0–3 and 0–11 m at Location 2, and 0–6 m at Location 3) were collected with a 2-cm-diameter PVC hose. Each sample was drained into a bucket and stirred gently, and a 1,000-ml subsample was removed. Three methods of preserving *M. rubrum* were initially compared: (a) 1% glutaraldehyde, (b) 0.5% Lugol's iodine (100 g of KI, 50 g of iodine, and 100 ml of glacial acetic acid in 1 l of distilled water), and (c) 2% formalin: acetic acid (50:50). Three subsamples of 30 ml were taken from each sample, filtered onto 1-μm-pore-size Nuclepore filters, and transferred to slides by freezing (Hewes and Holm-Hansen 1983, adapted by K. Pauley, unpubl. manuscript). Cells of *M. rubrum* were enumerated at 100× magnification. Estimates of *M. rubrum* concentration in the 3- to 8-m or 8- to 13-m zone were calculated by difference: for example, the value for 3–8 was obtained by subtracting the 0–3 m value from the 0–8 m estimate.

RESULTS

Phycoerythrin in Mussel Digestive Gland Tissue

Spectrofluorometric examination of samples of red-coloured digestive gland indicated the presence of the accessory photosynthetic pigment phycoerythrin. Excitation spectra of the chlorophyll fluorescence of digestive gland slurries possessed a principal peak at 545 nm with secondary peaks at 438 and 675 nm (Fig. 2a). The peak at 545 nm is characteristic of phycoerythrin absorption,

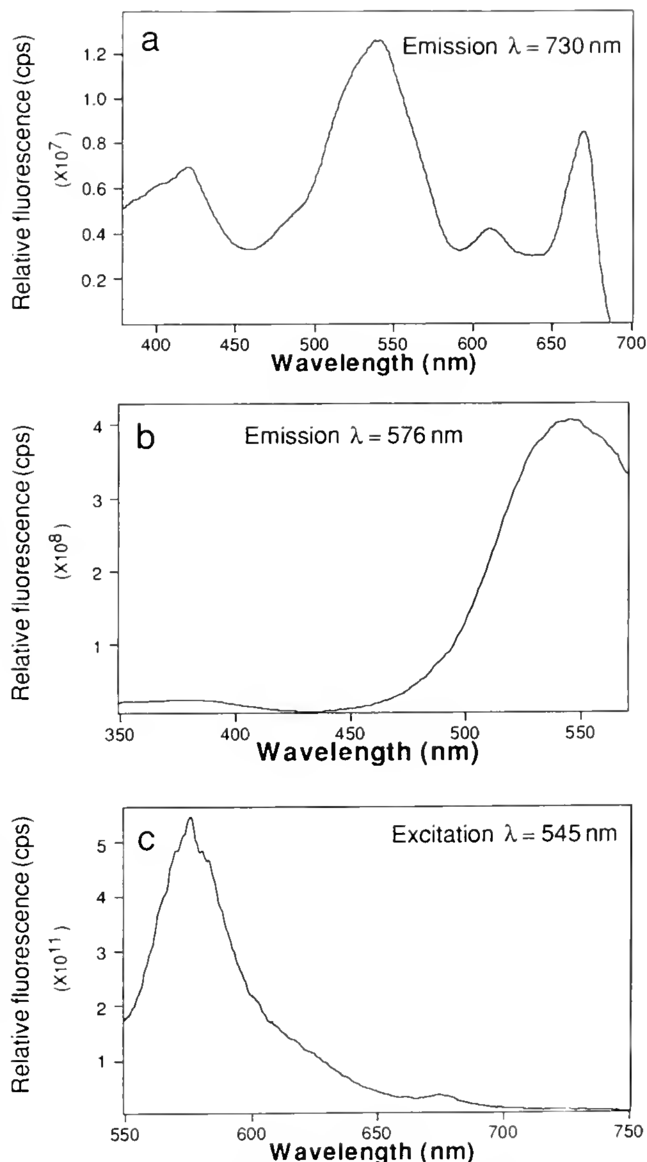

Figure 2. Fluorescence excitation and emission spectra of seawater extracts containing red-coloured pigment obtained from the digestive glands of mussels: (a) excitation spectrum of extract with fluorescence monitored at 730 nm, the wavelength chosen to detect chlorophyll *a* emission; (b) excitation spectrum of extract with fluorescence emission monitored at 576 nm, the emission maximum of phycoerythrin; the broad peak centered at 545 nm is characteristic of phycoerythrin absorption; (c) emission spectrum of extract using an excitation of 545 nm corresponding to the peak absorption of phycoerythrin; the emission peak at 576 nm is characteristic of phycoerythrin. cps = counts per second.

whereas the peaks at 438 and 675 nm are associated with chlorophyll absorption. The overall shape of this excitation spectrum is typical of phycoerythrin-bearing cryptophytes and closely resembled excitation spectra from cultures of the cryptophyte *Rhodomonas salina*. A fluorescence excitation scan of the red pigment confirmed an absorption maxima at 545 nm (Fig. 2b), whereas a third scan using an excitation wavelength of 545 nm showed a strong emission peak at 576 nm (Fig. 2c). This fluorescence excitation-emission signature was consistent with that of the accessory pigment phycoerythrin. Spectrofluorometric spectra of digestive gland tissue from the control mussels obtained from the Lunenburg site showed no evidence of phycoerythrin.

TABLE 1.

Mean rankings of red colouration based on the visual assessment of indigenous Ship Harbour mussels (shallow) and Lunenburg control mussels after 1 wk in Ship Harbour (shallow and deep).

Mussel Source	Location 1	Location 2	Location 3
Ship Harbour (shallow)	3.09	1.15	1.28
Control (shallow)	2.05	0.79	0.68
Control (deep)	1.55	1.85	0.85

Spatial and Temporal Variations in Red Colouration

During April and early May, mussel digestive glands were extremely swollen and had a distinctive russet-red colour. When the digestive gland was dissected, the stomach was found to contain a pink-stained crystalline style, as well as an abundance of red fluid. Visual examination of Ship Harbour mussels, as well as control mussels deployed for at least 1 wk in Ship Harbour, indicated considerable spatial variability in the extent of these characteristics (Fig. 3). Over the 8-wk period, the average ranking for the Ship Harbour mussels (shallow) was higher at Location 1 than at Locations 2 and 3 (Table 1). The control mussels deployed near the surface exhibited a similar pattern, although the mean values were consistently lower. A substantial variation in red colouration over depth was observed at Location 2, where the control mussels deployed in the deeper water had the higher ranking.

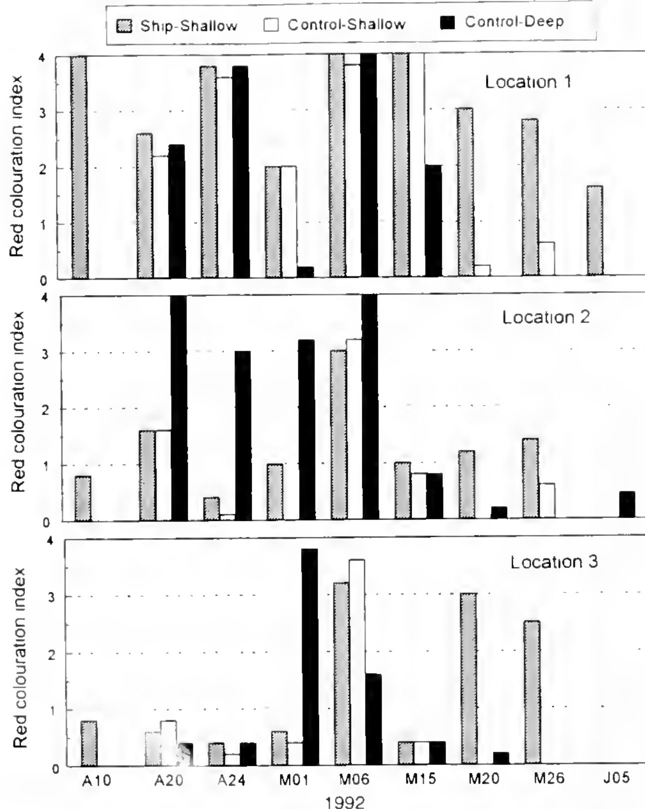

Figure 3. Red colouration ranking based on qualitative visual assessment of Ship Harbour mussels and Lunenburg control mussels after 1 wk in Ship Harbour. The shallow samples were taken from approximately 3 m below the surface, whereas the deep samples were from 8 m below the surface at Locations 1 and 2 and 5 m below the surface at Location 3.

Temporal variation in red colouration was also evident over the 8-wk period from April 10 to June 5 (Fig. 3). In April, mussels from Location 1 (shallow and deep) in the upper estuary and those deployed at depth at Location 2 showed the most intense colouration. In early May, there was generally an increase in the level of red colouration at all depths and locations. After May 15, the control mussels showed little evidence of phycoerythrin accumulation, whereas the Ship Harbour mussels continued to exhibit red digestive glands for several weeks. During the summer, mussels sampled from 8 m at Location 1 occasionally showed signs of red colouration, but samples from 3 m and from the other two locations consistently appeared normal.

Histological Assessment of Phycoerythrin

Histological sections of the red-coloured digestive glands of the Ship Harbour mussels were markedly different from those of control mussels when they were initially obtained from Lunenburg. In particular, they appeared to have larger digestive tubules and less space between the tubules. Particles of red-fluorescing pigment, probably cell fragments of *M. rubrum*, were often observed in the stomach area. In the short-term grazing experiment (May 1), control mussels showed signs of red-stained styles and red fluid in the style sac within 3 h of deployment. Phycoerythrin levels in the digestive tubules were assessed at 10% after 3 h and 20% after 4 h, indicating rapid accumulation of pigment. This was consistent with the observation that control mussels transferred into Ship Harbour typically resembled the adjacent Ship Harbour mussels within a week of deployment (Fig. 3; April 20 to May 15).

Ship Harbour mussels sampled from Location 1 (shallow) between April 10 and May 15 consistently had phycoerythrin levels of 90–100% (Fig. 4) By early June, pigment levels had declined to 80%, and there was no visual evidence of red colouration in the digestive gland. From mid-June to mid-July, phycoerythrin levels ranged from 27 to 56%, with considerable variation among individuals, possibly because of differences in depuration rates.

Interestingly, control scallops transferred into Ship Harbour (April 24 and May 1) with the control mussels showed no visual signs of red colouration after 1 wk. Examination of the digestive gland tissues under epifluorescence, however, indicated the presence of phycoerythrin. Levels were estimated at 60–70%, as opposed to the adjacent control mussels at 100%. At first, these results suggested that scallops might not accumulate sufficient phycoerythrin to display signs of red colouration. However, scallops from another nearby site (Country Harbour) that had higher concentrations of *M. rubrum* (80,000 cells $\cdot$ l^{-1}) did show red-coloured digestive glands (Fig. 5a).

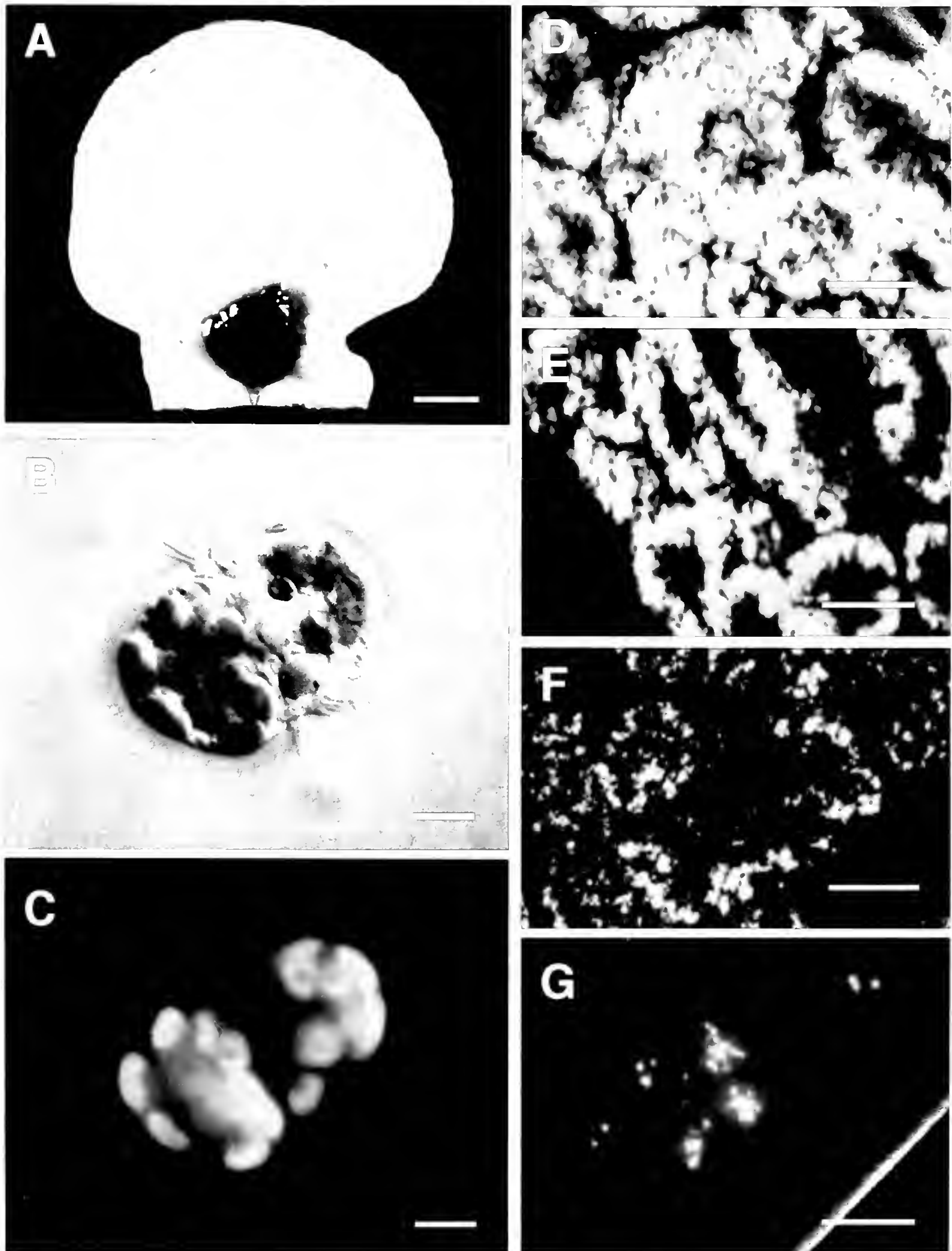

Figure 5. (a) Example of a cultured sea scallop with red-coloured digestive gland from Country Harbour, an estuary located 100 km east of Ship Harbour (concentration of *M. rubrum*, 80,000 cells l^{-1}); note that control scallops deployed in Ship Harbor accumulated phycoerythrin but not to the extent that they developed red colouration; scale bar = 15 mm; (b) *M. rubrum* under differential interference contrast, scale bar = 15 µm; (c) *M. rubrum* under green epifluorescent illumination showing the characteristic orange-red autofluorescence of the phycoerythrin pigment in the chloroplasts, scale bar = 15 µm; (d–g) cross-sections of mussel digestive gland illustrating the depuration of phycoerythrin over 3 wk; the relative phycoerythrin ranking declined from an initial value of 100% to 80% (Week 1), 40% (Week 2), and 10% (Week 3); scale bar = 100 µm.

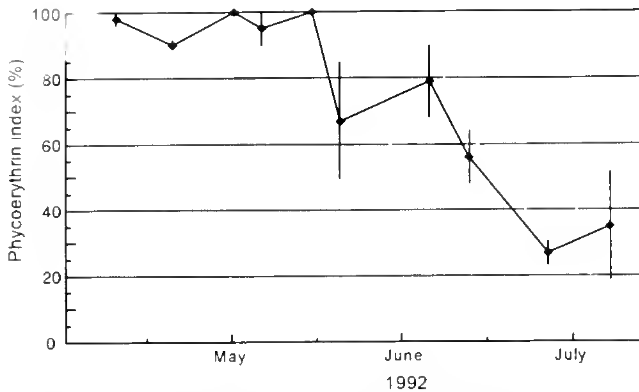

Figure 4. Phycoerythrin fluorescence index (%) for the digestive gland of Ship Harbour mussels from Location 1 (3 m).

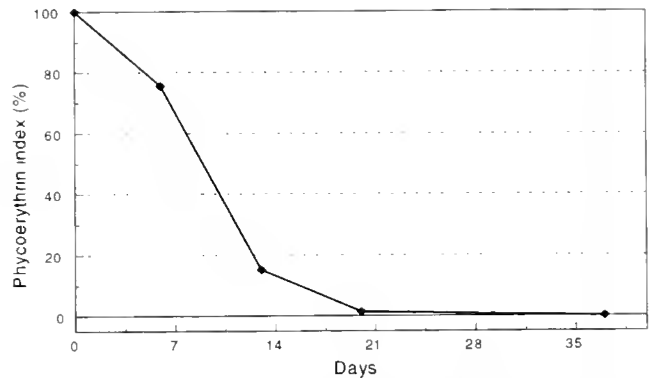

Figure 7. Phycoerythrin fluorescence index (%) for the digestive gland of mussels depurated in filtered seawater over 5 wk. See also Figure 5d–g.

Depuration Experiments

The depuration experiments were undertaken in an attempt to document the rate at which the red colouration disappeared from the digestive gland of the mussels. Visual assessment over time (Fig. 6) indicated a rapid change in the extent of the colouration; in particular, the red fluid and the pink colour of the style virtually disappeared after 20 h. The digestive gland, however, remained abnormally large relative to that of the control mussels; a noticeable decrease in size was only observed at 77 h. Assessment of phycoerythrin using epifluorescence indicated a slight decline over the duration of the experiment, but the fluorescence emission was still strong (i.e., 70–80%) after 77 h. Phycoerythrin apparently remains in the digestive gland long after visual evidence of pigment accumulation disappears. There was no significant difference in the rate of depuration between mussels in the ambient tank at 5°C and those in the 15°C tank.

In the long-term depuration experiment (Fig. 5d–g, Fig. 7), macroscopic evidence of phycoerythrin disappeared within 1 wk, but examination of mussel tissues under epifluorescent illumination indicated that phycoerythrin persisted in the digestive gland for approximately 3 wk. Depuration rates calculated from this trial suggested a decline in phycoerythrin levels of approximately 22% per day (r^2 = 0.92).

Temperature and Salinity Conditions in Ship Harbour

Preliminary measurements during April indicated a mean water column temperature of 3–5°C in Ship Harbour. Temperatures increased sharply from 4 to 6°C in early May (Fig. 8) to >10°C in early June. In general, the mean temperature at the deepest site (Location 1) near the head of the estuary was lower than that at the two more oceanic but shallower sites (Locations 2 and 3). Contour diagrams of temperature vs. depth over time and salinity vs. depth over time are presented for Location 1 (Fig. 9). Following the formation of a vertically stratified water column in early June, temperature in the 8- to 13-m zone remained below 10°C throughout the summer. Location 2 also showed <10°C temperatures at 8–10 m, but this cold layer was not evident at the more shallow Location 3. The salinity contour diagram indicated a warm low-salinity layer (>10°C, <28°/oo) in the upper 2 m from mid-May to mid-June, followed by a mixing event and the reestablishment of a stable halocline in late June. Profiles for Locations 2 and 3 showed a similar vertical structure, although the low-salinity surface layer was less evident towards the mouth of the estuary.

Abundance and Distribution of M. rubrum

Initial identification of the photosynthetic ciliate *M. rubrum* was based primarily on the presence of a distinct row of cilia at the juncture of the two body sections (Fig. 5b) and observations of phycoerythrin-containing chloroplasts under epifluorescent illumination (Fig. 5c). Initial attempts to fix *M. rubrum* with glutaraldehyde proved unsuccessful, and although Lugol's iodine was found to be quite effective in preserving the delicate cilia of this species, the best results were obtained with 2% formalin:acetic

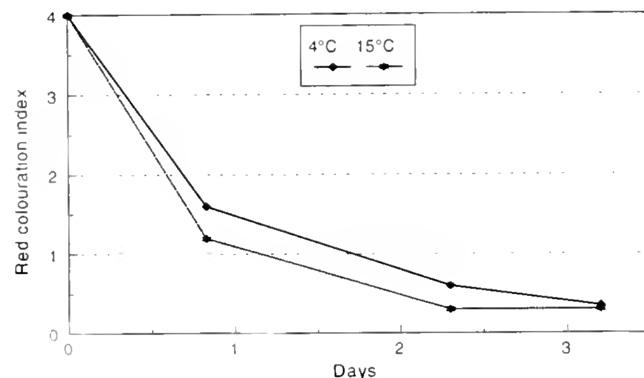

Figure 6. Decline in the red colouration of the digestive gland of mussels depurated in filtered seawater for 77 h.

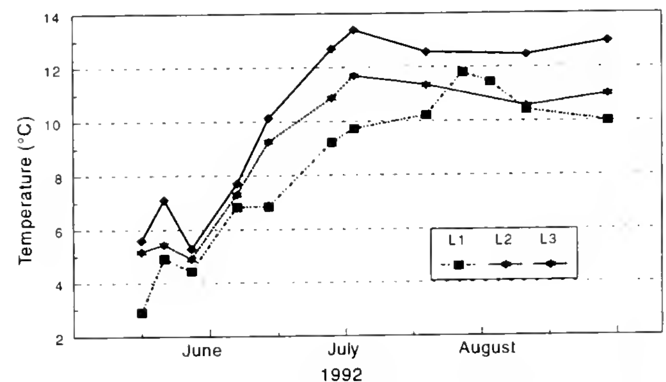

Figure 8. Mean water column temperature at the three locations in Ship Harbour.

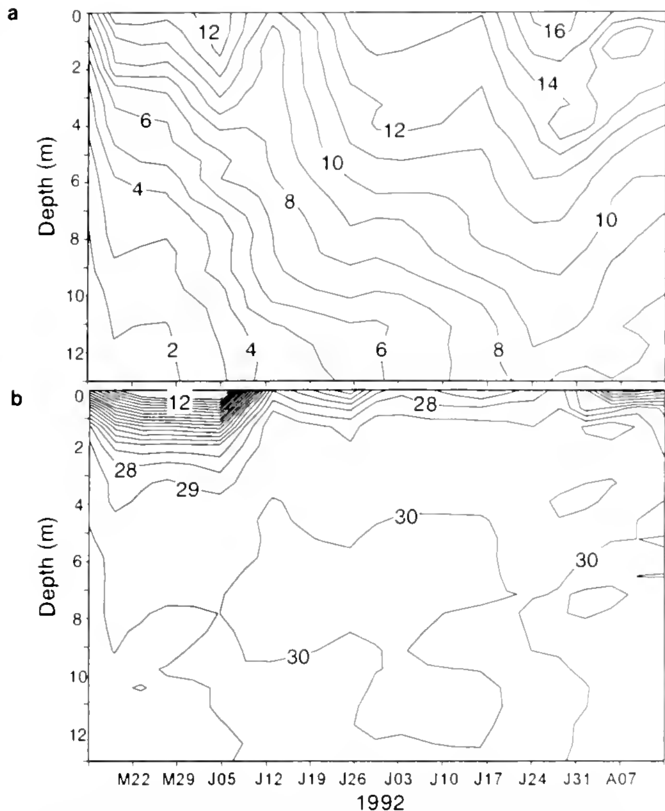

Figure 9. Contour plots of (a) the temperature structure and (b) the salinity structure of the water column at Location 1.

rubrum remained below 6,000 cells · l^{-1} through the summer with the exception of a brief bloom at Location 1 in late July.

Depth profile sampling indicated that *M. rubrum* was not evenly distributed through the water column; for example, on May 1, the concentration in the upper 3 m (3,700 cells · l^{-1}) was substantially lower than the average value for the water column (6,500 cells · l^{-1}), thus indicating that the species was relatively more abundant below 3 m. Further samples collected during June and July (Fig. 11) suggested that, as temperature increased, the population became confined to the deeper zones of the water column. By early July, most of the *M. rubrum* population was found in the 8- to 13-m zone at Location 1 or the 3- to 11-m zone at Location 2.

Temporal variability in the vertical distribution of *M. rubrum* may have been due to the horizontal movement of water masses of varying cell abundance or to the actual vertical movement of cells in response to light and/or tidal currents. For example, on May 6, water samples collected only 1 h apart showed substantial changes in cell distribution. To investigate short-term changes in the vertical distribution of *M. rubrum*, samples were taken every 2 h from 1130 to 2030 h at Locations 1 and 2 on May 20 (Fig. 12). Abundance estimates for the whole water column varied from 8,700 to 11,900 cells · l^{-1} at Location 1 and 9,100 to 16,500 cells · l^{-1} at Location 2. The vertical distribution of *M. rubrum* varied substantially over the 9-h period, but there was little evidence of a consistent tidal or diurnal migration pattern. At Location 1 during the ebb tide, there appeared to be a net movement of cells out of the surface layer (0–3 m) and the bottom layer (8–13 m) into the midwater zone (3–8 m). At Location 2, there appeared to be a

acid (50:50). Preservation of samples immediately after collection was also found to reduce the extent of cell disruption and minimize damage to the cilia.

Weekly estimates of *M. rubrum* concentration showed substantial temporal and spatial variability (Fig. 10). It should be noted that preliminary samples collected in April ranged from 5,000 to 50,000 cells · l^{-1}. In early May, the abundance of *M. rubrum* was relatively low (6,500–13,000 cells · l^{-1}), but numbers increased again in the week of May 6 (17,000–26,000 cells · l^{-1}). By May 15, *M. rubrum* was on the decline while other algal species were increasing in diversity and abundance. This change in the phytoplankton assemblage coincided with a decline in the phycoerythrin levels in the digestive glands of the mussels (Fig. 4). *M.*

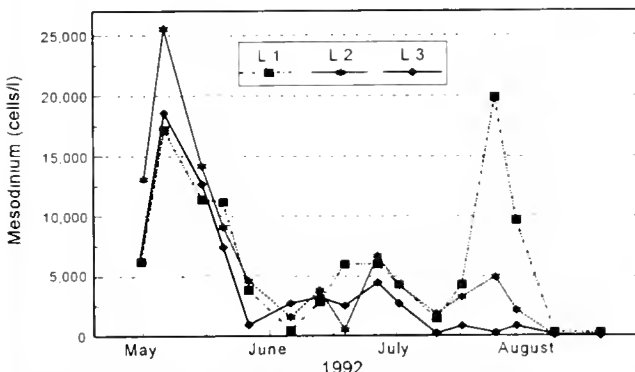

Figure 10. Abundance of *M. rubrum* (cells · l^{-1}) averaged over the whole water column at each location from May 1 to August 18.

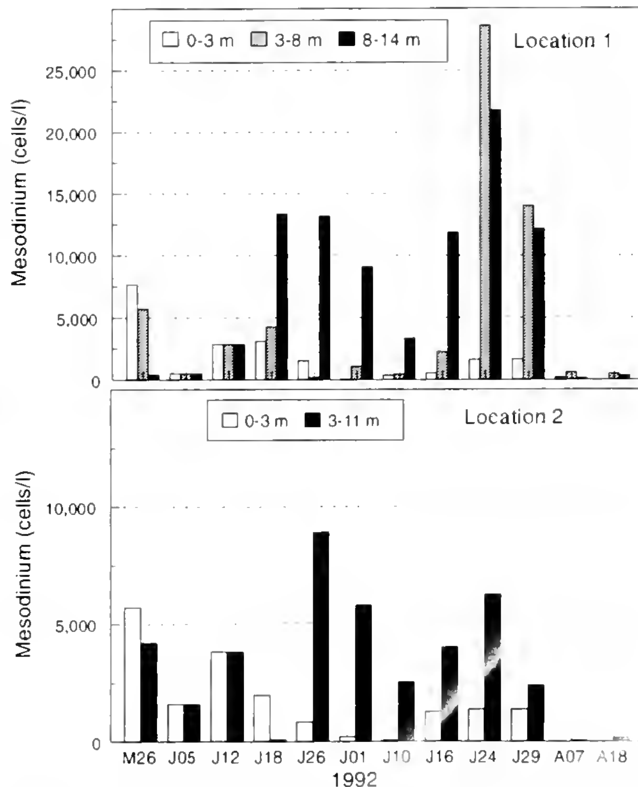

Figure 11. Vertical distribution of *M. rubrum* at Locations 1 and 2. Note that there were no stratified samples from June 5 and 12, and therefore, the single estimate from the whole water column was used for each layer.

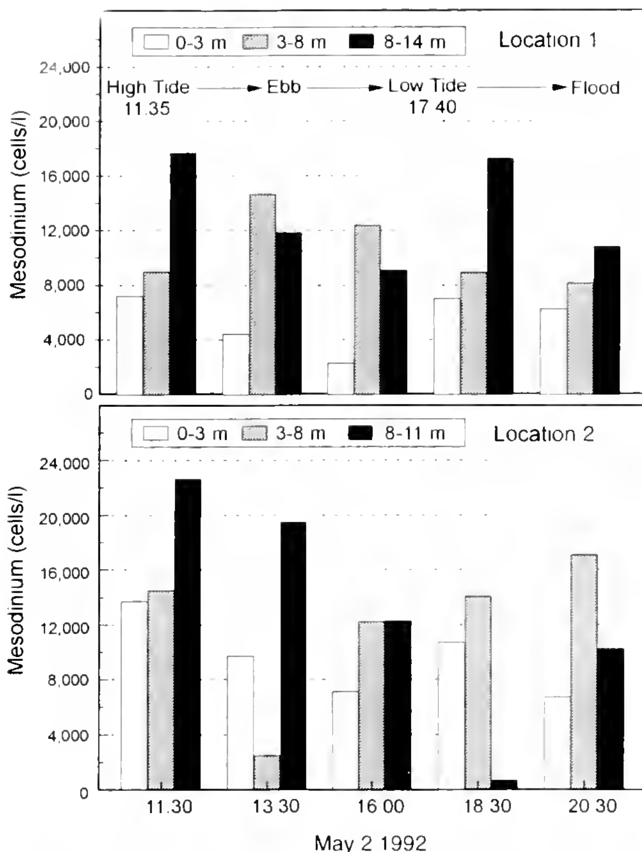

Figure 12. Vertical distribution of *M. rubrum* over a tidal cycle at Locations 1 and 2.

decline in the concentration of cells near the surface (0–3 m) during the ebb tide, but there was no consistent temporal pattern.

DISCUSSION

Accumulation and Depuration of Phycoerythrin

Spectrofluorometry was used to demonstrate that the red colouration in the mussel digestive gland was due to the presence of the accessory photosynthetic pigment phycoerythrin. The shape and the peaks of the chlorophyll excitation spectrum were typical of cryptophytes, or ciliates such as *M. rubrum*, which possess cryptophyte-type chloroplasts. Subsequent surveys of the phytoplankton assemblage using epifluorescence microscopy revealed that the most likely source of this red pigment was the photosynthetic ciliate *M. rubrum*. Although this study was initiated after the start of the *M. rubrum* bloom in mid-March (Phytoplankton Monitoring, unpubl. data), it did serve to clarify the temporal relationship between the presence of this species and the occurrence of red-coloured digestive glands in the mussels. In particular, the decline in the abundance of *M. rubrum* in the last 2 wk of May coincided with the disappearance of the red colouration and a decrease in phycoerythrin levels in the digestive gland. A concurrent increase in the availability of other nonphycoerythrin food sources may have contributed to the observed decline in pigment levels.

As expected, visual assessment of phycoerythrin levels was much less sensitive than microscopic evaluation under epifluorescent illumination. Only when phycoerythrin levels exceeded 80% was there macroscopic evidence of pigment accumulation in the digestive gland. For example, the apparent lack of red colouration in the digestive glands of the sea scallops initially suggested that this species was not feeding on *M. rubrum*, whereas in actuality, phycoerythrin levels were simply below the visual detection limit. Differences in the rate of uptake of phycoerythrin between scallops and mussels may indicate that scallops can select against *M. rubrum*, or that they are more effective at metabolizing and/or excreting the phycoerythrin pigment.

Control mussels deployed in Ship Harbour after May 15 rarely accumulated sufficient phycoerythrin to show signs of red colouration. The indigenous Ship Harbour mussels, however, continued to appear red for 2–3 wk, suggesting that depuration rates only slightly exceeded uptake rates. Laboratory depuration trials indicated that, even in cases where mussels exhibited strong colouration, these characteristics disappeared very rapidly in sand-filtered seawater. In particular, the red fluid from the style sac as well as the pink colouration of the style disappeared within 48 h. Full depuration of phycoerythrin may require a further 4–5 wk, but it appears that mussels could be marketed during this period. Thus, in some instances, short-term depuration of mussels with red-coloured digestive glands could prove a viable option for the shellfish industry.

It remains to be determined whether phycoerythrin, which accumulates in the digestive gland, is metabolized or excreted by mussels and/or scallops. Several researchers have used the autofluorescence of photosynthetic pigments and their breakdown products to investigate the nutritional history of bivalves (Bricelj and Malouf 1984, Hummel 1985, Robinson et al. 1989, Smith and Wikfors 1992). Because phycoerythrin is readily detectable by spectrofluorometry or epifluorescence microscopy, it may have some utility as a natural tracer for feeding, digestion, and/or energy storage studies. For example, the ratio of phycoerythrin to chlorophyll in the stomach relative to that in the phytoplankton may indicate whether bivalve shellfish are selecting for, or against, *M. rubrum*. Also, the rate at which phycoerythrin accumulates in, or disappears from, the digestive gland may provide insight into basic metabolic processes and how these differ among bivalve species.

Occurrence of M. rubrum *in Ship Harbour*

The high abundance of *M. rubrum* in Ship Harbour was consistent with the observation that this species tends to establish substantial populations at the head of estuaries (Crawford 1989). Crawford and Purdie (1992) suggested that *M. rubrum* actively disperses away from turbulent surface water on the ebb tide in order to avoid being flushed out of the estuary. The combination of this negative response to turbulence superimposed on a diurnal vertical migration pattern (Smith and Barber 1979, Crawford 1989) may account for some of the short-term variability in the distribution of *M. rubrum* in Ship Harbour. The complexity of this behaviour may also have confounded our attempt to identify tidal or diurnal migration patterns.

Although *M. rubrum* is reported to be positively phototactic (Crawford 1989), stratified sampling consistently indicated higher cell densities in the deeper zones of the water column.These depths were well below the preferred light intensity of this species, i.e., 50% surface illumination (cited in Smith and Barber 1979). As the surface water warmed, this pattern was accentuated; by late June, the only significant population of *M. rubrum* remained in the

8- to 13-m zone at Location 1. There are several other reports of *M. rubrum* populations existing under low-temperature and low-light conditions, e.g., under the permanent ice in Antarctica (Satoh and Watanabe 1991), deep in the aphotic zone in the Baltic Sea (Leppanen and Bruun 1986), and at the interface of the anoxic layer in a Finnish fjord (Lindholm and Mork 1990). It is probable that the ability of *M. rubrum* to vertically migrate allows it to seek out the optimal combination of light and nutrient conditions. This behavioural advantage may in turn explain the ability of *M. rubrum* to bloom in the early spring, 1–2 mo ahead of other phototrophic species in temperate estuaries (Revelante and Gilmartin 1987).

An increase in the abundance of *M. rubrum* was observed in the 3- to 8-m zone at Location 1 from July 24 to 30. During this period, however, it was noted that the cells of *M. rubrum* were 20–40 μm in diameter, as opposed to 30–80 μm during the spring. Montagnes and Lynn (1989) observed a similar seasonal variation in the size of *Mesodinium* in the Gulf of Maine. They suggested that smaller cells, with their higher surface-to-volume ratio, may have an advantage under nutrient-depleted summer conditions. On the other hand, Revelante and Gilmartin (1987) implied that there are two forms of *Mesodinium*, the phototrophic *Mesodinium rubrum* and a smaller, nanoplanktonic (<20-μm) ciliate that they labelled *Mesodinium* sp. In their study of the Damariscotta Estuary, the larger form dominated from December through April, whereas the smaller form prevailed from April through July. Lindholm and Mork (1990) also observed a wide range of *Mesodinium* phenotypes (20–60 μm) in a Baltic fjord; large forms (>40 μm) dominated in some years, and small forms (<30 μm) did so in other years.

An interesting discrepancy was observed between the spring plankton community at Ship Harbour and sites further south along the Atlantic Coast of Nova Scotia. In mid-April, the more southerly sites exhibited a classic spring bloom with a high abundance and diversity of algal species, whereas Ship Harbour continued to have very low levels of phytoplankton but high numbers of *M. rubrum* and other ciliated protozoans such as *Strombidium*, *Tintinnopsis*, and *Didinium*. Similar microzooplankton assemblages were observed in the spring in Flodevigen Bay in southern Norway (Dale and Dahl 1987) and in the Damariscotta Estuary in Maine (Revelante and Gilmartin 1987). One possibility is that the nutrient regime in Ship Harbour, particularly at the upper end where the rivers enter, favours the development of *M. rubrum*. Nutrient measurements from the spring period (P. Strain, Bedford Institute of Oceanography, unpubl. data) indicated unusually low nitrate levels, which translated into relatively high phosphate-to-nitrate ratios. Interestingly, Fonds and Eisma (1967) linked phosphate-rich upwelled waters with the occurrence of *M. rubrum* blooms in Holland.

Geographic Distribution of **M. rubrum**

Examination of mussels from several other aquaculture sites along the Atlantic Coast of Nova Scotia revealed no prominent cases of red colouration, with the exception of Country Harbour, where *M. rubrum* reached concentrations of 80,000 cells · l^{-1} in May–June 1992 (Carver, unpubl. data). Most sites showed relatively low levels of *M. rubrum* (500–5,000 cells · l^{-1}), although in a few instances, high numbers (>10,000 cells · l^{-1}) were observed for a brief period. The mussels at these sites sometimes exhibited a slight red colouration, but it was rarely sufficient to affect the marketability of the product.

Observations from the Phytoplankton Monitoring Program (Carver, unpubl. data) suggested that *M. rubrum* is a common component of the phytoplankton along the Atlantic Coast of Nova Scotia, particularly during the winter/spring period. It seems that the local strain of *M. rubrum* is unusual in that it blooms at low temperatures rather than in warm surface waters. For example, records of *M. rubrum* from other areas of the world suggest that this species is typically associated with "red tide" blooms during periods of upwelling in the late summer or autumn (Fenchel 1968, Taylor et al. 1971, Crawford 1989). One early survey suggested that *M. rubrum* rarely exceeds 1,000 cells · l^{-1} at water temperatures <10°C (Taylor et al. 1971). More recently, however, there have been several references to winter/spring blooms of *M. rubrum* in cold/temperate coastal waters (Table 2).

Given the ubiquitous distribution of *M. rubrum* and its association with "red tides," it is difficult to account for the scarcity of records on red colouration in the digestive glands of shellfish. For example, phytoplankton surveys in Passamaquoddy Bay, N.B. (Wildish et al. 1990), indicated that *M. rubrum* was the fifth most abundant phytoplankton species from 1987 to 1989. Yet, despite the long history of shellfish harvesting in this region, there are no documented accounts of red colouration. On the other hand, there

TABLE 2.

Estimates of the abundance and/or contribution of *M. rubrum* to the phytoplankton or microzooplankton community during the winter/spring period (water temperatures <10°C).

Location	Period	Abundance/Contribution	Source
North Sea	May 1981	Maximum: 50,000 cells · l^{-1}	Gieskes & Kraay 1983
Baltic Sea	Mar–Jun 1982	2% phytoplankton biomass, 10% primary production	Leppanen & Bruun 1986
FLodevigen Bay, southern Norway	May 1985	Maximum: 17,900 cells · l^{-1}	Dale & Dahl 1987
Saanich Inlet, British Columbia	Dec 1975–Feb 1976	43–85% microzooplankton biomass, 8% phytoplankton biomass; maximum: 12,000 cells · l^{-1}	Takahashi & Hoskins 1978
Damariscotta Estuary, Gulf of Maine	Dec 1981–Apr 1982	Major component of microzooplankton biomass (Dec–May)	Revelante & Gilmartin 1987
Isles of Shoals, Gulf of Maine	Dec–Jun 1986	Maximum: 1,200 cells · l^{-1} in March	Montagnes & Lynn 1989
Passamaquoddy Bay, New Brunswick	May 1988	Maximum: 10,600 cells · l^{-1}	Wildish et al. 1990
Ship Harbour, Nova Scotia	Apr–May 1992	Maximum: 30,000 cells · l^{-1}	This study

are anecdotal reports from the East Coast of the United States. John Hurst (pers. comm., 1996) noted that soft-shell clams (*Mya arenaria*) harvested in Maine during the spring often possess red-coloured digestive glands. Similar characteristics were also observed in surf clams (*Spisula solidissima*) harvested off New Jersey in August 1994. It seems that the probability of these characteristics developing depends on several factors other than the absolute abundance of *M. rubrum*. One is the relative importance of *M. rubrum* in the diet of the shellfish; i.e., in Ship Harbour in April-May 1992 *M. rubrum* not only occurred in high concentrations, but it dominated the phytoplankton biomass. Second, given the tendency of *M. rubrum* to concentrate in deeper, colder water (e.g., Location 1 at Ship Harbour), shellfish grown in suspended culture may be more likely to encounter this species than those harvested from wild beds in the intertidal zone. Third, on the basis of limited evidence, mussels appear to accumulate phycoerythrin more readily than do scallops grown under the same conditions. Thus, there may be species-specific differences in the rate of grazing on *M. rubrum* and/or variations in the ability to metabolize phycoerythrin. Finally, shellfish may depurate phycoerythrin more rapidly in the summer and autumn than during the spring, when their metabolic rates are lower. This may explain why records of *M. rubrum* blooms in warm waters have not been associated with red colouration, even when they occurred in the vicinity of large-scale mussel farms (e.g., MacKenzie et al. 1986).

Although there are few reports of *M. rubrum* negatively affecting the marketability of commercial shellfish species, the increasing nutrient enrichment of coastal waters may promote the growth of this species (Lindholm 1985, Lindholm 1992, Dale 1988). In South African waters, decaying blooms of *M. rubrum* caused a substantial deterioration in water quality that, in turn, negatively affected other organisms (Horstmann 1981). Furthermore, although there is no evidence to suggest that *M. rubrum* is toxic, Romalde et al. (1990) noted that potentially toxic bacteria (e.g., *Vibrio* spp.) may appear in association with *M. rubrum* blooms. It is thus possible that this species may eventually become a nuisance to coastal communities and the shellfish industry.

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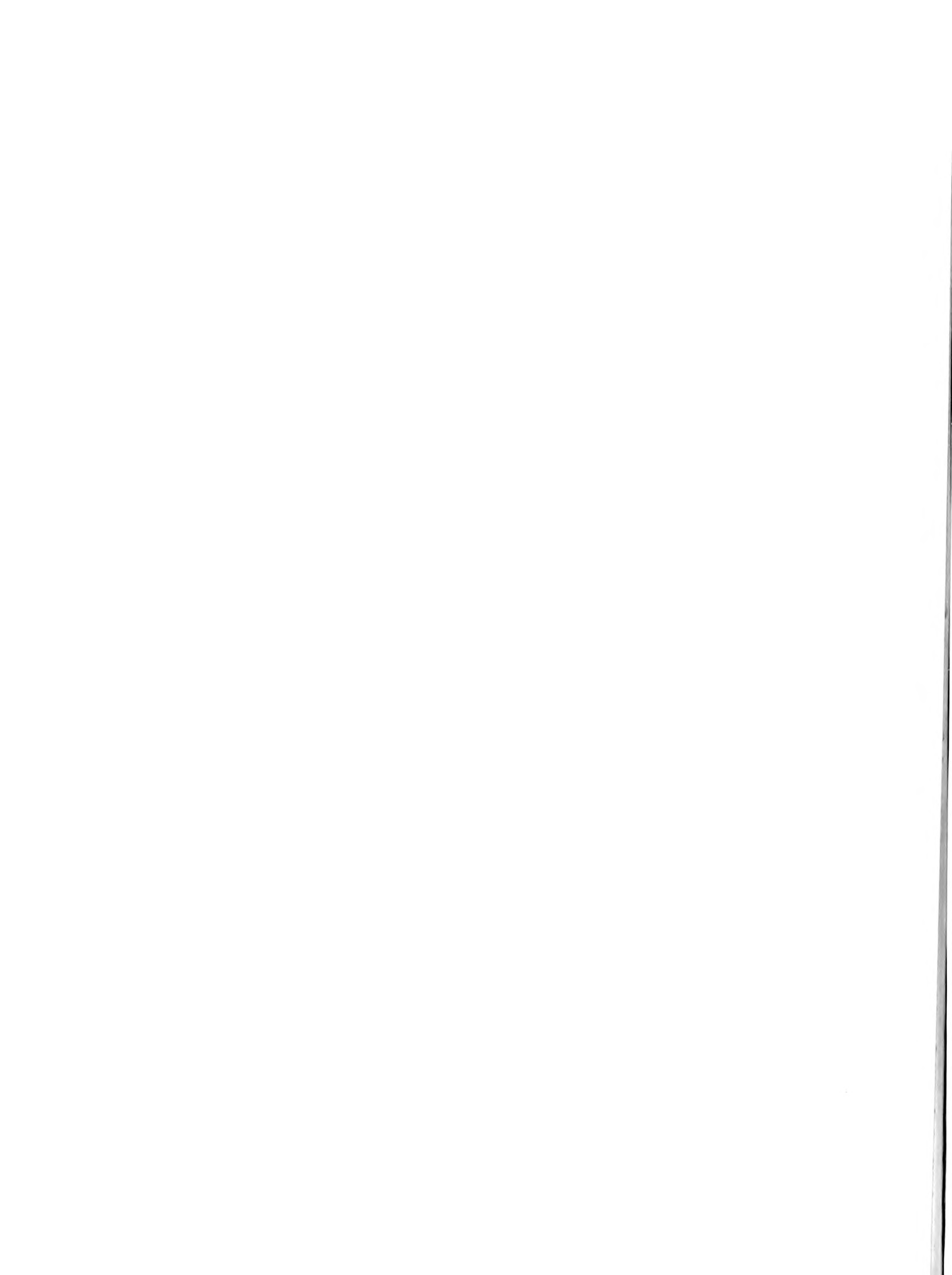

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NEOPLASIA AND BIOTOXINS IN BIVALVES: IS THERE A CONNECTION?

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ABSTRACT In the past 25 years, there has been an increase in the frequency of two major types of cancer in bivalves: disseminated neoplasia and germinomas, which cause debilitation and mortality in shellfish stocks. Disseminated neoplasia is common in softshell clams, *Mya arenaria*, the cockle, *Cerastoderma edule;* and blue mussels, *Mytilus trossulus;* and less common in edible oysters, *Ostrea edulis*, macomas, *Macoma balthica;* blue mussels, *Mytilus edulis;* and Olympia oysters, *Ostrea conchaphila*. Germinomas occur more frequently in northern quahogs, *Mercenaria mercenaria*, and softshell clams, *Mya arenaria*. Certain geographical locations, especially along the northwest Pacific and northeast Atlantic Coasts of North America and the Atlantic Coast of Europe, are "hot spots" for neoplasia. A genetic susceptibility of bivalves to tumor formation has been suggested, and the etiologies proposed include chemical carcinogens, viruses, and other transmissible agents. However, no clear cause-and-effect relationship has yet been conclusively demonstrated, nor has the potential role of biotoxins as etiological agents been examined. In the past 25 years, there has also been an increase in the frequency with which humans have been poisoned by consuming toxic bivalves. Filter-feeding bivalves accumulate biotoxins produced by toxic microalgal blooms. This study traces the worldwide distribution of paralytic shellfish poisoning (PSP), diarrheic shellfish poisoning, neurotoxic shellfish poisoning, amnesic shellfish poisoning, and venerupin shellfish poisoning and of the microalgae and bivalve species associated with the poisonings and then compares these distributions with the distribution of neoplasia in bivalves. The incidence of disseminated neoplasia in some affected bivalve species appears to parallel, both spatially and temporally, outbreaks of PSP that are associated with the toxigenic dinoflagellates *Alexandrium tamarense*, *A. minutum*, *A. fundyense*, and *A. catenella*. Shellfish that have accumulated potent saxitoxin and its derivatives (neosaxitoxin and gonyautoxins) produced by these dinoflagellates are highly toxic to humans. The presence of disseminated neoplasia parallels the presence of certain toxin derivatives in both the bivalve and the *Alexandrium* spp. to which the bivalves are exposed. Disseminated neoplasia is common in softshell clams, *M. arenaria*, that have apparently been exposed to and have accumulated gonyautoxins, (GTX), and in particular GTX1 and GTX4, that are produced by *A. tamarense* or *A. fundyense*. *M. mercenaria* is apparently not affected by disseminated neoplasia and does not usually accumulate toxins associated with *A. tamarense* or *A. fundyense*. Bivalves that accumulate high concentrations of saxitoxin or neosaxitoxin, such as butter clams, *Saxidomus giganteus;* surf clams, *Spisula solidissima;* sea scallops, *Placopecten magellanicus;* and California mussels, *Mytilus californianus*, are apparently not affected by disseminated neoplasia or germinomas. In *M. arenaria*, the incidence of germinomas appears to be related to the distribution of *Alexandrium* spp. blooms. In *M. mercenaria*, however, the distribution of germinomas is not related to those *Alexandrium* spp. that are commonly associated with PSP. The incidence of disseminated neoplasia and germinomas is not correlated with PSP outbreaks associated with *Pyrodinium bahamense* var. *compressum* or *Gymnodinium catenatum*. Although the epizootiological evidence presented here for a correlation between dinoflagellate toxin profiles, the deposition of toxins in bivalve tissues, and the presence of neoplasia in such bivalves is circumstantial, it should be investigated in field and laboratory experiments.

KEY WORDS: Bivalves, cancer, neoplasia, biotoxins, dinoflagellates, *Alexandrium*, epizootiology

NEOPLASIA AND BIVALVES

Occurrence and Type

Since the late 1960s, two main types of neoplasia in bivalves from marine and estuarine locations around the world have been reported with increasing frequency. The first type, disseminated neoplasia, affects some 15 species of bivalves (Tables 1a, 2a, 3a, and 4a) and can cause heavy mortalities (Elston et al. 1992). In disseminated neoplasia, tumor cells are initially found along with normal hemocytes in the circulating hemolymph. As the disease progresses, abnormal cells proliferate throughout the blood sinuses and connective tissue of the visceral mass, muscle, and mantle (Peters 1988). The pathogenesis of disseminated neoplasia is similar to that of vertebrate leukemia in the sense that the circulating tumor cells rapidly divide, ultimately invade the connective tissue, and in advanced stages, kill the host (Miosky et al. 1989). In bivalves, neither the ontogenesis of normal hemocytes nor that of the neoplastic (presumptively hemocytic) cells is known (Elston et al. 1992). Disseminated neoplasia in bivalves was reviewed by Lauckner (1983), Mix (1986a, 1986b), Peters (1988), and Elston et al. (1992) and, except for certain pertinent facts, will not be further discussed. Other types of neoplasia that have been documented, and are often confused with disseminated neoplasia, are gill carcinomas in *Macoma balthica* (L.) (Christensen et al. 1974, Farley 1976a) and epithelioma-like conditions in Australian rock oysters, *Crassostrea commercialis* (Iredale and Roughly) (Wolf 1976).

The second most common type of bivalve neoplasia, germinomas or gonadal tumors, affects 10 species and one hybrid (Table 5a). Tumors result from proliferation of the germinal epithelium, often completely filling the lumen of both male and female gonads (Hesselman et al. 1988, Peters et al. 1994). In germinomas, the affected gonadal follicles are filled with abnormal, hypertrophic cells. Metastasis to the circulatory system occurs in advanced stages (Elston et al. 1992).

Reports discussed here are based on verification of both kinds of neoplasia in the Registry of Tumors in Lower Animals (RTLA, Smithsonian Institution, Washington D.C.) according to Peters (1988) and Peters et al. (1994) and not necessarily as reported in original papers. Rare reports of neoplasia in a particular species, when based on one specimen from many thousands, may need further confirmation (Elston et al. 1992). Consequently, the report

TABLE 1a.

Distribution and prevalence of disseminated neoplasia in oysters with suspected etiology or conditions.

TABLE 1b.

Corresponding records of dinoflagellate blooms or shellfish toxicity events by nearest location and date.

Bivalve Species and Locality	Prevalence (%) (N =)	Date	Etiology	Reference	Toxicity/ Blooms	Site/Date	Bivalve	Reference
C. virginica								
Harris Creek	0.02 (5,000)	1960–1967	Genetic	Couch 1969, Farley 1969a, Otto and Farley 1976, Frierman 1976, Frierman and Andrews 1976	?*A. monilatum* *P. minimum*	?Chesapeake Bay 1970, 1971, 1973 Chesapeake Bay		Williams and Ingle 1972, Steidinger 1993, Seliger et al. 1975
York and James River Chesapeake Bay	0.01–0.075 (51,000)	1964–1973						
23 sites Chesapeake Bay	0.1 (20,000)	1974–1977		Harshbarger et al. 1979				
Apalachicola Bay, FL	0.27 (373)	1978?		Couch and Winstead (1979)	?*A. monilatum*	1978 FL, MS		Perry et al. 1979
Pensacola Bay, FL	0.04 (4,486)	8/78–8/80		Couch (1985)	*A. monilatum* fish kills	1978 FL, MS		Perry et al. 1979
Mobile Bay, AL	0.13 (2,336)							
Pascagoula Harbor, MS	0.44 (2,461)							
O. conchaphila								
Yaquina Bay, OR	7.0 (?) 0.0–2.0 (2,349)	1961–1970 ?1975		Farley and Sparks 1970, Mix 1975a, Mix 1975b, Mix et al. 1977	PSP *A. catenella*	1973 Yaquina Bay, OR	*M. galloprovincialis*	Nishitani and Chew 1988, Taylor 1984
T. chilensis								
Chiloe, Chile	2.0 (4) 1.0 (100)	2/78	Pristine waters	Mix and Breese 1980	DSP *D. acuta* PSP?	1980, 1984, 1991 Reloncavi estuary, Jacaf fiord, Chonos archipelago	*A. ater*, *M. chilensis*	Lembeye et al. 1993
New Zealand	1 case?	?		Peters 1988				
O. edulis								
Mali-Ston, Croatia	20.0–30.0 (?)	1975	Heavy mortality	Alderman et al. 1977	*L. polyedrum*, *A. minutum*	1980+, 1989		Marasović et al. 1995
Galicia, Spain	up to 35.0 (?)	1975			PSP *A. minutum*	1976 Galicia	*M. edulis*	Luthy 1979
Brittany, France	0.50 (69,476)	1975–1981	*C. gigas* was −ve (4,500)	Balouet et al. 1986	*P. minimum* PSP *A. minutum* DSP *D. acuta*, *D. acuminata*, *D. sacculus*	1986 1988–1990 1983–1990 Brittany	*M. edulis*, *O. edulis*	Berland and Grzebyk 1991, Erard Le-Denn 1991, Belin 1993

of a germinoma in *Mytilus trossulus* Gould, 1850 in British Columbia (Cosson-Mannevy et al. 1984) and reports of a disseminated neoplasia in *Crassostrea rhizophorae* in Brazil (Nasimento et al. 1986) and in *Crassostrea gigas* (Thunberg) in Japan (Farley 1969a) have not been included.

Neoplasia Distribution in Bivalves

The distribution and prevalence of bivalve neoplasia by type and by species are shown in Tables 1a to 5a. Neoplasia is common mostly in temperate regions (Figs. 1 to 3), particularly in northeastern and northwestern North America, the European Atlantic, the North Sea, and Scandinavia. A few cases have been documented in the Gulf of Mexico. Reports of neoplasia are rare in Australasia and the Mediterranean except for the Adriatic Sea near Croatia. In South America, only one case has been documented (Mix and Breese 1980). In the Middle East, Central America, Africa, and Asia, no reports are known except for one unconfirmed case in Japan (Farley 1969a).

Differences in the predisposition of bivalves to neoplasia are apparent in some families, genera, and species (Tables 1a to 5a). Oysters, mussels, clams, cockles, and macomas are affected, whereas scallops are not (or are rarely) affected. Oysters are heavily affected (*Ostrea edulis* L. and *Ostrea conchaphila* [Carpenter, 1857]), lightly affected (*Crassostrea virginica* and *Tiostrea chilensis*), or unaffected (*C. gigas*) by disseminated neoplasia (Table 1a). Both disseminated neoplasia (Table 1a) and germinomas (Table 5a) have been found in *C. virginica* in the Chesapeake Bay but were more common in the 1960s and 1970s than recently. Among the clams, *Saxidomus giganteus* (Deshayes) and *Spisula solidissima* (Say) are apparently unaffected by either disseminated neoplasia or germinomas. The northern quahog, *Mercenaria mercenaria* (L.), is unaffected by disseminated neoplasia (Table 2a) but is affected by germinomas (Table 5a). *Mya arenaria* L. is heavily affected by both types of neoplasia (Tables 2a and 5a). Blue mussels are affected by disseminated neoplasia in some geographical regions but not in others. Along the Pacific Coast of North America, *M. trossulus* is heavily affected and *Mytilus californianus* (Conrad) is unaffected by disseminated neoplasia (Table 3a). There have been no reports of disseminated neoplasia in *Mytilus edulis* L. from the northeast Atlantic Coast (North America) or in

TABLE 2a.

Distribution and prevalence of disseminated neoplasia in clams with suspected etiology or conditions.

TABLE 2b.

Corresponding records of dinoflagellate blooms or shellfish toxicity events by nearest location and date.

Bivalve Species and Locality	Prevalence (%) (N =)	Date	Etiology	Reference	Toxicity/ Blooms	Site/Date	Bivalve	Reference
M. arenaria								
Freeport, Harpswell Neck, ME	10.91 (440)	1967–1977	After oil spill in 1971	Yevich and Barzcsz 1976, Yevich and Barzcsz 1977	PSP *A. tamarense*	1972 York Harbor, ME	*M. edulis* *M. arenaria*	Twarog and Yamaguchi 1975
Jones Creek Annisquam River, MA	12.0 (50)	9/72	No obvious environmental relationship or viral etiology	Farley 1976a	*A. tamarense* first reported outbreak of PSP in the region at same time	9/10 1972 Annisquam River, Essex, and Eastham MA	*M. arenaria* *M. edulis* *A. irradians*	Hartwell 1975, Twarog and Yamaguchi 1975, Farley 1976a, Anderson et al 1982
Bourne, MA, Searsport, ME; Quonset, RI (10 sites)	0.0–64.0 (1,325)	1–9/76	Highest % at oil spill site? Viral	Brown et al 1976, 1977	PSP toxin closed shellfish beds	4–9/76 western ME	*M. edulis*	Hurst 1979
Allen Harbor, RI	20.0–40.0 (3,500)	7/77–3/79	*M. mercenaria* and *M. balthica* – ve	Cooper et al. 1982a, Cooper et al 1982b	PSP toxin closed shellfish beds	1979 Narragansett Bay, RI	*M. edulis*	Anderson et al 1982
New Bedford Harbor (NBH), Little Buttermilk Bay (LBB); Buzzard Bay, MA	73.2 NBH (407) 39.3 NBH (886) 17.0 LBB (881)	1/82–5/83 5/86–10/87	?PCBs* Environmental factors	Reinisch et al 1984, Leavitt et al 1990	*A. tamarense* *P. minimum*	1987–1988 NBH and 7 other stations		Borkman et al 1993, Pierce and Turner 1994
Long Island Sound (3 sites) Milford Point, CT	45.0–60.0 (3,963) 64.3 (2,121)	6/83–3/84 10/88–12/89	Unresolved	Brousseau 1987, Brousseau and Baglivo 1991a, Brousseau and Baglivo 1991b, Brousseau and Baglivo 1994	PSP *A. tamarense* *P. minimum*	1982–1983 Long Island 1986–1989 Long Island Sound, NY	*M. edulis*	Schrey et al 1984, Nuzzi and Waters 1993, Wikfors and Smolowitz 1993
Chesapeake Bay, MD (6 sites)	0.0–65.0 (3,584) 0.0–78.0 (?)	12/83–5/85 1990–1995	Was very rare in this location prior to 1984	Farley et al 1986, McLaughlin et al 1996	*P. minimum*	1978 1992		Seliger et al. 1979, Harding and Coats 1988, Marshall 1995
Shrewsbury River, NJ	0.0–19.0 (1,200)	9/86–8/87		Barber 1990	*A. tamarense* DSP *D. acuminata*	1987 Atlantic City, NJ 1980, 1983 NJ, NY		Cohn et al. 1988, Freudenthal and Jijina 1988
New Brunswick, Nova Scotia (22 sites), Canada	3.1–31.3 (688)	12/85–1/87		Morrison et al 1993	PSP *A. fundyense* (= *A. excavatum*) DSP *P. lima*	1986 New Brunswick, NS 1990 Atlantic NS	*M. edulis*, *M. arenaria* *M. edulis*	Martin et al 1990 Marr et al. 1992
Mya truncata								
Baffin Is., Canada	1.61 (856)	?1986	oil?	Neff et al 1987				
Ruditapes decussatus								
Galicia, NW Spain	1.3 (360)	2–12/93		Villalba et al 1995	DSP *Dinophysis* spp. *A. minutum* *G. catenatum*	1991–1993 Galicia	*M. galloprovincialis*	Blanco et al. 1995, Franco et al. 1994, Anderson et al 1989

* PCBs, polychlorinated biphenyls.

Mytilus galloprovincialis from the northwest Pacific Coast (North America), whereas there have been a few reports of this cancer in both species in Europe (Table 3a). Four species of macomas and one species of cockle have been reported with disseminated neoplasia (Table 4a). Scallops in the genera *Patinopecten*, *Placopecten*, and *Argopecten* are apparently unaffected by disseminated neoplasia, and only one case of germinoma has been reported in bay scallops, *Argopecten irradians* (Lamarck) (Table 5a).

TABLE 3a.

Distribution and prevalence of disseminated neoplasia in mussels with suspected etiology or conditions.

TABLE 3b.

Corresponding records of dinoflagellate blooms or shellfish toxicity events by nearest location and date.

Bivalve Species and Locality	Prevalence (%) (N =)	Date	Etiology	Reference	Toxicity/ Blooms	Site/Date	Bivalve	Reference
M. trossulus								
Yaquina Bay, OR	7.0–12.0 (100) 0.4–9.8 (2,934)	9/68–2/69 6/76–4/78	No virus found. Etiology remains unknown. PAH levels not significant	Farley 1969b, Mix 1983, Elston et al. 1988a, Moore et al. 1991, Cosson-Mannevy et al. 1984, Bower 1989, Emmett 1984	PSP *A. catenella*	1973 Yaquina Bay	*M. trossulus* *M. californianus*	Taylor 1984, Anderson 1984, Chiang 1988, Nishitani and Chew 1988
Puget Sound, WA	0.0–40.0 (40) 11.0 (660)	11/86 3/89–2/90			*A. catenella*	1988 Puget Sound, WA	*S. giganteus*, *Crassostrea gigas*	
Vancouver Island, BC	0.0–29.2 (166) 0.0–45.0 (278)	12/80–6/81 1988?			*A. catenella*	1980–1982 1985–1987 BC		
Departure Bay BC, Canada	10.0–36.0 (?)	10/83–9/84						
M. edulis								
Plymouth, England	1.61 (994)	1976–1978	Potentially carcinogenic PAHs in sediments*	Lowe and Moore 1978, Green and Alderman 1983	PSP *A. tamarense* ?*P. minimum*	1968, 1990 E. England	*M. edulis*	Wyatt and Saborido-Rey 1993
Morecombe Bay, N Wales and E. England	0.0–4.3 (4,000)	11/78–8/79						
Denmark*	0.2–0.8 (8,720)	10/83–9/84	Viral etiology and multifactorial hypothesis	Rasmussen et al. 1985, Rasmussen 1986	PSP *A. tamarense* ?*P. minimum* DSP *Dinophysis norvegica*	1987 1982	*M. edulis*	Moestrup and Hansen 1988, Kimor et al. 1985
Furuskar, Tvärminne, Finland	0.5 (205)	9/86		Sunila 1987	*A. tamarense*, *D. acuminata*, *D. acuta*, *P. minimum*, *G. catenatum*	1984		Kononen et al. 1993
M. galloprovincialis								
Humboldt Bay, CA	0.0 (40)	1988	—	Elston et al. 1988b.	*A. catenella*	1988	*M. galloprovincialis*	Price et al. 1991, Anderson et al. 1989,
Rias de Galicia, NW Spain	0.6 (170)	1986		Gutierrez and Sarasquete 1986	PSP *G. catenatum*, *A. minutum* DSP *Dinophysis acuta*, *D. acuminata*, *D. sacculus*	1976, 1981, 1984–1987 1978, 1981, 1983, 1987, 1991–1993	*M. galloprovincialis* *M. galloprovincialis*	Franco et al. 1994, Berland and Grzebyk 1991, Blanco et al. 1985, Blanco et al. 1995

* Elston et al. 1992 list this record as occurring in *M. trossulus*.

* PAH, polycyclic aromatic hydrocarbons.

The bivalves most commonly affected by disseminated neoplasia and in which prevalences of more than 20% have been consistently recorded are *M. arenaria* in the northeastern United States and Canada, *M. trossulus* in the northwestern United States and *Cerastoderma edule* (L.) in Ireland and France (Tables 2a, 3a, and 4a). Mortalities associated with disseminated neoplasia have been recorded in *O. edulis* (Alderman 1974), *O. conchaphila* (Farley and Sparks, 1970), *M. arenaria* (Cooper et al. 1982a, Farley et al. 1986, Leavitt et al. 1990, Brousseau and Baglivo 1991b), and *M. trossulus* (Cosson-Mannevy et al. 1984). Disseminated neoplasia caused mortalities of up to 78% in *M. arenaria* in New England. The disease may be contributing to recent population declines of *M. arenaria* in New England (Brousseau and Baglivo 1991b) and in the Chesapeake Bay (Brousseau and Baglivo 1991b, McLaughlin et al. 1996).

Prevalences of disseminated neoplasia generally change seasonally and are at their highest between October and March (Cooper et al. 1982a, Cosson-Mannevy et al. 1984, Farley et al. 1986, Rasmussen 1986, Brousseau 1987, McLaughlin et al. 1996), with minimum prevalences from April to August (Leavitt et al. 1990). Biphasic prevalences have also been noted: a second peak may occur from May to July (Cooper and Chang 1982, Cooper et al. 1982a, Barber 1990, McLaughlin et al. 1996) or from January to March (Leavitt et al. 1990). Low water temperatures may suppress the progression of neoplasia (Appeldoorn and Oprandy 1980) and reduce mortality (Brown et al. 1977).

In field studies, some species that were apparently unaffected by disseminated neoplasia have been found in the same location as other species that were heavily affected. For example, in northeastern North America, *M. arenaria* are heavily affected by disseminated neoplasia, whereas *M. mercenaria*, *M. edulis*, and *C. virginica* are unaffected.

The distribution of germinomas currently appears to be restricted to the East Coast of North America, the southern coast of Ireland, New Zealand, and Arctic Canada (Table 5a). *Mercenaria* spp. and *M. arenaria* are heavily affected by germinomas. Al-

TABLE 4a.

Distribution and prevalence of disseminated neoplasia in macomas and cockles with suspected etiology or conditions.

TABLE 4b.

Corresponding records of dinoflagellate blooms or shellfish toxicity events by nearest location and date.

Bivalve Species and Locality	Prevalence (%) (N =)	Date	Etiology	Reference	Toxicity/ Blooms	Site/Date	Bivalve	Reference
M. calcarea Baffin Island, Canada	0.19 (519)	?1986	oil?	Neff et al. 1987				
*M. balthica** Tvarminne, Finland	4.0–15.0 (1,748)	3/82–7/89	No apparent correlation with pollution	Pekkarinen 1993	*A. tamarense, D. acuminata, P. minimum, G. catenatum*	1984		Kononen et al. 1993
Macoma inquinata and *M. nasuta* Yaquina Bay, OR	5.0 (?)	?1975	PAH?	Farley 1976a	PSP *A. catenella*	1973 Yaquina Bay, OR	*M. californianus*	Nishitani and Chew 1988
C. edule Cork Harbor and coast, S Ireland (18 sites)	0.0–72.0 (1,356)	2/83–2/85	Environmental factors/infectious disease *M. edulis* were −ve	Twomey and Mulcahy 1984, Twomey and Mulcahy 1988a	DSP *Dinophysis acuminata, D. acuta, D. norvegica*	1984	*M. edulis*	Jackson and Silke 1995
					A. minutum	1987 Cork Harbour		Gross 1988
Brittany, France 4 sites	46.0 (?) 4.1 (752)	?1983	Reference site and site of Amoco oil spill, both had neoplasia	Poder et al 1983, Poder and Auffret 1986	DSP *D. acuta, D. acuminata, D. sacculus*	1983–1990 Brittany	*M. edulis*	Belin 1993
					PSP *Alexandrium minutum*	1988–1990 Brittany	*M. edulis, O. edulis*	Belin 1993
					P. minimum	1976, 1986		Berland and Grzebyk 1991

* This reference may not be a disseminated neoplasm but a gill carcinoma.

though *M. mercenaria* are distributed along the Atlantic Coast of North America, those with germinomas are more localized south of Rhode Island and are particularly prevalent along the southeast Atlantic Coast. Germinomas only occur in *M. arenaria* in Maine (Barber 1996). The prevalence of germinomas was highest during the warm summer months (Hessleman et al. 1988, Eversole and Heffernan 1993).

Germinomas are less common (Table 5a) than disseminated neoplasia (Tables 1a to 4a). In some incidences, both types of neoplasia were reported in the same species of bivalve at the same time and from the same location, for example, in *M. arenaria* in northeast North America, in *C. edule* in Ireland, and on rare occasions, in *Macoma calcarea* in northern Canada (Yevich and Barszcz 1976, Cosson-Mannevy et al. 1984, Twomey and Mulcahy 1984, Neff et al. 1987, Peters et al. 1994). In this situation, a common causative agent might be indicated.

Etiology

The etiology of bivalve neoplasia has been postulated to be related to various causative agents, but no clear cause-and-effect relationship or multifactorial sequence of events has yet been established. Tentative links between sublethal exposure to various pollutants and the presence of neoplasia have been postulated but not conclusively demonstrated. A systematic survey of shellfish during the NOAA Status and Trends mussel watch showed that the prevalence of neoplasia was not strongly correlated with chemical contamination (Hillman 1993). Smolowitz and Leavitt (1996) found no correlation between the distribution of disseminated neoplasia in *M. arenaria* and pollution in Boston Harbor and Cape Cod Bay, MA.

Hydrocarbon deposition associated with oil spills was tentatively linked to disseminated neoplasia in New England (Barry and Yevich 1975, Yevich and Barszcz 1976, 1977, Brown et al. 1977, 1979, Gilfillan et al. 1977, Harshbarger et al. 1979, Walker et al. 1981); Yaquina Bay, Oregon, (Mix et al. 1979, Mix 1988); Brittany, France (Auffret and Poder 1986, Poder and Auffret 1986); and northern Canada (Neff et al. 1987). The presence of neoplasia was demonstrated in areas where chemical contaminants were absent (Gilfallan et al. 1977) or were present at low background levels (Brown et al. 1977, Mix 1983, Cosson-Mannevy et al. 1984, Emmett 1984, Twomey and Mulcahy 1988a). Conversely, neoplasia was absent in areas where bivalves were exposed to extremely high concentrations of contaminants (Mix 1988).

Studies attempting to link the occurrence of neoplasia with contaminants have suggested a correlation between the high prevalence of neoplasia and pesticide use (Farley et al. 1991, Gardner et al. 1991b). An increased prevalence of disseminated neoplasia in *M. arenaria* was associated with and statistically correlated to elevated chlordane levels in the tissues (Farley et al. 1991). In recent epizootics, germinomas were observed in *M. arenaria* from Machiasport, Searsport, and Dennysville, ME (Table 5a). Herbicides and other agrochemicals were widely used in the extensive forestry and blueberry industries in the area. Gardner et al. (1991b) indicated that the estuaries at Dennysville had been contaminated by herbicides in a 1979 accidental spray overdrift during the aerial application of Tordon 101® to adjacent forests. Herbicide contamination was the only identified common denominator at all three sites where *M. arenaria* with germinomas were found (Gardner et al. 1991b). Other field studies could not correlate the dis-

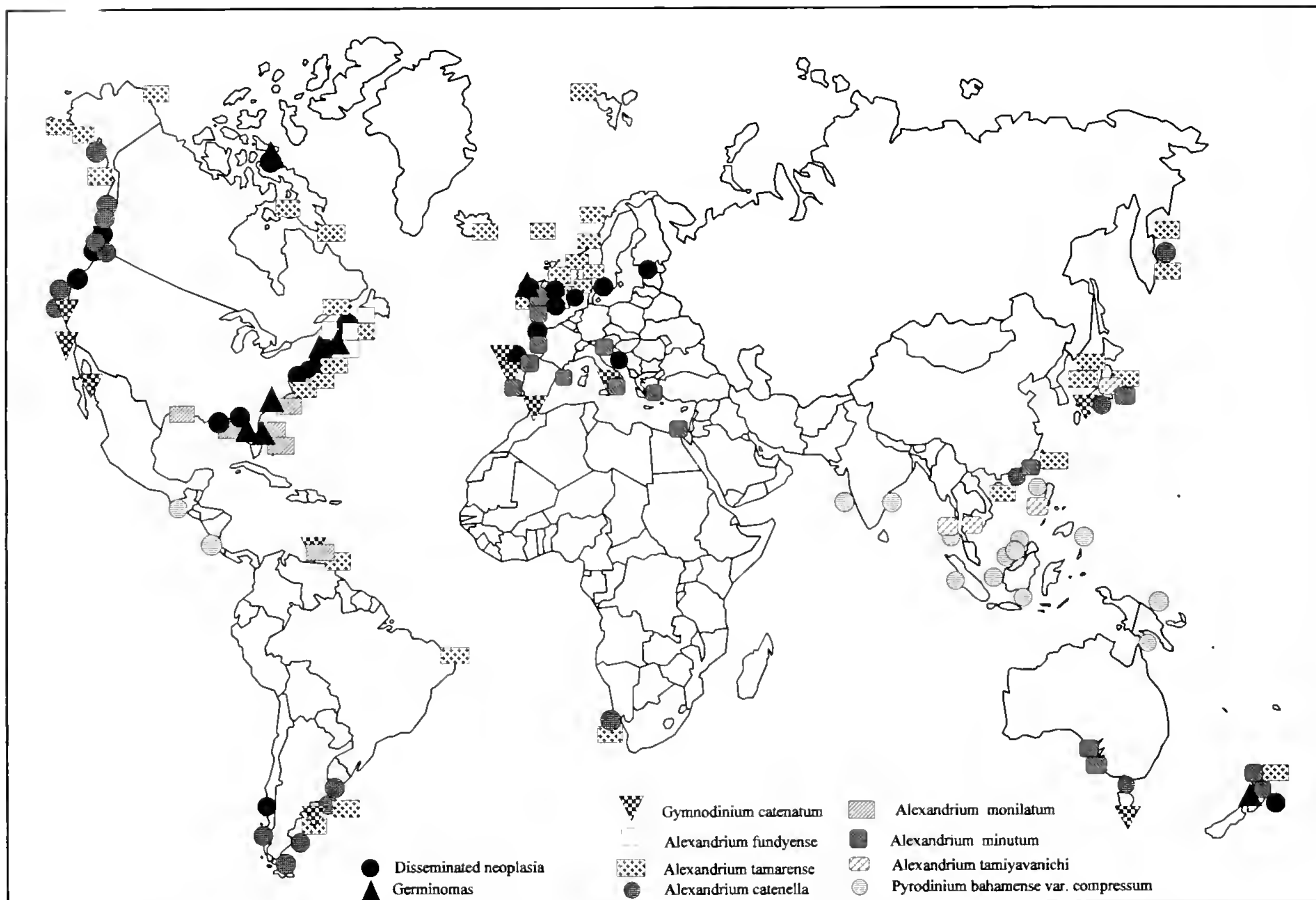

Figure 1. The distribution of dinoflagellates associated with PSP and the distribution of disseminated neoplasia and germinomas in bivalves.

tribution of carcinogenic pollutants with the development of germinomas (Yevich and Barry 1969, Barry and Yevich 1972, Hesselman et al. 1988).

Bivalves have been exposed to various chemical pollutants in laboratory exposures (Rasmussen et al. 1985, Rasmussen 1986), but no disseminated neoplasia or germinomas have been induced (Elston et al. 1992). Exposure to chemicals has induced numerous lesions (Farley 1977, Rasmussen et al. 1985) and, rarely, other types of neoplasia in bivalves. Thirty days after *C. virginica* or *M. edulis* were exposed to particulate suspensions or solid sediments from Black Rock Harbor, Bridgeport, CT, benign tumors were documented in the kidney, gastrointestinal tract, gonad, heart, and neural tissue (Gardner et al. 1991a).

Evidence for a viral etiology for disseminated neoplasia has only been demonstrated in *M. arenaria* (Brown 1980, Oprandy et al. 1981, Oprandy and Chang 1983). Normal *M. arenaria* that were exposed to water that had passed over infected *M. arenaria* developed neoplasia, thus suggesting that a transmissible agent was involved (Brown 1980). When virus-like particles from *M. arenaria* with neoplasia were injected into normal *M. arenaria*, these clams subsequently developed neoplasia. Virus-like particles were then reisolated from the newly induced neoplasia, conforming to Koch's postulates (Oprandy et al. 1981). A virus similar to an RNA tumor virus was isolated from *M. arenaria* with neoplasia, and after the injection of the purified virus into normal *M. arenaria*, neoplasia was induced. Because the virus was not isolated from any of the nonneoplastic samples, it was reasoned that a virus was the etiological agent of disseminated neoplasia (Oprandy and Chang 1983). The chemical 5-bromodeoxyuridine was used to induce retrovirus expression and replication as well as disseminated neoplasia in *M. arenaria*. Oprandy and Chang (1983) suggested that the clam tumor–inducing retrovirus may be endogenous in the cells of normal *M. arenaria*. A retrovirus was also found in the hemocytic cells of *M. arenaria* with disseminated neoplasia (Cooper and Chang 1982). Virus-like particles have been demonstrated in disseminated neoplasia (Rasmussen 1986), and a viral agent has been suggested as the probable cause of neoplasia in mussels (Elston et al. 1988a). However, ultrastructural examinations of tissues from *C. edule* (Auffret and Poder 1986), *O. edulis* (Cahour and Balouet 1984), *M. arenaria* (Farley 1976b, Cooper and Chang 1982, Medina et al. 1993), and *M. trossulus* (Mix et al. 1979) with disseminated neoplasia have failed to reveal the presence of virus. Since the earlier studies demonstrating retrovirus in *M. arenaria*, a viral etiology has not been confirmed despite numerous attempts (Elston et al. 1992).

A viral etiology in the development of germinomas is also unconfirmed. Intranuclear inclusions have been reported in germinoma cells of *M. arenaria* (Harshbarger et al. 1979; Hesselman et al. 1988), but electron microscopy of the same tissue, which is deposited at the RTLA, did not reveal virus (Peters et al. 1994).

An infectious etiology has also been postulated. Disseminated neoplasia appears to be transmissible if neoplastic cells are injected into disease-free bivalves (Farley 1987, Elston et al. 1988b, Twomey and Mulcahy 1988b). However, in several experiments, controls were also diagnosed with neoplasia (Farley 1987, Elston et al. 1988b). Kent et al. (1991) attempted to transfer disseminated

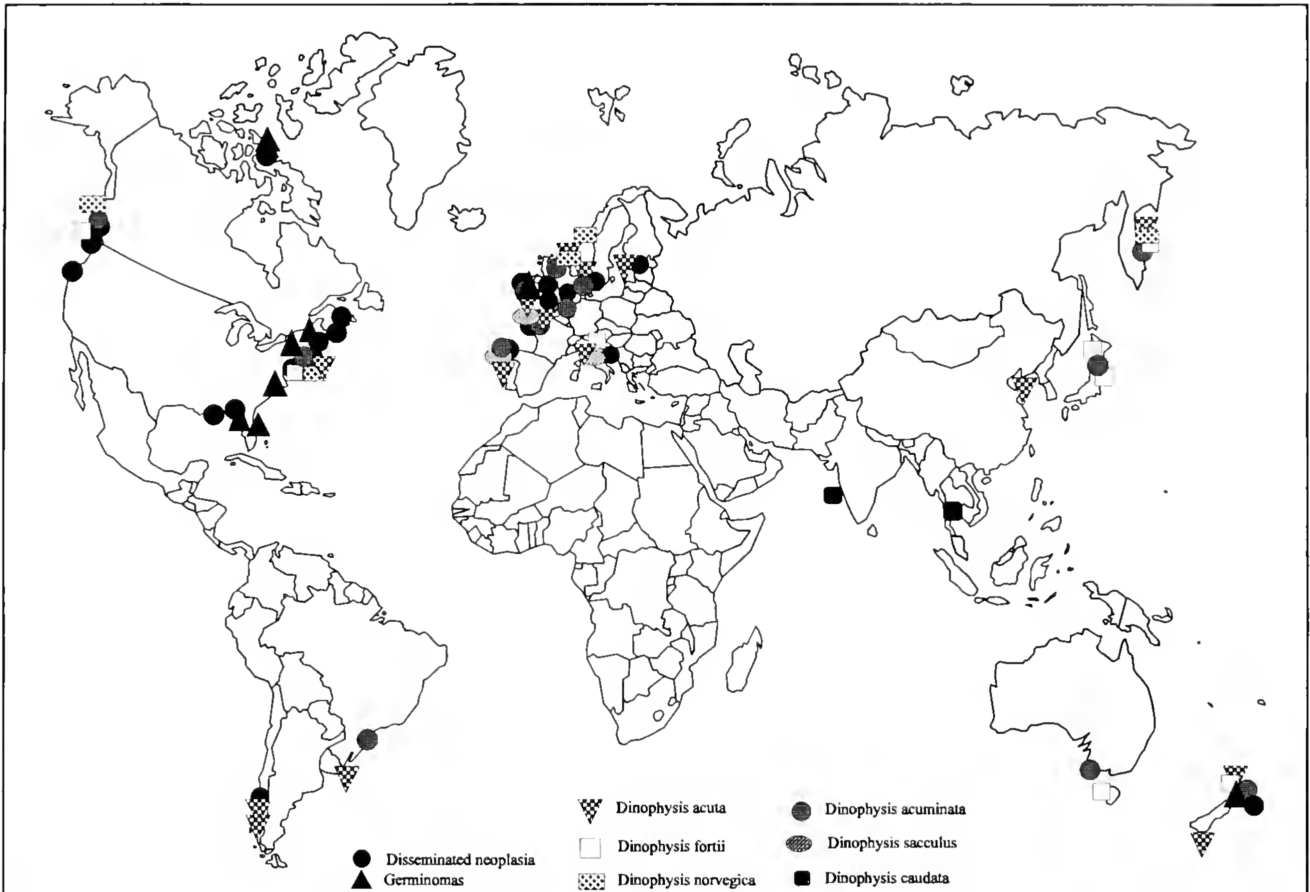

Figure 2. The distribution of dinoflagellates associated with DSP and the distribution of disseminated neoplasia and germinomas in bivalves.

neoplasia by injecting blood from heavily affected *M. trossulus* into *M. arenaria*, *O. edulis*, *O. conchaphila*, and other *M. trossulus*. After 152 days, only the injected *M. trossulus* were showing signs of disseminated neoplasia.

BIOTOXINS AND BIVALVES

In coastal areas where toxigenic microalgae occur, bivalves pose a public health risk because they accumulate a variety of biotoxins by filter feeding on phytoplankton. Exposures to toxic microalgae are usually acute, and high levels of toxins in bivalves prone to toxin accumulation can be reached within days or after only a few weeks. Biotoxins in shellfish are transferred to humans (and other predators) through consumption. The most common poisonings of humans from the consumption of shellfish are paralytic shellfish poisoning (PSP), diarrheic shellfish poisoning (DSP), neurotoxic shellfish poisoning (NSP), and more recently, amnesic shellfish poisoning (ASP). Venerupin shellfish poisoning (VSP) has rarely been documented. Biotoxins causing human shellfish poisonings are usually associated with dinoflagellates or, in the case of ASP, with diatoms.

In addition to the public health risk associated with eating toxic bivalves, the bivalves themselves may be affected by toxin exposure. The accumulation of biotoxins in bivalves varies between species, with geography, with the toxicity of specific dinoflagellates, and in the localization of toxins in bivalve tissues. Bivalve feeding behavior may be one of the principal factors controlling toxin levels. Some bivalves show immediate behavioral responses to avoid the consumption of toxic dinoflagellates (Gainey and Shumway 1988, Shumway 1990). Some species typically burrow into and feed on sediments, whereas others filter plankton from the water. Toxigenic dinoflagellates can produce benthic cysts and/or vegetative planktonic stages, so bivalves may be differentially exposed to toxins because of their feeding modes.

Although some studies have evaluated the effects of short-term toxin exposure on bivalve behavioral and physiological responses, other effects of biotoxins on bivalve health are generally unknown. Despite the frequent exposure of bivalves to biotoxins, no apparent associated pathological effects have been reported (Prakash et al. 1971). The detrimental effects of dinoflagellates and their toxins on bivalves have only recently been considered (Shumway 1990, Shumway et al. 1990, Wikfors and Smolowitz 1993, 1995, Smolowitz and Shumway 1996).

The tissues that accumulate toxins and their different components are known to vary both geographically and temporally among bivalve species, but the effects of chronic exposures are unknown. The majority of available information is on dinoflagellates known to be producers of toxins that are lethal or deleterious to mammals. The existence of biotoxins or toxic components that are potentially lethal or sublethal to molluscs should be considered. Recent evidence has shown that dinoflagellates that are apparently not toxic to mammals may be pathogenic to bivalves (Wikfors and Smolowitz 1995).

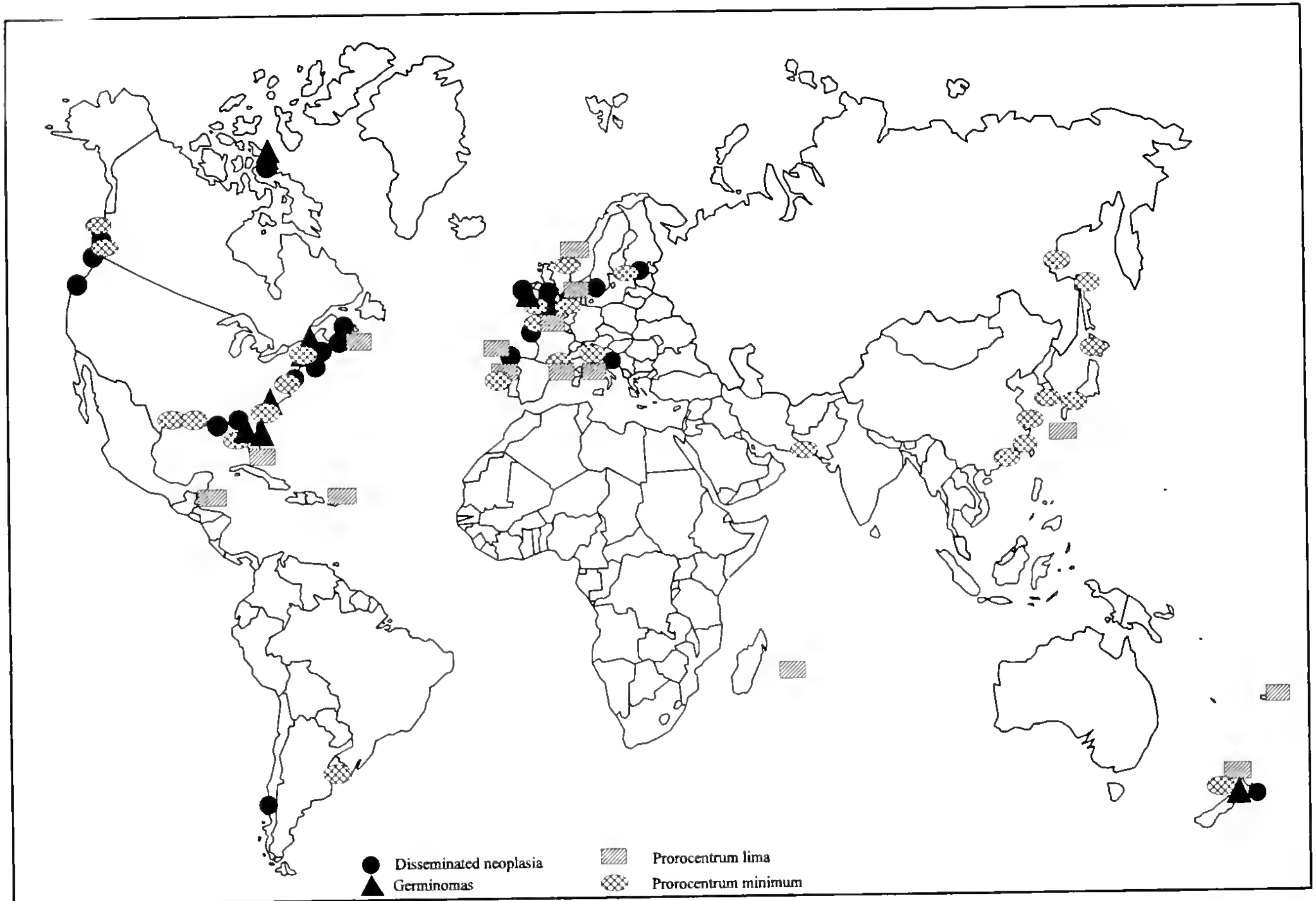

Figure 3. The distribution of *Prorocentrum* spp. implicated in shellfish toxicity and the distribution of disseminated neoplasia and germinomas in bivalves.

ASP

The first outbreak of ASP, in 1987 in Prince Edward Island, Canada, occurred after humans consumed toxic bivalves exposed to a bloom of the diatom *Pseudo-nitzschia multiseries* (Hasle) (Bates et al. 1989). Although the effects of diatoms and their toxins on bivalves have not been well documented, ASP is not considered here to be involved with the initiation of bivalve neoplasia. For the remainder of this article, only biotoxins associated with dinoflagellates will be considered.

NSP

NSP associated with brevetoxins has been documented from the Gulf of Mexico, the eastern United States, and New Zealand and is produced principally by toxins of *Gymnodinium breve* Davis and *Gymnodinium* spp. (Steidinger 1993, Chang 1995). Given that the known distribution of NSP is restricted to these areas, brevetoxins are not considered to play a role in the etiology of bivalve neoplasia. Although brevetoxin is well known for its role in fish kills (Steidinger 1993), its effects on molluscs are less well documented.

PSP

Since the 1970s, there has been a steady increase in the distribution of PSP worldwide (Hallegraeff 1993). Outbreaks of PSP are now common in temperate regions, particularly in North and South America, Europe, South Africa, Japan, and Australasia, and in equatorial regions in the Far East, Central Americas, northern South America, and India (Fig. 1). Outbreaks of PSP are related to a series of factors, including dinoflagellate distributions, environmental conditions favoring high concentrations of cells, population toxicity levels, bivalve distributions, and differential toxin uptake and accumulation by bivalves (Shumway 1990, Hallegraeff 1993). Many bivalve species accumulate PSP toxins, and these species pose a high public health risk during particular seasons and at certain geographical locations.

Dinoflagellate Distribution

Dinoflagellate species associated with the production of paralytic shellfish toxins (saxitoxin [STX] and its derivatives) are *Alexandrium acatenella* (Whedon and Kofoid), *Alexandrium catenella* (Whedon and Kofoid), *Alexandrium fundyense* (Balech), *Alexandrium lusitanicum* (Balech), *Alexandrium minutum* (Halim), *Alexandrium ostenfeldii* (Paulsen), *Alexandrium tamarense* (Lebour), *Alexandrium tamiyavanichi* (Balech), *Gymnodinium catenatum* Graham, *Pyrodinium bahamense* var. *compressum* (Böhm), and possibly, *Alexandrium monilatum* and *Lingulodinium* (=*Gonyaulax*) *polyedrum* (Stein) (Steidinger 1993). Some cyanobacteria are associated with the production of STX (Mahmood and Carmichael 1986), and bacteria have been implicated in paralytic shellfish toxin production (Kodama et al. 1990). How-

TABLE 5a.
Distribution and prevalence of germinomas in bivalves with suspected etiology or conditions.

TABLE 5b.
Corresponding records of dinoflagellate blooms or shellfish toxicity events by nearest location and date.

Bivalve Species and Locality	Prevalence (%) (N =)	Date	Etiology	Reference	Toxins/ Blooms	Site/Date	Bivalve	Reference
Arctica islandica								
Newport, RI	1 case (?)	?		Peters et al 1994				Hurst 1979
A. irradians								
Massachusetts	1 case (?)	?		Peters et al 1994				Hurst 1979
M. calcarea								
Baffin Island, Canada	1 case	?1986	oil?	Nett et al 1987, Peters 1988				
C. edule								
Cork Harbor, Ireland	0.15 (1,356)	2/83–2/85		Peters et al 1994	DSP *Dinophysis* spp *A. minutum*	1984 1987	*M. edulis*	Jackson and Silke 1995, Gross 1988
C. virginica								
Delaware Bay, DE, Chesapeake Bay, MD, Black Rock Harbor, CT	2.0 (50) 0.01 (20,000) 0.23 (420)	8/69 1974–1977 1985		Farley 1976a, Harshbarger et al 1979, Gardner et al 1986	*P. minimum* *A. tamarense*	1978 1986–1989		Seliger et al 1975, Nuzzi and Waters 1993
T. chilensis								
New Zealand	21 cases	?		Peters et al 1994	PSP *A. minutum*, *A. tamarense*	1993		Chang et al 1995
M. arenaria								
Searsport, Machiasport, Dennysville, ME; Washington County, ME	6.0–12.5 (2,125) 6.4 (204), <22.0 (?) 32.0–40.0 (300) 10.0–43.0 (?)	1971–1976 1979 1993	oil spill, virus herbicides	Yevich and Barszez 1976, 1977, Brown et al 1977, Harshbarger et al 1979, Gardner et al 1991b, Barber 1996	PSP *A. tamarense*	1972 York Harbor, ME	*M. edulis* *M. arenaria*	Twarog and Yamaguchi 1975
M. edulis								
New York	1 case	? 1987		Peters et al 1994	*A. tamarense*	NY 1986–1989		Nuzzi and Waters 1993
M. mercenaria								
Narragansett Bay, RI, Indian R Lagoon, FL; Charleston, SC	0.23 (1,300) 2.3–2.7 (539) 3.3–31.5 (1,263) 6.5 (708) 42.0 (?) 58.0–75.0 (440)	summer 68 summer 69/70 5/85–6/87 9/87–8/88 9/87–10/88 1988–1992	No relationship with water quality	Yevich and Barry 1969, Barry and Yevich 1972, Hesselman et al 1988, Bert et al 1993, Eversole and Heffernan 1993, Eversole and Heffernan 1966	*D. acuminata* *A. monilatum*	1984 1978 Indian R. Lagoon, FL		Maranda and Shimuzu 1987, Norris 1983
Mercenaria campechiensis								
Tampa Bay, FL; Indian R Lagoon, FL; Charleston, SC	7.7 (26) 11.8 (85) 42.0 (?) 58.0–75.0 (440)	9/86 9/87–8/88 9/87–10/88 1988–1992		Hesselman et al 1988, Bert et al 1993, Eversole and Heffernan 1993, Eversole and Heffernan 1996	*A. monilatum*	1978 Indian R. Lagoon, FL		Norris 1983
M. campechiensis × *M. mercenaria*								
Indian R. Lagoon, FL, Charleston, SC	21.6 (75) >42.0 (?) 100.0 (440)	9/87–8/88 9/87–10/88 1988–1992		Bert et al. 1993, Eversole and Heffernan 1993, Eversole and Heffernan 1996	*A. monilatum*	1978 Indian R. Lagoon, FL		Norris 1983

ever, the majority of outbreaks worldwide have been attributed to dinoflagellates.

The taxonomy of *Alexandrium* has been in flux. Balech (1995) synonymized *Alexandrium excavatum* with *A. tamarense* and synonymized *Alexandrium ibericum* (Balech) with *A. minutum*. Balech (1994) named a new species *A. tamiyavanichi* that had been previously identified as *Alexandrium cohorticula* in the Far East (Kodama et al. 1988, Ogata et al. 1990, Pholpunthin et al. 1990, Wisessang et al. 1991, Han et al. 1992). In this article, the most updated references have been used (Anderson et al. 1994, Balech 1995). I acknowledge that some records that are based on the original authors' descriptions may be inaccurate. When the taxonomy has changed, the original designation has been noted wherever possible.

Figure 1 shows the distribution of blooms of the more common toxic dinoflagellate species associated with PSP. In the majority of cases, PSP outbreaks are associated with *A. tamarense*, *A. fundyense*, *A. catenella*, *A. minutum*, *G. catenatum*, and *P. bahamense* var. *compressum* (Fig. 1). Particular species have distinct distribution patterns: *P. bahamense* var. *compressum* is tropical and is common in Asia and Central America; *G. catenatum* is common along the West Coast of North America, the European Atlantic, southeastern Australia, New Zealand, southern South America, and Japan; *A. tamarense* is common in northwestern and northeastern North America and in Europe, New Zealand, Argentina, and the Far East; and *A. catenella* is common from Alaska to north-central California, central and southern Chile, southeastern Australia, New Zealand, and South Africa but is rare in southern California and Central America (Taylor 1984, Balech 1995).

Dinoflagellate Toxicity

No natural toxigenic dinoflagellate population has been found to contain all naturally occurring PSP toxin derivatives, so the toxin profile is considered to be characteristic of the dinoflagellate strain (Cembella et al. 1993). Some of the PSP toxin derivatives are highly toxic (as sodium channel–blocking agents in mammals) and include the carbamate toxins, saxitoxin (STX), neosaxitoxin (NEO), and the gonyautoxins (GTX1, GTX2, GTX3, and GTX4). The decarbamoyl analogues (dcSTX, dcNEO, dcGTX1, dcGTX2, dcGTX3, and dcGTX4) and deoxydecarbamoyl analogues (doSTX, doGTX2, doGTX3) are of intermediate toxicity. The least toxic derivatives are the *N*-sulfocarbamoyl toxins B1 (GTX5), B2 (GTX6), C1, C2, C3, and C4 (Sullivan 1988, Oshima 1995). GTX1/GTX4, GTX2/GTX3, C1/C2, and C3/C4 are pairs in an epimeric relationship: GTX1, GTX2, C1, and C3 are the α-epimers, and GTX3, GTX4, C2, and C4 are the β-epimers. Essentially, these pairs are in equilibrium with each other, but different physicochemical conditions can shift the ratio of the α- and β-forms (Shimizu 1987). In some assays, the epimer pairs are combined because of inconsistent epimerization and are thus represented as a combined mol%.

The toxin profiles of the more common dinoflagellate species associated with PSP are different (Table 6). By species, the individual toxin components (mol%) are quite varied. In *P. bahamense* var. *compressum*, there is a lack of C1 to C4 and GTX1 to GTX4; in *A. minutum*, only GTX is present, with high levels of GTX1 and GTX4 in strains from Spain and Australia and only GTX2 and GTX3 in strains from France (Table 6); in *G. catenatum*, there are zero to trace levels of GTX1 to GTX4; in *A. tamarense*, there are trace to low levels of STX, B1, and B2 and high levels of NEO and GTX1 to GTX4; in *A. fundyense*, there are low levels of GTX1, GTX2, and GTX4 and high levels of GTX3; and in *A. catenella*, there are high levels of NEO, GTX4, B1, and B2 and low levels of GTX1, GTX2, and GTX3 (Table 6). Toxin profiles for *A. monilatum* are unknown (Schmidt and Loeblich 1979).

Toxin composition in dinoflagellate species and strains can vary with geographical range and can be influenced by environmental factors or experimental conditions (Cembella et al. 1988, Anderson et al. 1990, Anderson et al. 1994). *Alexandrium* strains

TABLE 6.

Toxin profiles of dinoflagellates associated with PSP.

Toxin (mol%)	Dinoflagellate species									
	A tamarense	*A. minutum*	*A minutum*	*A catenella*	*A. fundyense*	*A ostenfeldii*	*A. tamiyavanichi*	*A. lusitanicum**	*G. catenatum*	*P. bahamense var. compressum*
STX	0.0–3.2			trace–2 8	26.8		0 4–23.0		0 2	0 0–15.6
NEO	0.3–30 1			trace–22.8	13.2				0 1–3.8	10.5–68.0
GTX1	0.9–20.3	5.0–45 2	0.0	trace–3 9	0 6		1 1–3.8	26.0–41.0		
GTX2	0.1–23.0	<3 0–15.7	18.0	0.1	1.5	0.6	0.3–3 9	6.0	trace	
GTX3	0.3–86.0	<3 0–10.8	80.0	trace–0.9	50 1	0.1	2.2–10.2	12.0	trace	
GTX4	12.1–80 5	28.3–90 0	0.0	trace–26.2	5.1		36 8–72.8	41.0–53.0	0.8	
B1 (GTX5)	trace			trace–35.5			7.2–13.3		0.3–20 0	26.0–69.4
B2 (GTX6)				trace–57.3		91 6			0.1–36.0	4.0–8.0
C1	1 2–3.2			0.6–3.1	1/2 2.7	1/2 7.7	0.1–7.5		1.2–11.1	
C2	49.0–69 1			15.9–70.9	+	+	0.4–2.2		6.3–52.2	
C3				0.5–2.3			1 9–2.9		6.3–31.3	
C4	0 7–1.8			0.2–10.3			5.1–15.0		30.5–68.4	
dcSTX	0.7–3 0			0.1					0.1–4.0	0.0–4.5
dcGTX2				0.1					2/3 0.1–9.2	
dcGTX3	trace			0.1					+	
Location of isolate	Japan, Korea	Australia, Spain	France	Australia, Korea	USA	Denmark	Thailand Japan	Portugal	Australia, Japan, Spain	Malaysia
Reference	Lassus et al 1989. Lee et al 1992, Kim et al 1993	Hallegraeff et al 1991, Franco et al 1994	Erard-Le-Denn 1991	Hallegraeff et al. 1991, Kim et al 1993	Bricelj et al. 1990	Hansen et al. 1992	Wisessang et al. 1991, Oshima et al. 1990	Mascarenhas et al. 1995	Oshima et al. 1987, 1990, Oshima et al 1993	Oshima et al. 1987, Usup et al. 1995

* Considered to be a synonym of *A minutum* by Franco et al. 1995

can vary from highly toxic to nontoxic (Anderson 1990). The original isolate of *A. tamarense* from the River Tamar, Plymouth, England, and other strains from La Jolla, CA, were found to be nontoxic (Schmidt and Loeblich 1979). The toxicity of *A. tamarense* strains increases northwards along the northeast Atlantic Coast of North America (Maranda et al. 1985, Cembella et al. 1988) and northwards in Japan (Kim et al. 1993). This toxicity gradient in isolates from the more northerly latitudes is a reflection of the increased proportion of the highly potent carbamate toxins (STX, NEO, and GTX1 to GTX4) in *A. tamarense* (Anderson et al. 1982, Anderson et al. 1994). The proportion of the less toxic *N*-sulfocarbamoyl fractions such as C1, C2, B1, and B2 is higher in the more southern areas (Anderson 1990, Anderson et al. 1994). The presence of *A. tamarense* has been documented in southern New England and Long Island, but PSP outbreaks are rarer in these areas than they are in the more northerly regions of New England and Canada (Anderson et al. 1982). Bricelj et al. (1991) also pointed out that blooms of *A. tamarense* are typically less dense in the southern region of its geographical range, which may explain the relative lack of shellfish toxicity in the Long Island area. Analyses of the toxin composition and morphology of 28 strains of *A. tamarense* and *A. fundyense* indicate that although the two species are interspersed geographically from New Jersey to the St. Lawrence estuary and Newfoundland, Canada, only *A. fundyense* occurs in the Gulf of Maine (Anderson et al. 1994). The north-south trend in toxicity in these isolates was not as distinct as that described by Maranda et al. (1985), but this finding can be partially explained by the fact that high-toxicity isolates from northern areas were not tested (Anderson et al. 1994).

The toxin profiles that are discussed in outbreaks of PSP typically refer to those of the bloom-forming vegetative stages. However, the cysts of *Alexandrium* spp. are known to be more toxic than the vegetative cells. When newly formed, the cysts can be up to 1,000 times more toxic than the vegetative cells and are 10 times more toxic even after several months of dormancy (Dale and Yentsch 1978). Benthic bivalves such as *M. arenaria* could therefore be exposed to high levels of toxins at all times if sediments are filtered during feeding.

Toxicity in Bivalves

The distribution of paralytic shellfish toxins in bivalves varies among species and individuals. This variation occurs initially because of differences in dinoflagellate bloom duration, density, and inherent toxicity. The exposure of bivalves to paralytic shellfish toxins can result in increased mucus and pseudofeces production, modification of valve activity, change in filtration rate, impaired burrowing activity, and altered byssus production, cardiac activity, and oxygen consumption (Shumway and Cucci 1987, Gainey and Shumway 1988, Shumway 1990). In the presence of *A. tamarense*, *M. mercenaria* close the shell valves (Shumway 1990). This response may partly explain the absence or low level of PSP in this species (Table 7). Other species, like *M. arenaria*, retract the siphon (Shumway and Cucci 1987) or, like *C. gigas*, reduce pumping rates (Dupuy and Sparks 1967) when exposed to *A. tamarense* and *A. catenella*, respectively. PSP toxicity levels for *C. gigas* are lower than those of *Placopecten magellanicus* (Gmelin) and *Patinopecten yessoensis* (Jay) (Table 8), and levels for *M. arenaria* are lower than those of *M. edulis* (Tables 7 and 9), which may partly be the result of these behavioral adaptations. Further differences in uptake dynamics and detoxification mechanisms, in anatomical localization, and in physiological breakdown or transformation mechanisms determine the persistence of the toxins in the bivalve tissue (Shimuzu and Yoshioka 1981, Maruyama et al.

TABLE 7.

Selected examples of maximum toxicity levels reported in clams and the associated dinoflagellate species involved in the PSP outbreak.

Bivalve	Date and Location	Toxicity (μg of STXeq 100 g^{-1})	Dinoflagellate	Tissues	Reference
M. mercenaria	1972 Eastham, MA;	0	*A. fundyense*/*A. tamarense*	Whole body	Twarog and Yamaguchi 1975,
	1975 Monhegan Island, ME	1,113	*A. fundyense*/*A. tamarense*	Whole body	Shumway pers. comm.
M. arenaria	1972 York Harbor, ME;	1,726	*A. fundyense*/*A. tamarense*	Whole body	Twarog and Yamaguchi 1975,
	1972 Merrimack River Estuary, MA;	9,600	*A. fundyense*/*A. tamarense*	Whole body	Twarog and Yamaguchi 1975,
	1972 Essex, MA	3,500	*A. fundyense*/*A. tamarense*	Whole body	Twarog and Yamaguchi 1975
S. solidissima	1981 Phippsburg, ME;	7,934	*A. tamarense*	Viscera	Shumway et al. 1988,
	1990 Georges Bank, ME	6,423	?*A. tamarense*	Whole body	White et al. 1993
S. giganteus	1985 British Columbia, Canada	9,600	*A. catenella*	Whole body	Chiang 1988
S. nuttalli	1980 Campbell Cove, CA	14,000	*A. catenella*	Whole body	Price et al. 1991
Meretrix meretrix	1988 Indonesia	1,400	*P. bahamense* var. *compressum*	Whole body	Adnan 1993
Callista chione	1989 Mediterranean Coast, Spain	200	*G. catenatum*	Whole body	Bravo et al. 1990
Arctica islandica	1985 Jonesport, ME;	>1,895	*A. tamarense*	Whole body	Shumway et al. 1988,
	1990 Georges Bank, ME	1,218	?*A. tamarense*	Whole body	White et al. 1993

TABLE 8.

Selected examples of maximum toxicity levels reported in oysters, scallops, and cockles and the associated dinoflagellate species involved in the PSP outbreak.

Bivalve	Date and Location	Toxicity (μg of STXeq 100 g^{-1})	Dinoflagellate	Tissues	Reference
A. irradians	1972 Eastham, MA	2,040	*A. fundyense/A. tamarense*	Whole body	Twarog and Yamaguchi 1975
C. virginica	1972 Eastham, MA,	0	*A. fundyense/A. tamarense*	Whole body	Twarog and Yamaguchi 1975;
	1988 Gulf of St. Lawrence, Canada	214	*A. fundyense/A. tamarense*	Whole body	Worms et al. 1993
C. gigas	1972 British Columbia, Canada;	1,900	*A. catenella*	Whole body	Chiang 1988,
	1980 Marin County, CA;	5,500	*A. catenella*	?Whole body	Nishitani and Chew 1988,
	1986 Okeover Inlet, BC, Canada	9,929	?*A. catenella*	Whole body	Shumway pers. comm.
Crassostrea iridescens	1989 SE Mexico	811	*Pyrodinium bahamense* var. *compressum*	Whole body	Cortés-Altamirano et al. 1993
O. edulis	1986 Harpswell, ME;	1,300	*A. tamarense*	Whole body	Shumway et al. 1990,
	1988 Brittany, France	282	*A. minutum*	Whole body	Belin 1993
Cerastoderma sp.	1986 Obidos Lagoon, Portugal	1,096	*G. catenatum*	Whole body	Franca and Almeida 1989
P. yessoensis	?1984 Japan	220,000	?*A. tamarense*	Digestive gland	Noguchi et al. 1984
P. magellanicus	1978 Bay of Fundy, Canada;	150,000	*A. tamarense* (=*A. excavatum*)	Digestive gland	Jamieson and Chandler 1983,
	1990 Georges Bank, ME;	14,775	*A. tamarense*	Whole body	White et al. 1993,
	1992 Bay of Fundy, Canada	6,180	*A. fundyense*	Digestive gland	Waiwood et al. 1995

1983, Beitler and Liston 1990, Bricelj et al. 1990, Bricelj et al. 1991, Cembella et al. 1993, White et al. 1993, Cembella et al. 1994, Shumway et al. 1994, Cembella and Shumway 1995).

STX was first isolated from toxic butter clams, *S. giganteus* (Schantz et al. 1957, Schantz 1960), and it and at least 20 derivatives (Oshima 1995) in various combinations and concentrations have been associated with PSP. The total toxicity of shellfish meat is usually represented as the integrated potency of all toxins present in the sample and expressed in micrograms of STXeq (STX equivalents) per 100 g (Sullivan et al. 1985, Anderson et al. 1984). Shellfish-monitoring standards have an acceptable safety level of 80 μg STXeq 100 g^{-1} in raw shellfish soft tissues, and toxicities above this level are considered to pose an immediate public health risk (Clem 1975). A range of STX toxicity levels is found in different bivalves (Tables 7–9): *P. yessoensis*, *P. magellanicus*, and *Mytilus* spp. become highly toxic (Tables 8 and 9); *M. arenaria* have intermediate toxicity levels (Table 7); and *M. mercenaria* and *C. virginica* tend not to accumulate or have low levels of toxin (Tables 7 and 8). In general, toxicity levels in bivalves exposed to the various dinoflagellates can range from high to low: high when exposed to *A. tamarense* and *A. catenella*, medium when exposed to *A. fundyense* and *G. catenatum*, and low when exposed to *A. minutum* and *P. bahamense* var. *compressum* (Tables 7–9). When exposed to *A. catenella*, maximum toxicity levels (in micrograms of STXeq per 100 g) in bivalves varied from 9,929 in *C. gigas*, 14,000 in *Saxidomus nuttalli*, 30,360 in *M. trossulus*, and 127,000 in *Mytilus chilensis* (Tables 7–9).

Bivalve species have different toxin profiles, primarily because of the toxin profile and toxigenicity of the dinoflagellate species to which they are exposed (Tables 10 and 11) and secondarily because of their inherent and differential abilities to accumulate and to bioconvert, depurate, or otherwise modify the various PSP toxins. Bivalves exposed to *P. bahamense* var. *compressum* or *G. catenatum* accumulate very low levels of GTX, whereas some species that are exposed to *Alexandrium* spp. accumulate high GTX levels (Tables 10 and 11). Different bivalve species acquire totally different toxin profiles when exposed to the same dinoflagellate species (e.g., *A. tamarense;* Table 12). Additionally, individuals of the same bivalve species can have totally different toxin profiles, depending on the particular dinoflagellate species and strain to which they are exposed and the location and season of exposure. For example, *M. edulis* accumulate >20 mol% of the derivatives NEO, GTX1, GTX2, GTX4, and C1 when exposed to *A. tamarense;* >20% of the derivatives GTX1, GTX2, GTX3, and GTX4 when exposed to *A. minutum;* and >20 mol% of the derivatives STX and GTX2 when exposed to *A. fundyense* (Table 10).

TABLE 9.

Selected examples of maximum toxicity levels reported in mussels and the associated dinoflagellate species involved in the PSP outbreak.

Bivalve	Date and Location	Toxicity (μg of STXeq 100 g^{-1})	Dinoflagellate	Tissues	Reference
M. edulis	1972 York Harbor, ME;	10,092	*A. fundyense*/ *A. tamarense*	Whole body	Twarog and Yamaguchi 1975,
	1972 Merrimack River Estuary, ME;	7,392	*A. fundyense*/ *A. tamarense*	Whole body	Twarog and Yamaguchi 1975,
	1972 Essex, MA,	7,200	*A. fundyense*/ *A. tamarense*	Whole body	Twarog and Yamaguchi 1975,
	1980 Argentine Sea, Argentina;	50,000	*A. tamarense* (=*A. excavatum*)	?Whole body	Carreto et al. 1985,
	1981 SW Norway;	42,000	*A. tamarense* (=*A. excavatum*)	Whole body	Langeland et al. 1984,
	1986 Harpswell, ME;	2,100	*A. tamarense*	Whole body	Shumway et al. 1990
	1990 Georges Bank, ME;	24,417	?*A. tamarense*	Whole body	White et al. 1993
	1988 Brittany, France	401	*A. minutum*	Whole body	Belin 1993
Mytilus planulatus	1986 S Tasmania, Australia;	8,350	*G. catenatum*	Whole body	Hallegraeff et al. 1989,
	1986/1987 Adelaide, S Australia	2,700	*A. minutum*	Whole body	Hallegraeff et al. 1989
M. trossulus	1978 Puget Sound, WA;	30,360	*A. catenella*	?Whole body	Nishitani and Chew 1988,
	1982 British Columbia, Canada;	30,000	*A. catenella*	Whole body	Chiang 1988,
	1987 Kodiak, AK	>5,000	?*A. catenella*	?Whole body	Nishitani and Chew 1988
M. galloprovincialis	1976 Vigo, Spain;	6,000	*G. catenatum*	Whole body	Lüthy 1979,
	1984 Galicia, Spain	445	*A. minutum*	Whole body	Bianco et al. 1985
M. californianus	1980 Marin County, CA	16,000	*A. catenella*	Whole body	Price et al. 1991
Mytilus sp.	1986 NW Portugal	1,600	*G. catenatum*	Whole body	Sousa et al. 1995
Mytilus chilensis	1992 S Chile	127,200	*A. catenella*	?Whole body	Benavides et al. 1995
Chloromytilus palliopunctatus	1989 SW Mexico	542	*P. bahamense* var. *compressum*	Whole body	Cortés-Altamirano et al. 1993
Perna viridis	1988 Indonesia	1,054	*P. bahamense* var. *compressum*	?Whole body	Adnan 1993
Perna perna	1989 Venezuela	1,309	*G. catenatum*	?Whole body	La Barbera-Sanchez et al. 1993
Modiolus modiolus	1990 Georges Bank, ME	5,016	?*A. tamarense*	Whole body	White et al. 1993

Tissue Deposition of Toxins

Bivalve toxin profiles vary by geographic region (Tables 7–9), by season, and in the distribution of toxic components in different tissues (Beitler and Liston 1990, Cembella et al. 1993, Cembella et al. 1994, Shumway et al. 1994). Some of these differences are reflected in the ability of bivalves to convert toxins both from highly toxic carbamates (STX, NEO, GTX1, GTX2, GTX3, GTX4) to mildly toxic decarbamoyl analogues (dcSTX, dcGTX1, dcGTX2, dcGTX3, dcGTX4) and vice versa or in the ability to store less toxic *N*-sulfocarbamoyl toxins (Tables 10 and 11). The ability to convert carbamates to decarbamoyl derivatives has been demonstrated in *S. solidissima, Protothaca staminea* (Conrad), *Peronidia venulosa*, and *Mactra chinensis* (Sullivan et al. 1983, Bricelj and Cembella 1995, Oshima 1995, Bricelj et al. 1996). Bivalves may therefore have different toxin profiles from those of the dinoflagellate to which they were exposed, and their toxin profiles can vary as a function of time since exposure (Cembella et al. 1994). Depuration times vary between different species. Most species can naturally eliminate PSP toxins within weeks (Shumway 1990). Pacific oysters, *C. gigas*, are able to depurate toxins from their tissues in less than 9 wk (Shumway et al. 1990). However, *S. giganteus, P. magellanicus*, and *S. solidissima* are known to retain high levels of toxins for long periods of time (from months up to 3+ y) (Shumway and Cembella 1993, Shumway et al. 1994, Shumway pers. comm.). In *S. giganteus*, the siphons are the main sites of toxin accumulation (Beitler and Liston 1990), and toxins are stored as STX, NEO, GTX2, and GTX3 (Kitts et al. 1992). In *P. magellanicus* and *P. yessoensis*, the majority of the toxins is concentrated in the digestive gland, with toxicity levels in the gills and gonads typically less than 80 μg of STXeq 100 g^{-1} (Shumway and Cembella 1993). In *S. solidissima*, toxicity levels of more than 20,000 μg of STXeq 100 g^{-1} were recorded in the gills (Shumway et al. 1994). Tissue storage of toxins can vary by season and by concentration (Cembella et al. 1994). Differences in toxin accumulation in individual bivalves exposed to PSP ranged from 40 to 3,213 μg of STXeq 100 g^{-1} in September (White et al. 1993).

The bivalve accumulation of particular toxins and the deposition of these toxins in different tissues have been studied during laboratory-controlled exposures (Lassus et al. 1989, Bricelj and Cembella 1995). Bricelj and Cembella (1995) exposed *S. solidissima* to *A. minutum* even though *S. solidissima* would not typically be exposed to this dinoflagellate, which is rare in North America. The toxins of the *A. minutum* strain to which the bivalves were exposed were exclusively GTX1/GTX4 (96.9 mol%) and GTX2/GTX3 (3.1 mol%). After 40 days, the deposition of GTX1/GTX4 in the gills and viscera of the bivalves had declined to less than 5.0

TABLE 10.

Toxin concentrations (mol%) in dinoflagellate species and clams and mussels associated with PSP outbreaks. Where possible, concentration ranges have been provided to reflect the dynamics of toxin sequestration, conversion, and depuration.

Bivalve/ Dinoflagellate	Carbamates						*N*-sulfocarbamoyls			Decarbamoyls		Reference
	STX	NEO	GTX1	GTX2	GTX3	GTX4	B1/B2	C1/C2	C3/C4	dcSTX	dcGTX2/3	
M. edulis	1.3–9.7	1.4–50.2	2.0–45.7	8.8–36.1	2.1–7.0	2.6–26.3		13.5–42.9				Lee et al.
A. tamarense	0.3–0.5	1.3–2.2	17.7–20.3	7.0–7.5	1.9–2.1	12.5–13.5		43.6–44.1				1992
M. edulis	0.0–0.5	2.0–9.0	2.0–7.0	8.0–13.0	9.0–51.0	0.0–6.0		17.0–24.0				Lassus et
A. tamarense	0.0	0.3–1.1	1.1–2.1	7.0–23.0	70.0–86.0	2.4–4.0		0.8–1.4				al. 1989
M. edulis			38.8–42.4	21.9–30.7	<0.1–30.7	25.9–76.8						Oshima et
A. minutum			40.5	5.2	1.3	52.9						al. 1990
M. edulis	40.0	10.0	1/4 13.0	2/3 48.0				2.7				Bricelj et
A. fundyense	26.8	13.2	0.6	1.5	50.1	5.1						al. 1990
M. trossulus		+	+	+	+	+	+					Shimuzu
?*A. catenella*												et al. 1978
M. californianus	60.9	30.4	1–4 8.7									Whitefleet-
A. catenella												Smith et al. 1985
M. planulatus	0.1–0.3		0.5–3.0	0.3	0.2	3.6–5.2	1.5–2.7	8.9–18.5	55.9–79.4	5.3–16.2		Oshima et
G. catenatum	0.2			trace	trace	0.8	0.3–0.8	7.5–63.3	36.8–99.7	0.3–1.2		al. 1987
M. galloprovincialis	5.0	0.0	1/4 0.0	2/3 0.0			38.5–42.0	42.0	11.0			Anderson et
G. catenatum	6.0	2.0	1/4 2.0	2/3 0.0				36.0	17.0			al. 1989
M. mercenaria	25.4–30.2	12.2–12.6	1/4 9.3–9.6	2/3 46.0–49.6				1.8–3.9				Bricelj et al. 1991
A. fundyense	25.8	13.8	8.9	47.8				3.7				
M. arenaria	+	+	+	+	+	+		+				Martin et
A. fundyense												al. 1990
M. arenaria	23.3	16.2	19.2	17.4	15.6	5.4	0.3	2.7				Hurst et al.
A. tamarense												1985
R. phillipinarum	0.0–2.0	0.0–2.0	0.0–5.0	1.0–17.0	2.5–60.0	0.0–5.0		2.0–13.0				Lassus et
A. tamarense	0.0	0.3–1.1	1.1–2.1	7.0–23.0	70.0–86.0	2.4–4.0		0.8–1.4				et al 1989
P. viridis	0.2	1.8	30.6	13.4	4.7	49.3						Wisessang
A. tamiyavanichi	0.4	0.0	7.0	0.7	8.5	56.4						et al 1987
P. viridis	46.7	8.6					25.5			19.1		Oshima et
P. bahamense var. *compressum*	15.6	10.5					69.4			4.5		al. 1987
Spondylus butleri	+	+					+	+		+		Oshima et
P. bahamense var *compressum*												al. 1990
Spisula sp.	89.0	9.1	4.6	4.1	2.3		0.6	6.8				Hurst et al.
A. tamarense												1985
Meretrix casta	0.4	0.1	22.8	17.8	5.9	12.9		27.9	0.3		1.2–1.8	Karunasagar
?*A. tamiyavanichi*												et al 1990

+, present but no value given.

mol% toxin concentration, and GTX2 and GTX3 had been converted to dcGTX2 and dcGTX3. Exposures of *M. mercenaria* to *A. tamarense* and *A. fundyense* (Bricelj et al. 1991) indicated that *M. mercenaria* could accumulate toxins (Table 10), even though toxin accumulation may not occur in the field (Table 7). Cells of the high-toxicity *A. fundyense* isolate were only consumed if supplemented with a nontoxic diatom, *Thalassiosira weissflogii* (Bricelj et al. 1991).

Effects of PSP on Bivalves

In the short term, bivalves are not usually affected by paralytic shellfish toxins (Kao 1993) because their neuromuscular functions operate mainly by voltage-gated calcium channels. STX and its derivatives block only the voltage-gated sodium channels, which function in mammalian nerves and skeletal and cardiac muscle fibers (Kao 1993). High levels of STX are therefore typically not considered to be lethal or pathogenic to bivalves (Prakash et al. 1971). However, the effects of the chronic exposure of bivalves to STX and its derivatives are unknown. Paralysed *M. arenaria* were reported during the PSP outbreak in western Maine and Massachusetts in 1972, whereas toxic *M. arenaria* in eastern Maine and Canada showed no effects (Prakash et al. 1971). Paralysed *M. arenaria* are seen in Maine regularly (Shumway pers. comm.). Morbidity and mortality of shellfish were associated with a PSP outbreak in eastern England in 1968 (Adams et al. 1968, Ingham et al. 1968). Eighty percent of *C. gigas* that had been exposed to 10×10^6 *A. monilatum* cells l^{-1} died within 48 h (Sievers 1969).

The various combinations of the individual toxins described above determine the toxic potential of the shellfish to humans and the physiological damage expected to occur in the bivalves in which they accumulate. The total STX toxicity is the most important measure for public health concerns, yet the relative proportions of these toxic derivatives and their distribution in different tissues are not always considered. The long-term effects of these exposures on molluscan health needs critical evaluation.

TABLE 11.

Toxin concentrations (mol%) in dinoflagellate species in scallops and oysters associated with PSP outbreaks. Where possible, concentration ranges have been provided to reflect the dynamics of toxin sequestration, conversion, and depuration.

Bivalve/ Dinoflagellate	Carbamates						*N*-sulfocarbamoyls			Decarbamoyls		Reference
	STX	NEO	GTX1	GTX2	GTX3	GTX4	B1/B2	C1/C2	C3/C4	deSTX	dcGTX2/3	
P. magellanicus	20.0	1.0	3.0	58.0	11.0	<1.0						Fix Wichmann et al. 1981
A. tamarense (= *A. excavatum*)	0.0	11.0	9.0	9.0	41.0	30.0						
P. magellanicus	11.5	8.4	4.6	39.2	23.8		2.3	7.7	+			Hurst et al. 1985
A. tamarense												
P. yessoensis	2.7	34.2		5.4	0.6		0.4	76.8		0.3	2.3–4.8	Oshima et al. 1990
A. catenella												
P. maximus	0.0–1.5	0.0–2.0	0.0–5.0	1.5–29.0	4.0–40.0	0.5–3.5		3.0–21.0				Lassus et al. 1989
A. tamarense	0.0	0.3–1.1	1.1–2.1	7.0–23.0	70.0–86.0	2.4–4.0		0.8–1.4				
C. gigas	0.0–2.0	2.0–7.0	0.0–3.5	2.0–22.0	1.5–46.0	0.0–4.0		7.0–35.0				Lassus et al. 1989
A. tamarense	0.0	0.3–1.1	1.1–2.1	7.0–23.0	70.0–86.0	2.4–4.0		0.8–1.4				
C. gigas	+	+	+	+	+	+	+					Onoue et al. 1981
A. catenella												
C. gigas	0.2	0.0	0.7	0.2	0.1	4.0	3.1	10.0	79.8	2.1		Oshima et al. 1987
G. catenatum				trace	trace	0.8	0.3–0.8	7.5–63.3	36.8–99.7	0.3–1.2		
Crassostrea cucullata	0.7	0.0	13.5	52.5	10.1	4.7		14.2		0.0	4.2	Karunasagar et al. 1990
?*A. tamiyavanichi*												

DSP

DSP is associated with the consumption of shellfish that have been exposed to the dinoflagellates *Dinophysis* spp. (Fig. 2) and *Prorocentrum lima* (Ehrenberg) (Fig. 3). DSP outbreaks are most commonly reported in temperate areas in Europe, the Far East, South America, and Australasia (Fig. 2) (Lassus and Marcaillou-Le Baut 1991, Aune and Yndestad 1993). Recently, DSP was documented in eastern Canada (Quilliam et al. 1993). Bivalves currently implicated in DSP outbreaks are *M. edulis*, *Mytilus coruscum*, *M. galloprovincialis*, *P. yessoensis*, *Chlamys nipponensis*, *Tapes japonica*, *Gomphira melanaegis*, *M. mercenaria*, *Aulacomya ater*, and *M. arenaria* (Lassus and Marcaillou-Le Baut 1991, Lembeye et al. 1993).

Dinophysistoxins (DTXs) (DTX-1, DTX-2, and DTX-3) and okadaic acid (OA) are the major toxins currently known to be involved with DSP. DTXs have been found in *Dinophysis acuminata* (Claparède and Lachmann), *Dinophysis acuta* (Ehrenberg), *Dinophysis caudata* (Saville-Kent), *Dinophysis fortii* (Pavillard), *Dinophysis norvegica* (Claparède and Lachmann), *Dinophysis sacculus* (Stein) (Lee et al. 1989), and *P. lima* (Marr et al. 1992). OA, which is found in some benthic dinoflagellates in tropical regions (Steidinger 1993) and is suspected to have a role in ciguatera poisoning, has also been found in the planktonic *P. lima* (Jackson et al. 1993) and *Dinophysis* spp. (Lassus and Marcaillou-Le Baut 1991). OA and DTX-1 have been experimentally shown to induce skin tumors in mice (Fujiki et al. 1988, Suganuma et al. 1988).

The accumulation and metabolism of DTXs in bivalves have not been well investigated, and the effects on molluscan health are unknown. The exposure of mussels to high concentrations of *P. lima* resulted in reduced filtration rates and was attributed to toxicity associated with inhibitory or cytotoxic effects (Pillet and Houvenaghel 1995). *M. edulis* that were experimentally exposed to *P. lima* accumulated OA and DTX-1 in the hepatopancreas. No mortality was associated with exposure (Pillet et al. 1995). Clearance rates of juvenile and adult *Argopecten irradians* were not inhibited by exposure to toxigenic *P. lima*, and no mortalities were observed. Toxin saturation levels were attained within the first 2 days of exposure, but toxin retention efficiency was low (Bauder et al. 1996).

Dinophysis spp. and *P. lima* are widely distributed (Figs. 2 and 3), and the effects of the exposure of bivalves to low-level concentrations of these dinoflagellates should be investigated. The presence of OA in the planktonic *P. minimum* has not been confirmed (see VSP).

TABLE 12.

Comparative toxin profiles of selected bivalves after exposure to *A. tamarense*. Where possible, concentration ranges have been provided to reflect the dynamics of toxin sequestration, conversion, and depuration.

Bivalve	STX (mol%)	GTX1/GTX4 (mol%)	GTX2/GTX3 (mol%)
C. gigas	0.0–2.0	0.0–7.5	3.5–68.0
M. edulis	0.0–9.7	0.0–72.0	17.0–64.0
P. maximus	11.5	0.0–8.5	5.5–69.0
P. magellanicus	11.5–20.0	<1.0–7.6	0.0–81.8
Spisula sp.	89.0	4.6	6.4
R. phillipinarum	0.0–2.0	0.0–10.0	3.5–77.0
M. arenaria	23.3	24.6	33.0

VSP/Prorocentrum minimum

VSP has been associated with the consumption of shortnecked clams, *Venerupis semidecussata*, and Pacific oysters, *C. gigas*, and was coincidental with blooms of the dinoflagellate *Prorocentrum minimum* in Japan (Akiba and Hattori 1949). VSP is rare, and its true role in shellfish poisonings has been the subject of some discussion. Because of its association with VSP, the widespread distribution of *P. minimum* (Fig. 3) will be reviewed here. *P. minimum* is considered to consist of strains that are largely non toxic to humans (Taylor 1984), but toxins that could be pathogenic to bivalves have been isolated (Okaichi and Imatomi 1979). Other shellfish toxicity events associated with *P. minimum* have been documented in *M. edulis* in Norway (Tangen 1983), in *C. edule* and *Venerupis decussatus* (Silva 1985) in Portugal, and in *M. mercenaria* in northeastern North America (Freudenthal and Jijina

1988) in Chesapeake Bay, blooms of *P. minimum* appear to be fairly common (Sellner et al. 1993) (Tables 1b to 5b) and have recently been associated with shellfish mortalities (Luckenbach et al. 1993).

Recent studies have shown pathological effects, inhibition of feeding, and mortality in shellfish exposed to *P. minimum* (Bardouil et al. 1993, Luckenbach et al. 1993, Wikfors and Smolowitz 1993, Wikfors and Smolowitz 1995). *M. mercenaria* and *A. irradians* were fed *Prorocentrum micans*, *P. minimum*, and *Isochrysis* sp. in single-species and mixed-species tests (Wikfors and Smolowitz 1993). *M. mercenaria* survived well in all experiments, but in *A. irradians*, none of the diets supported good growth. A mixed diet of *Isochrysis* and *P. minimum* caused 100% mortality in 1–4 wk. *A. irradians* ingested *P. minimum*, but histopathological observations showed poorly developed digestive diverticula, attenuation of the epithelium with abnormal vacuolation and necrosis, and large thrombi in the heart and in the open vascular system of the mantle, digestive diverticula, gill, and kidney tissues (Wikfors and Smolowitz 1993). All juvenile oysters, *C. virginica*, exposed to 100% *P. minimum* bloom density died within 14 days, and 43% exposed to 33% bloom density died within 22 days, but oysters exposed to 5% bloom density had good shell growth and no mortality (Luckenbach et al. 1993). Wikfors and Smolowitz (1993) suggested that *P. minimum* produces an enterotoxin that gradually affects absorptive cells, an effect that was indicated by the development of thrombi throughout the vascular system. Spat of *C. virginica* exposed to *P. minimum* had an abnormal accumulation of lipids in the stomach epithelium (Wikfors and Smolowitz 1995).

A *Prorocentrum* species has recently been implicated in mass mortalities of flat oysters, *Ostrea rivularis*, in southern China (Yomgjia et al. 1995). The pathology was consistent with a systemic toxicosis resulting from the absorption of toxins by the digestive gland. Interestingly, the most intense lesion was formed by hemocytes that accumulated in and around the hemolymph channels, infiltrated the walls of the blood sinus, and formed intravascular thrombi. This pathology appears to be similar to that found in *C. virginica* by Wikfors and Smolowitz (1993). These studies suggest that *Prorocentrum* spp. may induce pathological effects in the hematopoietic system of oysters. If *P. minimum* produces toxins that are important in neoplasia development, could the chronic exposure of oysters to low-level concentrations of *P. minimum* induce neoplasia of the hematopoietic system?

A COMPARISON OF BIVALVE NEOPLASIA AND BIOTOXIN DISTRIBUTION

The epizootiology of disseminated neoplasia and germinomas in bivalves appears to closely parallel, both spatially and temporally, the distribution of blooms of dinoflagellate species associated with PSP or VSP (Tables 1–5; Figs. 1 and 3). The correlations noted here are conservative because they reflect only the coincidences of acute bloom formations and high concentrations of toxins in bivalves. They do not take into account the distribution of low levels of dinoflagellate concentrations and thus do not address the potential effect of chronic exposure of bivalves to toxins. These correlations need to be experimentally and statistically verified. A relationship between the distributions of neoplasia and DSP is not currently indicated (Tables 1–5; Fig. 2).

My working theory that certain dinoflagellate toxins induce neoplasia in bivalves is based on the currently available toxin profiles of bivalves and dinoflagellates. I recognize that there are gaps in the data and inconsistencies between studies in techniques used; dinoflagellate species, strains, and geographical isolates examined; and time elapsed between bivalve exposures to dinoflagellate blooms and subsequent analysis of their toxin profiles. However, patterns and trends in the relationship between biotoxins and neoplasia may still be recognized.

One of the earliest descriptions of disseminated neoplasia in bivalves in North America mentioned that an outbreak of PSP had been going on in the area at the same time (Farley 1976a) (Table 2). During the first red tide that led to a major PSP outbreak from southern Maine to Cape Ann, MA, in September 1972 (Hartwell 1975, Mulligan 1975), *M. edulis* and *M. arenaria* were the most prone to PSP (Tables 7 and 9) and they remained toxic until April 1973 (Hartwell 1975). *M. arenaria* was heavily affected by disseminated neoplasia and germinomas, but *M. edulis* was refractory (Tables 2a, 3a, and 5a), even in locations where *M. arenaria* and *M. edulis* had high toxin levels (Twarog and Yamaguchi 1975) (Tables 7, 9). *M. mercenaria* and *C. virginica* did not accumulate toxin (Tables 7 and 8), and they were also refractory to disseminated neoplasia and germinomas (Tables 1a, 2a, and 5a). PSP outbreaks coincided with several reports of disseminated neoplasia in *M. arenaria* in Maine during 1972–1975 (Table 2) (Farley 1976a). In August 1986, Morrison et al. (1993) found disseminated neoplasia in 3.1% of *M. arenaria* from Lepreau Harbor, New Brunswick, a month after PSP had been found there in the same species (Martin et al. 1990). Numerous parallel temporal and spatial occurrences of PSP and disseminated neoplasia are shown in Tables 1–4.

With the exception of the Gulf of Mexico, it appears that the distribution of disseminated neoplasia in bivalves is restricted to comparatively temperate regions in both the northern and the southern hemispheres. Disseminated neoplasia has not been reported in Asia, California, Africa, the Middle East, central and northern South America, or the tropics (Tables 1a to 4a; Fig. 1). Thus, for the most part, the distribution of disseminated neoplasia in bivalves more closely parallels the distribution of *Alexandrium* spp. associated with PSP (Tables 1–4; Fig. 1) than that of *P. bahamense* var. *compressum* or *G. catenatum*. PSP outbreaks in Asia are usually associated with *P. bahamense* var. *compressum*, and similar associations have recently been reported in Guatemala and Venezuela (Fig. 1). However, toxicity levels in shellfish associated with *P. bahamense* var. *compressum* are typically low (Tables 7–9) and are associated with the toxins STX and NEO and their less potent derivatives (Tables 6 and 10). Unlike some *Alexandrium* spp., this dinoflagellate lacks toxin derivatives such as GTX that might be potential inducers of neoplasia (Table 6). Currently, there are no documented cases of bivalve neoplasia in areas where *P. bahamense* var. *compressum* occurs (Fig. 1).

G. catenatum has trace levels of GTX (Tables 6, 10, and 11). Shellfish toxicity associated with exposure to *G. catenatum* typically tends to be low (Tables 7–10) and is usually associated with high levels of the nontoxic components B1, B2, and C1 to C4 (Tables 10 and 11). PSP outbreaks associated with *G. catenatum* have been reported to occur in Europe, particularly along the Atlantic Coasts of France, Spain, and Portugal, and in Tasmania, Argentina, and California (Fig. 1). In some cases, this distribution of *G. catenatum* parallels that of disseminated neoplasia, but the dinoflagellate has not been reported to occur in northeastern and northwestern North America or in Scandinavia, areas in which there is a high prevalence of disseminated neoplasia. The distri-

butions of neoplasia and *G. catenatum* therefore do not seem to be highly correlated (Fig. 1). In areas where *G. catenatum* and disseminated neoplasia do co-occur, I think that the correlation is more likely to be caused by the presence of *Alexandrium* spp., which co-occurs with *G. catenatum* in those areas (Fig. 1).

Analyses of the toxin compositions of PSP-causing dinoflagellate species (Tables 6, 10, and 11) show a possible connection between the presence of disseminated neoplasia and exposure to the highly toxic GTX. It is postulated here that the combination of specific toxins will, in some cases, initiate neoplastic development in bivalves. Dinoflagellate species with distributions that parallel that of disseminated neoplasia on a worldwide basis and that have toxin profiles with high levels of GTX are *A. tamarense*, *A. minutum*, *A. catenella*, and *A. fundyense* (Table 6).

If there is a relationship between disseminated neoplasia in bivalves and their toxin profiles and concentrations, then high STX or NEO levels do not appear to be as important as other combinations of STX derivatives. It generally appears that when >20 mol% of the gonyautoxins GTX1/GTX4 are present, then disseminated neoplasia is also present (Tables 10–13). When >20 mol% STX or NEO is present, then disseminated neoplasia is generally absent (Table 13). In *M. arenaria*, after exposure to *A. tamarense*, >20.0 mol% of STX, GTX1/GTX4, and GTX2/GTX3 are present (Table 12). However, after exposure to *A. fundyense*, *M. arenaria* had low levels of STX and high levels of GTX1, GTX3, and GTX4 (Martin et al. 1990). In this case, the common toxin derivative associated with the presence of disseminated neoplasia in *M. arenaria* appears to be GTX and not STX. Bivalves such as *M. trossulus* and *M. arenaria* that store highly potent GTXs are affected by disseminated neoplasia, whereas those species that store STX, such as *S. giganteus*, *S. solidissima*, *P. magellanicus*, *P. yessoensis*, *Spondylus butleri*, and *M. californianus* are unaffected by disseminated neoplasia (Table 13).

Recent appearances of *Alexandrium* spp. with high levels of GTX such as *A. tamarense* and *A. tamiyavanichi* (Balech 1995) (identified as *A. cohorticula*) in Thailand, Korea, and Japan in the 1980s (Ogata et al. 1990, Pholpunthin et al. 1990, Han et al. 1992) and *A. minutum* in Australasia (Hallegraeff et al. 1991) may foreshadow the appearance of disseminated neoplasia in predisposed bivalves in these areas. There is a noticeable absence of dissem-

TABLE 13.

Geographic distribution of neoplasia in various bivalves associated with PSP (high-risk *Alexandrium* spp.), DSP, and VSP and distribution of toxins (at least > 20 mol%).

Bivalve	Germinomas	Distribution	Disseminated Neoplasia	Distribution	PSP					DSP		VSP
					C1/C2	NEO	STX	GTX1/4	GTX2/3	DTX1	OA	
M. edulis	+	NE North America	+ +	Europe	+	+	–*	+	+	+	+	+
M. trossulus	0	NW North America	+ + +	NW North America	?	–	–	+	+	ND	ND	ND
M. galloprovincialis	0		+	Europe	+	–	–	?+	–	+	+	ND
M. californianus	0		0		–	+	+	–	–	ND	ND	ND
M. arenaria	+ + +	NE North America	+ + +	NE North America	–	–	+	+	+	?+	?+	ND
M. truncata	0		+	N Canada	ND	ND	ND	ND	ND	ND	ND	ND
C. gigas	0		0		+	–	–	–	+	ND	+	+
C. virginica	+	E North America	+	E North America, Gulf of Mexico	–	–	–	–	–	ND	ND	?+
T. chilensis	?+	New Zealand	+	SW South America, New Zealand	ND	ND	ND	ND	ND	ND	ND	ND
O. edulis	0		+ +	Europe	ND	ND	ND	ND	ND	ND	ND	ND
O. conchaphila	0		+ +	NW North America	ND	ND	ND	ND	ND	ND	ND	
P. magellanicus	0		0		–	–	+	–	+	ND	+	ND
P. yessoensis	0		0		+	+	–	–	–	+	+	ND
A. irradians	+	NE North America	0		ND	ND	+	ND	ND	ND	ND	ND
Mercenaria mercenaria	+ + +	E/SE North America, Gulf of Mexico	0		–	–	–	–	–	?+	?+	+
C. edule	?+	Western Europe	+ + +	Western Europe	ND	ND	ND	ND	ND	ND	+	+
S. solidissima	0		0		–	–	+	–	–	ND	ND	ND
S. butleri	0		0		–	+	+	–	–	ND	ND	ND
A. islandica	+	NE North America	0		ND	ND	ND	ND	ND	ND	ND	ND
M. balthica	0		+ +	Scandinavia	ND	ND	ND	ND	ND	ND	ND	ND
M. casta	0		?		+	–	–	+	+	ND	ND	ND
S. giganteus	0		0		–	–	+	–	–	ND	ND	ND

+ + +, high risk; + +, medium risk; +, low risk; 0, no risk.

* STX values for exposures to *A. fundyense* are >20.0 mol% (Table 10) (ND, no data).

inated neoplasia in bivalves in California, which correlates with and may have been influenced by the absence of *A. tamarense* in this area, by the presence of low-toxicity *G. catenatum*, or by the fact that California mussels, *M. californianus*, retain high STX levels when exposed to *A. catenella* (Fig. 1; Table 10).

From a public health standpoint, the toxicity of individual, nonconsumable bivalve organs is not usually considered because it is the total toxicity value that is important for safety standards. Toxicities that are reported as micrograms of STXeq per 100 g of shellfish meat (Prakash et al. 1971) are a composite of the total toxicity of the shellfish tissues that are typically consumed by humans. Even though the toxicity of individual organs can be much higher than the overall toxicity of the shellfish meat (Martin et al. 1990), these individual values are only relevant from a human health perspective when particular organs, such as adductor muscles from scallops, are consumed (Shumway and Cembella 1993). From a molluscan health perspective, however, the distribution of toxins and derivatives in individual organs may be critical. If neoplastic induction requires a particular period of chronic exposure to one or more toxins, then the deposition of the various toxin derivatives, their concentrations, and their persistence in different organs may play a significant role. At present, both the sites for hematopoiesis and the cellular origin of disseminated neoplasia in bivalves are unknown (Elston et al. 1992). Likely organ sites could include those with open blood sinuses such as the gills, heart, kidney, and brown gland, whereas those such as the adductor muscle and mantle might be less likely.

If there is a correlation between the tissue deposition of the highly toxic carbamate gonyautoxins and the prevalence of neoplasia, then it may be apparent in current bivalve data (Tables 10 and 11). In New England, *M. arenaria* is affected by both disseminated neoplasia and germinomas (Tables 2a and 5a). Martin et al. (1990) showed that the toxicity of whole *M. arenaria* extracts had a typical seasonal pattern, with a maximum of 2,103 μg of STXeq 100 g^{-1} present in July 1986 in Lepreau Harbor, New Brunswick. Toxicities for some individual tissues were far higher than the total maximum toxicity levels reported (Martin et al. 1990). Levels of approximately 10,000 μg of STXeq 100 g^{-1} were present in the digestive gland; 6,500 μg of STXeq 100 g^{-1} in the heart, kidney, and brown gland; 500 μg of STXeq 100 g^{-1} in the gills; 300 μg STXeq 100 g^{-1} in the gonad; and 120 μg of STXeq 100 g^{-1} in the muscle. Could the deposition of PSP toxins, and particularly the gonyautoxins, in tissues such as the gills, kidney, heart, or brown gland trigger the development of disseminated neoplasia? Could the deposition of these same toxins in the gonad trigger germinoma development? After *M. arenaria* were exposed to *A. fundyense* blooms, PSP toxins were transferred rapidly from the digestive gland to the kidney, where they were retained for extensive periods of time (Martin et al. 1990). Morrison et al. (1993) found disseminated neoplasia in *M. arenaria* from the same area (Lepreau Harbor) as those *M. arenaria* studied 1 month previously by Martin et al. (1990). Presumably *M. arenaria* had retained high levels of GTX in susceptible tissues during that period. The presence of disseminated neoplasia appears to be more than coincidental, and verification of such a cause-and-effect scenario is critical.

In contrast, *P. yessoensis* are known to be highly contaminated by toxins during PSP outbreaks (Table 8) but are refractory to disseminated neoplasia. Toxin-profile studies of the scallop *Pecten maximus* show that the accumulation of STX, NEO, and GTX occurs mostly in the digestive gland. The accumulation and subsequent transformation of these toxins in the gonad, kidney, and adductor muscle of the scallop *P. maximus* (L.) lead to an almost complete absence of GTX1 and GTX4 in these tissues 15 days after experimental exposure to *A. tamarense*. However, the digestive glands still contained GTX1 to GTX4 and NEO after 35 days (Lassus et al. 1992). Cembella et al. (1994) reported seasonal variation in toxicity profiles of *P. magellanicus* tissues. In the digestive glands, GTX2 and C1/C2 were the main components; in the gill, NEO; in the mantle, GTX2 and GTX3; and in the gonads, C1/C2, GTX2, GTX3, and NEO. Levels of GTX1 and GTX4 were negligible. The low level or complete absence of GTX1 and GTX4 might again explain the absence of disseminated neoplasia and germinomas in scallops (Cembella et al. 1994). The ability to transform toxic PSP carbamates to their corresponding nontoxic decarbamoyl derivatives, as demonstrated by *S. solidissima*, *P. staminea*, *P. venulosa*, and *M. chinensis* (Bricelj and Cembella 1995, Oshima 1995, Bricelj et al. 1996), may also correlate with a lack of neoplasia. Again, species with high STX concentrations and low levels of GTX appear to be unaffected by disseminated neoplasia—or at least less affected by disseminated neoplasia than are those species that retain high levels of GTX.

Although *M. edulis* is heavily affected by PSP in northeastern North America, the incidence of disseminated neoplasia has not been recorded in this species in this region. However, *M. edulis* from northern Europe (England and Scandinavia) are affected by disseminated neoplasia (Table 3a). Several factors could help to explain these geographical differences. In northern Europe, *M. edulis* are more than likely to be exposed to *A. tamarense* or *A. minutum* and, in general, have high GTX levels (>60 mol% GTX1/GTX4) (Table 10). In northeastern North America, *M. edulis* are typically exposed to *A. tamarense* or *A. fundyense*. In Maine, where a high prevalence of disseminated neoplasia and germinomas is documented in *M. arenaria* (Tables 2a and 5a), *M. edulis* are more than likely to be exposed to *A. fundyense* (Anderson et al. 1994). When exposed to *A. fundyense*, more than 40 mol% of STX but only about 13 mol% of GTX1/GTX4 is retained in *M. edulis* (Table 10). In this situation, the high levels of STX and the low levels of GTX may explain the absence of disseminated neoplasia in *M. edulis* in this region. I postulate that high levels of GTX1/GTX4 are required to trigger neoplastic development. If *M. edulis* are usually exposed to *A. fundyense* in Maine and this results in the deposition of low levels of GTX1/GTX4, then the absence of disseminated neoplasia in *M. edulis* in New England can be explained.

The worldwide distribution of germinomas is more localized than that of disseminated neoplasia (Figs. 1–3). If *Alexandrium* spp. are involved in tumor induction, then it might be expected that there would be a parallel distribution of germinomas and disseminated neoplasia in bivalves. In some cases, this situation holds true, as for example, in *M. balthica* in northern Canada, *M. arenaria* in New England, and *C. edule* in Cork, Ireland. However, in most cases, this situation does not occur (Table 13). The absence of germinomas in bivalves from most of Europe and their rare occurrences along the Pacific Coast of North America suggest that in these areas, toxins from the dinoflagellates *A. tamarense*, *A. fundyense*, *A. minutum*, *A. catenella*, and *G. catenatum* are not necessarily involved in germinoma induction. Alternatively, if these dinoflagellates are involved, then the toxin components required for germinoma induction are probably different than those required for the induction of disseminated neoplasia. The high prevalence of germinomas in *M. mercenaria* and the fact that their

exposure to toxic *Alexandrium* spp. under natural conditions may not always result in toxin accumulation (Table 7) suggest an alternative hypothesis for germinoma induction in this species.

There may be a possible correlation between the distribution of neoplasia in bivalves in the Gulf of Mexico and southeastern North America and the distribution of toxigenic *A. monilatum* or *P. minimum*. These dinoflagellates have been documented to occur throughout the Gulf of Mexico, the Caribbean, and southeastern and mid-Atlantic North America as far north as the Chesapeake Bay (Steidinger 1993). A potential relationship between the distribution of *P. minimum* and *A. monilatum* and neoplasia could also be postulated for the bivalves *C. virginica*, *T. chilensis*, *M. mercenaria*, and *Mercenaria campechiensis* (Figs. 1 and 3; Tables 1, 5, and 13). The distribution of disseminated neoplasia in oysters appears to be more related to the presence of *P. minimum* or *A. monilatum* than to other toxic dinoflagellates in the genus *Alexandrium* (Table 1). Most accounts of oyster exposure to *Alexandrium* spp. report low or no toxicity (Table 8), whereas toxins from *Prorocentrum* spp. cause pathological effects and have been associated with oyster mortalities (Wikfors and Smolowitz 1993, Wikfors and Smolowitz 1995, Yomgjia et al. 1995). Currently, there is no information on the uptake of toxins from *P. minimum* or *A. monilatum* by, or their toxicity to, *M. mercenaria*, although *P. minimum* cells were found in *M. mercenaria* in Nassau County, NY in 1985 after an outbreak of human shellfish poisoning (Freudenthal and Jijina 1985).

Although there are some incidences of neoplasia that parallel the distribution of DSP (Fig. 2), there are few records of DSP from the East and West Coasts of North America or the Gulf of Mexico, where neoplasia is prevalent. Dinoflagellate species with associated OA and DTX-1 would appear to be likely candidates for causing tumors in bivalves, yet the existing epizootiology of bivalve neoplasia does not appear to parallel the known distribution of these dinoflagellates (Fig. 2). However, the focus of this article has been to review the distribution of toxicity outbreaks typically associated with high-density planktonic blooms and acute exposure to bivalves. Therefore, if the long-term, low-level exposure of bivalves to OA or DTX is occurring through the continual consumption of *Dinophysis* spp. and *P. lima*, then field data comparing high-density bloom distributions and neoplasia incidence may not be pertinent.

The influence of anthropogenic chemical carcinogens on the induction of neoplasia in invertebrates has been well investigated (Mix 1986a). Many bivalves have been exposed to highly contaminated sediments containing chemical carcinogens known or suspected to affect aquatic organisms (Gardner and Yevich 1988). In most cases, a direct cause-and-effect relationship between bivalve exposure to carcinogens and the induction of disseminated neoplasia or germinomas could not be clearly demonstrated (see Etiology). However, in a few examples, benign tumors developed (Gardner et al. 1991a). One could speculate that if there is a connection between bivalve exposure to particular dinoflagellate toxins and neoplasia, then the neoplasia found in chemical carcinogen exposure studies could have been caused by exposure to sedimentary biotoxins. The majority of sediment exposure studies were carried out using sediments from high-risk PSP areas in New England such as Narragansett Bay, RI; Long Island Sound, Blackport, CT; Searsport, Freeport, and Dennysville, ME; and New Bedford Harbor, MA (Yevich and Barscsz 1976, Yevich and Barscsz 1977, Gardner and Yevich 1988) (Table 2b)—all areas where *Alexandrium* spp. cysts are known to be widespread in the sediments (Anderson et al. 1982, Maranda et al. 1985). It has been documented that the total toxin concentration in cysts of *A. tamarense* is six-fold higher than that in the natural population of vegetative cells (Oshima et al. 1992). Further, these cyst toxins comprised approximately 80 mol% of GTX compared with approximately 69 mol% of GTX in vegetative cells (Oshima et al. 1992). Theoretically, if these cysts were present in sediments from high-risk PSP areas during the exposure of bivalves to chemical contaminants, then bivalves could also have ingested toxic cysts along with other contaminated sediment particles.

There is little or no information about the potential role of natural biotoxins in the induction of tumors in aquatic organisms. OA and DTX-1 produced by *Dinophysis* spp. and *P. lima* can, in addition to causing DSP, promote tumors in mammals. The fact that bivalves accumulate toxins associated with *Dinophysis* and *Prorocentrum* is unequivocal, but the role of OA and DTX in inducing tumors in aquatic animals is currently unknown. It remains to be seen as to whether they can trigger neoplasia development in bivalves. Long-term studies to investigate the relationship between toxin exposures and neoplasia should be initiated.

A multifactorial etiology of neoplasia development in bivalves could be hypothesized, but before such a step can be made, the role of biotoxins in tumor induction should be defined and clearly demonstrated. In at least one species of bivalve (*M. arenaria*) known to be affected by disseminated neoplasia, the presence of a retrovirus has been demonstrated (Oprandy et al. 1981). Retroviruses may be endogenous in certain bivalve species and strains such as *M. arenaria* and *Mytilus* spp. The proliferation of these viruses could be triggered by exposure to natural carbamate toxins. Carbamate toxins could act directly as mutagens. Different bivalves may also be predisposed to viral or cellular oncogenes. Genetic differences in species predisposition to neoplasia may be significant (Van Beneden et al. 1993). Genetic susceptibility to germinomas was determined for *M. mercenaria*, *M. campechiensis*, and their hybrids. Hybrids were more affected by germinomas, which could be explained by decreased genetic fitness (Bert et al. 1993).

Although this concept is highly speculative at present, the geographic association of shellfish toxicity events, dinoflagellates, and neoplasia certainly represents strongly circumstantial evidence. If gonyautoxins induce disseminated neoplasia, then information on the chronic deposition of these toxins in different bivalve species and tissues may be indicative of the differences in species' predisposition to neoplasia. Table 13 shows the geographical distribution of disseminated neoplasia and germinomas, the species affected, and the typically high toxin concentrations (>20 mol%) that some bivalves accumulate. These data can be used to generate a theoretical risk assessment for the geographic distribution of bivalves with disseminated neoplasia (Table 14) and germinomas (Table 15).

Hypotheses

1. Disseminated neoplasia and germinomas can be induced in bivalves by toxins produced by dinoflagellates; a bivalve's predisposition to neoplasms is dependent on genetic, behavioral, physiological, environmental, and geographic factors that may operate in sequence.
2. Certain species, such as the softshell clam, *M. arenaria*, and the cockle, *C. edule*, are affected by both disseminated neoplasia and germinomas, but only in specific geographic locations and at certain times of the year. Other species,

TABLE 14.

Predisposition and theoretical risk of bivalve species to disseminated neoplasia by geographic region and by exposure to dinoflagellate species.

Bivalve	North America	Central/ South America	Europe	Africa	Asia/ Far East	Australia/ New Zealand	PSP: *A. tamarense*	PSP: *A. catenella*	PSP: *A. fundyense*	PSP: *A. minutum*	PSP: *G. catenatum*	PSP: *P. bahamense* var. *compressum*	VSP *P. minimum*
M. edulis	0		+ +		0		+ +	0	0	? +	0	0	? + +
M. planulatus						? + +	? +			+ +			
M. trossulus	+ + +						+ + +	+ + +			0		?
M. galloprovincialis	0		+				? +	0		+	0		? + +
M. californianus	0							0			0		
M. arenaria	+ + +						+ + +		+ + +				? + +
M. truncata	+						+						
A. ater		0						0					
C. gigas	0		0		0		0	0		0	0	0	0
C. virginica	+						0		0				? + +
T. chilensis		+				+	+ +	? + +		+ + +	0		? + +
O. edulis	0		+ +				0		0	+ +	0		? + +
O. conchaphila	+						+	? +					
P. magellanicus	0						0		0				
P. yessoensis					0		0	0		?0		0	
A. irradians	?0						0			0			
M. mercenaria	0						0			0	0		
C. edule			+ + +				? + + +			+ + +	0		? + +
S. solidissima	0						0		0	0			
S. butleri	0												
A. islandica	+						? +						
M. balthica	0		+				? +						? + +
M. casta					?0							0	
P. viridis					0							0	
S. giganteus	0						0	0					

+ + +, high risk; + +, medium risk; +, low risk, 0, no risk

such as the blue mussels, *M. edulis* and *M. trossulus*, and the eastern oyster, *C. virginica*, are rarely affected by both types of neoplasia. Some species, such as *M. mercenaria*, are apparently affected only by germinomas, and others, such as *O. edulis*, are affected only by disseminated neoplasia. The butter clam, *S. giganteus;* the Japanese scallop, *P. yessoensis;* the sea scallop, *P. magellanicus;* the surfclam, *S. solidissima;* and the California mussel, *M. californianus*, are apparently unaffected by either disseminated neoplasia or germinomas.

3. The absence of disseminated neoplasia and germinomas in bivalves from particular geographic regions is likely to be correlated with the absence of dinoflagellate species with high-risk toxins, such as GTXs, or with the resistance to neoplasia of particular bivalve species.
4. Disseminated neoplasia is prevalent in most geographic regions where PSP and *Alexandrium* spp. occur, but only in certain species of bivalves. More specifically, only certain species of *Alexandrium*, such as *A. tamarense*, *A. minutum*, and *A. fundyense*, are potential etiological agents of tumor induction.
5. Even though dinoflagellates such as *P. bahamense* var. *compressum* and *G. catenatum* cause PSP, they are unlikely to induce disseminated neoplasia or germinomas.
6. The decreasing toxicity of *A. tamarense* along a north-to-south gradient in the coastal United States could explain the higher prevalence of disseminated neoplasia in bivalves in more northerly regions.
7. *M. mercenaria* is apparently unaffected by disseminated neoplasia and does not usually accumulate toxins associated with *A. tamarense* or *A. fundyense*. *M. mercenaria* is, however, affected by germinomas. Toxins from known PSP-producing dinoflagellates do not appear to play a role in the development of germinomas in this species. In *M. arenaria*, the incidence of germinomas appears to be related to the distribution of *Alexandrium* spp. blooms.
8. *A. monilatum* and *P. minimum* may be potential etiological agents of neoplasms in some bivalves because of their parallel geographic distributions and their implicated toxicity. The toxin profiles of these dinoflagellate species are unknown.
9. There is a seasonal variation in the prevalence and intensity of neoplasia that parallels the seasonal variation of dinoflagellate blooms and the changing toxin profiles of high-risk toxins in particular tissues. In general, there appears to be a time lag of a few weeks after exposure to dinoflagellate toxins before disseminated neoplasia appears in bivalves.
10. In the development of disseminated neoplasia, bivalves could be sufficiently stressed by toxin exposure to render them susceptible to virus infection. Certain species may have cellular and viral oncogenes that are triggered by toxin exposure.
11. Certain bivalves that were historically affected by neoplasia in particular geographic areas may no longer be affected because the bloom-forming toxic dinoflagellate species are no longer dominant there.

TABLE 15.

Predisposition and theoretical risk of bivalve species to germinomas by geographic region and by exposure to dinoflagellate species.

							PSP							
Bivalve	**North America**	**Central/ South America**	**Europe**	**Africa**	**Asia/ Far East**	**Australia/ New Zealand**	***A. tamarense***	***A. catenella***	***A. fundyense***	***A. minutum***	***G. catenatum***	***A. monilatum***	***P. bahamense* var. *compressum***	**VSP *P. minimum***
M. edulis	+		0		0		?+			?0	0		0	?+
M. planulatus						0	0			?0				
M. trossulus	?+						?+	?0						
M. galloprovincialis	0		+					0		+	0			
M. californianus	0							0			0			
M. arenaria	+ +						+ +		?+					?+ +
M. truncata	+						+							
C. gigas	0		0		0			0					0	
C. virginica	+						0		0			?+		?+
T. chilensis		?0				+ +	0	0		+ +	0			
O. edulis	0		0							0				
O. conchaphila	0						0	0						
P. magellanicus	0						0		0					
P. yessoensis					0		0			0	0		0	
A. irradians	+						?+		?+					
M. mercenaria	+ + +						0		0			?+ +		?+ +
C. edule			+ +				?+ +			?+ +				?+ +
S. solidissima	0						0		0					
A. islandica	+						?+							
M. balthica	0		+ +				?+ +							
S. giganteus	0							0			0			

+ + + = high risk, + + = medium risk, + = low risk, 0 = no risk.

12. There is a strong likelihood that toxic microalgae play a role in both chronic disease and mortality in aquatic organisms.

Future Research

No published surveys have been specifically targeted at evaluating the prevalence of neoplasia in relation to known biotoxin distributions. Evidence presented here suggests that such a study should be conducted. Field data can be obtained through the routine monitoring of both affected and unaffected bivalves from sites where neoplasia is known to be prevalent. In addition to sampling bivalves, water quality and the presence of dinoflagellate species in water and sediment samples should be monitored. There should be comparative studies on bivalve behavior and feeding strategies during dinoflagellate exposure. Toxin profiles and the tissue deposition of toxic derivatives in bivalves should be confirmed for a range of species. Toxin profiles of suspect dinoflagellate species such as *A. monilatum*, *P. minimum*, and *L. polyedrum* should also be established. This information should be collected on both short- and long-term bases and in parallel with routine diagnostic procedures for monitoring molluscan health, disease, pathologies, and the distribution of neoplasia.

In addition to potential effects on bivalves, consideration should be given to the effects that chronic exposure to dinoflagellate biotoxins may have on other organisms. This consideration should include the evaluation of toxin exposure through dietary transfer and the distribution and epizootiology of neoplasia in organisms that consume molluscs. The long-term effects of biotoxin exposure at all levels of the food chain should be investigated. Criteria for public health exposure may also require reappraisal.

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SURVEY OF PARALYTIC SHELLFISH POISON AND DOMOIC ACID IN PUGET SOUND PREDATORY GASTROPODS

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ABSTRACT Two predatory gastropods, moonsnails (*Polinices lewissi*) and frilled dogwinkles (*Nucella lamellosa*), were collected in the Puget Sound basin in the summer and fall of 1994 and in the spring of 1995. Analyses indicated the presence of paralytic shellfish poison (PSP) toxins in these gastropods in all sampling periods; however, no domoic acid was detected in any of the samples. Other species of molluscan shellfish and a species of crustacea, considered possible prey and/or indicators of PSP in the area, were also collected. In September 1994, levels of PSP in moonsnails collected from Agate Passage averaged 145 μg of saxitoxin (STX) equivalents (equiv.)/100 g, butter clams taken from the same area at the same time averaged 73 μg of STX equiv./100 g. In October 1994, when a local monitoring station indicated the presence of PSP in Mystery Bay, a collection and PSP analyses of molluscan shellfish yielded the following average values: dogwinkle (*N. lamellosa*) averaged 72 μg of STX equiv./100 g; blue mussels (*Mytilus edulis*), 652 μg of STX equiv./100 g; Pacific oyster (*Crassostrea gigas*), 45 μg of STX equiv./100 g, northern horse mussel (*Modiolus modiolus*), 48 μg of STX equiv./100 g; and snails (*Searlesia dira*), 50 μg of STX equiv./100 g. In April 1995, another collection and analyses of shellfish from Agate Passage showed that moonsnails averaged 48 μg of STX equiv./100 g, and butter clams (*Saxidomus giganteus*) averaged 35 μg of STX equiv./100 g (range, 0–77 μg of STX equiv./100 g); Pacific littleneck clams (*Protothaca staminea*) and red rock crab (*Cancer productus*) did not contain PSP toxin. Modifications of the sample preparation methods required for the analyses of samples are described.

KEY WORDS: Paralytic shellfish poison, gastropods, domoic acid

INTRODUCTION

In the Puget Sound area of Washington, the consumption of marine invertebrates has increased, in part, because of an increase in ethnically more diverse population and a desire on the part of consumers to try an increased variety of seafoods (Carney and Kvitek 1991). Recent site surveys and beach interviews of shellfish diggers show that predatory marine snails are collected from Puget Sound beaches (Carney and Kvitek 1991). It was found that the moonsnail (*Polinices lewissi*) and other gastropods, such as the dogwinkle (*Nucella lamellosa*), were being noncommercially harvested. In particular, the moonsnail probably because of its size and accessibility to shore gathering, is a popular harvested species. In their 1990 survey, it was estimated that the annual recreational harvests of the moonsnail and dogwinkle, *Nucella* sp., were 21,000 and 119,000 individuals, respectively (Carney and Kvitek 1991).

In the Puget Sound, the Washington Department of Health monitors shellfish from beaches and caged mussel stations for the presence of paralytic shellfish poison (PSP). Beaches are closed to harvesting when PSP toxin levels equal or exceed 80 μg of saxitoxin (STX) equivalent per 100 g (80 μg of STX equiv./100 g) of shellfish tissue. In humans and higher vertebrates, the PSP toxins are potent neurotoxins.

Since predatory marine snails prey on clams that are known to acquire the marine biotoxins associated with PSP, it is not unreasonable to expect that they too could accumulate these toxins. However, little is known about the human risk of PSP intoxication due to the consumption of these animals. Two recent reviews (Matter 1994, Shumway 1995) discussed PSP and its distribution in the marine food web, with emphasis on predatory gastropods. White et al. (1993a and 1993b) reported toxicity at levels near 3,000 μg of STX equiv./100 g tissue in northern moonsnails (*Euspira heros*) and waved whelk (*Buccinum undatum* L.) taken from the Georges Bank. The northern moonsnails were slow to depurate the toxin, and there was a high individual variability of toxicity (White et al. 1993b). Worms (1993) also detected PSP toxins in the northern moonsnails and suggested that their slow depuration rate may be attributed to their slow metabolic rate. He also suggested that predatory snails may be able to maintain detectable toxin levels throughout the year by feeding on bivalve species with a toxin level that falls under the detection limit. The rough whelk (*B. undatum*) was implicated in an outbreak of PSP poisoning in Quebec (Prakash et al. 1971, Medcof 1972). Although 12 people developed symptoms, there were no deaths. Studies following the outbreak showed that the whelk accumulated toxin in its digestive gland after feeding on contaminated bivalve molluscan tissue.

Although no specific large-scale studies have been conducted,

there are a small number of references to PSP in gastropods from the Puget Sound basin. Quayle (1969) reported the detection of PSP in moonsnails (*P. lewissi*) in nearby British Columbia, above the closure limit of 80 µg of STX equiv./100 g tissue. He also showed that nontoxic moonsnails became toxic when fed butter clams containing PSP toxins. MacDonald (1970) reported that two species of whelks, *Thais* (=*Nucella*) *lima* and *Thais lamellosa*, found at Agate-Crescent Beach area on the Strait of Juan de Fuca, were reported to contain PSP toxins; however, levels of toxin were not given.

In response to the increased consumer demand for marine invertebrates, an interagency meeting was sponsored in May 1994 by the Environmental Protection Agency (EPA) to assess the available risk management data on PSP in Puget Sound predatory gastropods. Participants included the U.S. Food and Drug Administration, the Washington State Department of Health (DOH), the Washington State Department of Fisheries and Wildlife and the National Marine Fisheries Service (NMFS). It was understood that predatory marine snails were reported capable of accumulating significant amounts of PSP toxins at other locations in the world. However, specific data on the PSP content of Puget Sound gastropods were lacking. In a similar manner, the diatom genus *Pseudonitzschia* is frequently observed in Puget Sound (Dr. Rita Horner, University of Washington School of Oceanography, Seattle, WA, personal communication 1994), indicating a possible risk of domoic acid poisoning.

In order to assess the PSP and domoic acid risk within the Puget Sound basin, a limited survey was undertaken of moonsnails, other predatory snails, and clams collected from various sites where routine monitoring by the Washington DOH documented PSP occurrences. Samples were collected by the Region 10 EPA Dive Team and NMFS personnel in the summer and fall of 1994 and the winter of 1995, and the NMFS provided PSP and domoic acid analyses of the samples. The purpose of this analytical survey was to document whether or not moonsnails and other predatory snails become PSP contaminated.

METHODS

Sample Collection

The collection of moonsnails was accomplished by gleaning from available beaches and by the EPA Region 10 Dive Team. Sampling locations were identified that were expected to be moonsnail habitat and were also in the vicinity of Washington DOH–monitoring sites. Sample collection sites are shown in Figure 1. On September 1, 1994, moonsnails (*P. lewissi*), butter clams (*Saxidomus gigantus*), soft shell clams (*Mya arenaria*), and Pacific little neck clams (*Protothaca stamina*) were collected from the same beach in Agate Pass. On September 2, 1994, six moonsnails were collected from the beach at Double Bluff. On October 28, dogwinkles (*N. lamellosa*), blue mussels (*Mytilus edulis*), Pacific oysters (*Crassostrea gigas*), omnivorous snails (*Searlesia dira*), and horse mussels (*Modiolus modiolus*) were collected from Mystery Bay. On April 13, 1995, moonsnails, Pacific littleneck clams, and red rock crab (*Cancer productus*) were collected at Agate Pass. All animals were stored frozen in the shell until they were processed for PSP and domoic acid analyses. These marine toxins are stable under frozen conditions.

PSP Analyses

PSP analysis was performed by the use of modifications to the AOAC Official Method (1990). All meat was cleaned from the

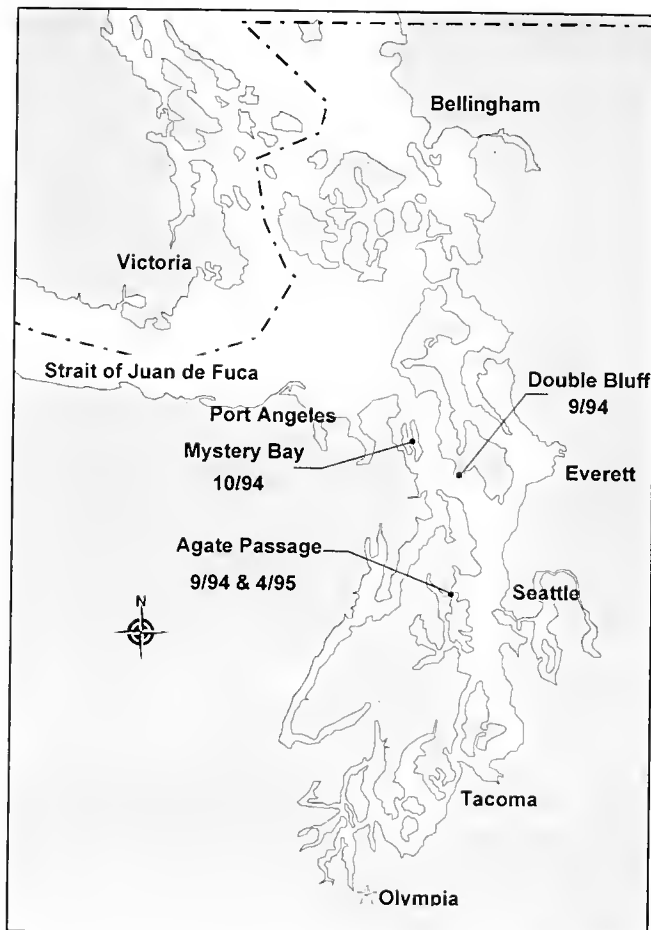

Figure 1. Sample collection sites in Puget Sound, WA.

shells. Individual body weights were determined for large specimens. Because of their small size, dogwinkles, blue mussels, and Pacific littlenecks were composited. For crab samples, only hepatopancreas were analyzed for PSP content. Each moonsnail was divided into two samples, the foot tissue and the viscera portion, which included both the digestive gland and gonadal tissue. Moonsnail foot tissue was extremely tough and difficult to homogenize, requiring some modification of the standard procedures. It was necessary to mince the tissue first with a scalpel blade, cutting the tissue into thin strips and then dicing the strips into small pieces. Moonsnail foot tissue samples were combined with an equal amount of water and mixed in a Sorvall Omnimixer-SE (Omni International, 6530 Commerce CT., Gainesville, VA) for 30 s to form smooth homogenates (corrections for PSP content were necessary because of sample dilution, i.e., 1:1). For the PSP analysis, 10 mL of the homogenate was adjusted to pH 4 by the dropwise addition of concentrated 5N HCl, usually one to two drops. Because variations in the bioassay results were small, only two animals were used per sample. Moonsnail samples, collected on April 13, 1995, were divided into two portions: viscera (digestive gland/gonadal tissue) and meat (all other tissue). The viscera portion was identical to that taken in the September survey, whereas the meat portion in this sampling consisted of the foot, musculature, and other edible material. Because of the small sample size and soft tissue, the *Nucella* samples were pooled and homogenized. It was unnecessary to add water to samples of California mussels, blue mussels, and Pacific little necks for homog-

enization, because samples were of adequate size and had soft tissues.

Twenty grams of each 1:1 homogenate was weighed into 50-mL screw-cap glass centrifuge tubes. The pH was determined and adjusted to about pH 4 by the dropwise addition of 5N HCl. Most tissues required only about 1–5 drops of 5N HCl; however, moonsnail viscera took up to 20 drops of the acid solution. Unexpectedly, moonsnail viscera produced an odorless frothing and bubbling homogenate after the addition of the acid, presumably as the result of the release of carbon dioxide. For samples that were not homogenized with water, 10 g of tissue was weighed into a centrifuge tube and 10 mL of 0.1N HCl was added. The pH was then determined and adjusted to the correct range with 5N HCl. All samples were then heated in a constant temperature water bath, containing 50% ethylene glycol, at 105°C for 10 min to denature the proteins. Samples were cooled by either immersion into cold tap water or by air cooling, and the pH was again determined and readjusted if necessary to not higher than pH 4.5. The samples were transferred into polycarbonate centrifuge tubes and spun at 15,000 rpm (27,000 RCF) for 15 min. The separated liquid was decanted and, for extra cleanup, filtered through a Millex-HA 0.45-μm-pore-size filter connected to a plastic disposable syringe into labeled scintillation vials. A Millipore glass fiber prefilter was used for the *Nucella* sample, to avoid clogging the HA filter. The filtrate was then used for the PSP assay according to the AOAC method.

Domoic Acid Analysis

Moonsnail samples were analyzed for domoic acid by the use of the high-performance liquid chromatography method of Hatfield et al. (1994). .

RESULTS

September 1994 Sampling

Samples from Double Bluff and Agate Passage were found to contain PSP in the viscera tissue. PSP was detected in every sample collected from Agate Passage in September 1994. Levels averaged 145 μg of STX equiv./100 g of tissue, with a standard deviation of ±45 and a coefficient of variation of 31% (Table 1). PSP levels ranged from nondetectable to a high of 95 μg of STX equiv./100 g of tissue in the Double Bluff samples; however, two of the samples were nontoxic. When PSP was present in the viscera of moonsnails, it appeared lower in the Double Bluff samples than in the Agate Passage samples. In the Double Bluff moonsnail viscera sample, 940902-5, the symptoms demonstrated in the mice were not thought to be consistent with PSP (i.e., respiratory paralysis).

In the September 1994 samplings, foot tissue appeared free of PSP toxins. For example, in the viscera sample containing the highest detected levels of PSP toxins, sample 940901-6-msv (214 μg of STX equiv./100 g) from Agate Passage, no toxin was found in the corresponding foot tissue (940901-6-msf) from the same animal. Because of this lack of toxicity observed in the foot tissue, only viscera tissues were tested for toxicity in the remaining samples.

When moonsnails were collected at the Agate Passage site, a small sampling of other shellfish was also collected. These shellfish included butter clams, soft shell clams, and Pacific little neck clams (Table 2). Only the butter clams were found to contain PSP toxins, averaging 73 μg of STX equiv./100 g of tissue and ranging from 45 to 94 μg of STX equiv./100 g. PSP was not detected in either soft shell and little neck clam samples.

TABLE 1.

PSP toxicity in moonsnails from Double Bluff and Agate Passage collected in September 1994.

Sample	Toxicity (μg STX equiv./100g tissue)
Agate Passage	
940901-1-viscera	171
940901-2-viscera	164
940901-3-viscera	114
940901-4-viscera	155
940901-5-viscera	116
940901-6-viscera	213
940901-7-viscera	80
Viscera average	**145**
940901-6-foot	ND*
Double Bluff	
940902-1-viscera	ND
940902-2-viscera	95
940902-3-viscera	ND
940902-4-foot	ND
940902-4-viscera	ND
940902-5-foot	ND
940902-5-viscera	DNQ**
940902-6-foot	ND
940902-6-viscera	54

* ND, not detected, treated as 0 μg of STX equiv./100g of tissue for calculations.
** DNQ, toxicity detected, not quantified. No deaths, but animals were distressed, response was not characteristic of PSP.

October 1994 Sampling

Washington DOH monitoring indicated elevated concentrations of PSP in Mystery Bay in October. During sample collection, moonsnails were not present in this bay; therefore, other shellfish

TABLE 2.

Other shellfish collected at Agate Passage in September 1994.

Sample*	Toxicity (μg of STX equiv./100g of tissue)
Butter clams	
940901-bc-1	45
940901-bc-2	90
940901-bc-3	86
940901-bc-4	94
940901-bc-5	75
940901-bc-6	46
Softshell clams	
940901-ss-1	ND
940901-ss-2	ND
Pacific littleneck clams	
940901-ln-1	ND
940901-ln-2	ND

* Abbreviations: bc, butter clams (*S. gigantus*); ss, soft shell clams (*M arenaria*); ln, Pacific littlenecks (*P. stamina*).

species were collected: the predatory dogwinkle (*N. lamellosa*), an omnivorous snail (*S. dira*), Pacific oysters, and two species of mussels. Because of their size, the Pacific oysters and the horse mussel (*M. modiolus*) were analyzed individually, whereas the meats from the blue mussel samples and dogwinkles were composited. Blue mussels were highly toxic (652 μg of STX equiv./100 g of tissue); all of the other species collected also contained PSP toxins (Table 3), although at very reduced levels. With the exception of the mussels, all of the other samples were below the health guideline of 80 μg of STX equiv./100 g. Nevertheless, the dogwinkle sample contained the second highest levels of PSP (72 μg of STX equiv./100 g). Because mussels are known to both accumulate and depurate PSP toxins rapidly, such high levels of PSP in mussels indicated a probable bloom of PSP-producing phytoplankton.

April 1995 Sampling

Approximately 8 months after the initial moonsnail collection (September 1994), another survey was made in the same area (Agate Passage), on April 13, 1995. PSP levels were expected to be low during this period in Puget Sound. In addition to moonsnails, samples of butter clams, littleneck clams, and two red rock crab (*C. productus*) were collected and analyzed for PSP. PSP levels are reported in Table 4. As expected, the early spring collection produced moonsnail and butter clam samples with relatively low PSP levels. All moonsnail viscera examined contained PSP activity, ranging from 43 to 53 μg of STX equiv./100 g. However, only four of six butter clam samples were found to contain PSP activity. In contrast, the September 1994 sampling found all of the butter clam samples to be toxic. Toxin levels in clams collected in the spring 1995 survey ranged from 40 to 77 μg of STX equiv./100 g. Littleneck clams and red rock crab hepatopancreas were not toxic.

Domoic Acid Analyses

All samples subjected to PSP analysis were also analyzed for domoic acid. No domoic acid was detected in any sample. At the time and places of collection, no blooms of *Pseudonitzschia* sp. were observed or reported.

Mineral Composition

Mineral analyses of the moonsnail viscera homogenates were undertaken to gain a better understanding of the degassing ob-

TABLE 3.

PSP assay of whole-body shellfish samples collected October 28, 1994, at Mystery Bay, WA.

Sample	Toxicity (μg of STX equiv./100g of tissue)
941028-dogwinkle (*N. lamellosa*)	72
941028-blue mussel (*M. edulis*)	652
941028-1-Pacific oyster (*Crassostrea virginicus*)	41
941028-2-Pacific oyster (*C. virginicus*)	44
941028-3-Pacific oyster (*C. virginicus*)	48
941028-4-Pacific oyster (*C. virginicus*)	47
941028-1-horse mussel (*M. modiolus*)	45
941028-2-horse mussel (*M. modiolus*)	51
941028-1-snail (*S. dira*)	50

TABLE 4.

PSP levels in moonsnails, littleneck clams, and red rock crab collected on April 13, 1995, at Agate Passage.

Sample*	Tissue Analyzed	Toxicity (μg of STX equiv./100g of tissue)
Moonsnails (*P. lewissi*)		
950413-msv-1	Viscera	45
950413-msm-1	Foot	0
950413-msv-2	Viscera	49
950413-msm-2	Foot	0
950413-msv-3	Viscera	54
950413-msm-3	Foot	0
950413-msv-4	Viscera	49
950413-msm-4	Foot	0
950413-msv-5	Viscera	44
950413-msm-5	Foot	0
950413-msv-6	Viscera	47
950413-msm-6	Foot	0
Butter clams (*S. gigantus*)		
950413-bc-1	Whole body	47
950413-bc-2	Whole body	76
950413-bc-3	Whole body	40
950413-bc-4	Whole body	44
950413-bc-5	Whole body	0
950413-bc-6	Whole body	0
Pacific littleneck (*P. stamina*)		
950413-ln-1	Whole body	0
Red rock crab (*C. productus*)		
950413-rch-1	Whole body	0

* Abbreviations: msv, moonsnail viscera; msm, moonsnail foot tissue; bc, butter clam; ln, littleneck.

served during the acidification step of the PSP assay. Mineral composition is reported on both a dry and a wet weight basis in Table 5. Marked differences were noted in the average moisture content of the moonsnail samples collected in September 1994 (84.5% moisture) and April 1995 (74.2% moisture), which, in turn, was apparently reflected in the mineral composition of these samples (Table 5). Ash content, expressed on a wet weight basis, was significantly different between the 1994 (1.4%) and the 1995 (2.7%) samples; however, on a dry weight basis, no significant differences were observed between the samples (9.4% for 1994 and 10.5% for 1995). On both dry and wet weight bases, sodium was present at the highest level, as expected. Magnesium was present at the second highest level in both the 1994 and the 1995 samples. On a wet weight basis, the macromineral levels of sodium and potassium varied considerably between the 1994 and the 1995 samples, with 1995/1994 ratios of 1.86 and 2.20, respectively. However, when expressed on a dry weight basis, these sodium and potassium ratios were less dramatic, 1.07 and 1.29, respectively. Magnesium concentrations, on a dry weight basis, were similar in both the 1994 and the 1995 samples. However, on a wet weight basis, the September 1994 samples were almost one-half of the April 1995 level. On a dry weight basis, calcium levels were similar in both samples, 3,444 and 3,564, respectively. However, on a wet weight basis, the 1995 samples were 1.8 times higher than the 1994 samples. The concentrations of the trace minerals (chromium, copper, manganese, and zinc) were similar between the 1994 and the 1995 samples on a wet weight basis. As expected for shellfish, zinc levels were high in both 1994 and 1995, 102 and 116 ppm on a wet weight basis, respectively.

TABLE 5.

Elemental composition, presented on both a dry and a wet weight basis, of moonsnail viscera collected in September 1994 (940901) and April 1995 (950413).

Element	950413 Average (ppm) Dry Wt. (N = 6)	940901 Average (ppm) Dry Wt. (N = 6)	Ratio 950413/940901	950413 Average ppm Wet Wt.	940901 Average ppm Wet Wt.	Ratio 950413/940901
Ca	3,564	3,444	1.0	930	522	1.8
Cr	1.8	2.8	0.62	0.44	0.43	1.1
Cu	35	56	0.64	9.0	8.5	1.1
Fe	323	316	1.0	82	48	1.7
K	7,270	5,618	1.29	1,880	854	2.2
Mg	15,801	14,231	1.11	4,113	2,205	1.9
Mn	23	33	0.68	5.8	5.2	1.1
Na	18,330	17,104	1.1	4,598	2,474	1.9
P	6,482	5,828	1.1	1,682	885	1.9
Sr	37	35	1.0	9.4	5.3	1.8
Zn	458	606	0.76	116	102	1.1

DISCUSSION

Both the moonsnail, *P. lewissi*, and the frilled dogwinkle, *N. lamellosa*, are predatory marine carnivores (Keep 1911, Ricketts and Calvin 1968, Barth and Brushears 1982) and attack their prey using a special boring device, a radula (Russel 1993, Carriker 1961). Both species are commonly found from British Columbia to at least southern California, with *Nucella* preferring rocky beaches, whereas moonsnails prefer sandy bottoms (Ricketts and Calvin 1968, Rice 1968). The favored prey for these species includes mussels and clams (Ricketts and Calvin 1968), but *N. lamellosa* also feeds on barnacles (*Balanus* sp.) (Emlen 1966). Mussels and clams are phytoplankton feeders and have the highest risk of containing marine biotoxins, e.g., domoic acid (from the consumption of *Pseudonitzschia* spp.) or PSP (from consuming *Alexandrium* spp.). Boring into their prey, these carnivores consume the viscera, which potentially carry the highest levels of toxins. Because these animals live in a zone that frequently experiences blooms of *Alexandrium* capable of producing PSP, and prey on shellfish that are phytoplanktonic feeders, it is not surprising that they can and do accumulate PSP toxins.

Until the mid- to late 1970s, PSP was not commonly found within the Puget Sound basin. Before then, monitoring and closures were common along the Strait of Juan de Fuca, on beaches west of the city of Port Angeles (Fig. 1). However, in 1977, we and others measured very high levels (>14,000 μg of STX equiv./100 g) of PSP toxins in mussels from Puget Sound (J. Wekell, unpublished data, 1977). Since that time, PSP has become a constant risk throughout the basin. Predatory snails and other potential human food items besides bivalve shellfish have not been routinely monitored for the presence of PSP or other marine toxins.

In the Puget Sound basin, PSP levels in shellfish generally begin to rise in May and continue through the summer and early fall months, although high levels of PSP have been noted as late as November and December. Because of their rapacious nature, it is not surprising that PSP levels in moonsnails and other predatory gastropods follow a pattern similar to that observed in other shellfish in Puget Sound. Butter clams are known to accumulate and retain PSP toxins consistently for long periods along the coasts of Oregon, Washington, British Columbia, and Alaska. Although the levels of PSP in predatory snails found in Puget Sound appear to be low, our September 1994 survey found levels well in excess of the regulatory closure limit of 80 μg of STX equiv./100 g; this indicates that the consumption of these shellfish does represent a potentially serious human health risk.

Unlike data reported by Shumway (1995) and White et al. (1993a and 1993b) for *E. heros* (= *Polinices*) taken from Georges Bank, we found PSP activity restricted to the viscera of the Puget Sound moonsnail (*P. lewissi*) in both samplings (fall of 1994 and spring of 1995). In the Georges Bank survey, *Euspira* PSP levels were considerably higher (>2,500 μg of STX equiv./100 g of tissue) than the toxicities observed in our studies (i.e., <250 μg of STX equiv./100 g of tissue). In addition, the sampling in this survey was considerably smaller than the sampling conducted in the Georges Bank survey; however, the variation of PSP levels from the September sampling at Agate Passage is similar to that reported by White et al. (1993b) for moonsnails taken from Georges Bank. Nagashima et al. (1995) reported that the trumpet shell (*Charonia lampas*) collected from the Galician Coast in Spain contained PSP toxins in the digestive gland in a range similar to those we found in Puget Sound snails. Interestingly, the toxin suite found in the Galician shells was composed of mainly the decarbamoyl derivatives of STX (dcSTX), gonyautoxin2 (dcGTX2), and gonyautoxin3 (dcGTX3), with only minor amounts of GTX2 and GTX3.

In order to obtain some understanding about the variation at each collection site, individual shellfish were analyzed. With these constraints, modifications to the AOAC method were necessary because of the nature of the tissues and the amounts available for analysis. Samples of snails from the Double Bluff area were used to test and develop modifications to the PSP assay procedure, i.e., tissue sampling and handling. Moonsnail viscera presented a problem in adjusting the pH. Because viscera consisted of soft tissue, homogenizing with an equal volume of water was unnecessary. For the viscera analysis, 1 part homogenized viscera was mixed with 1 part 0.1N HCl, but in order to achieve the desired pH 4 with some 1994 samples, considerably more acid was required because a higher than expected initial pH (pH 7.6–8.6) was observed. In order to achieve the desired pH, as required in the AOAC procedure, it was necessary to add a more concentrated acid, i.e., 5 N HCl, to the mixtures (10 mL), requiring 20–25 drops or about 1 mL. Because this amount increased the volume nearly 10%, adjustment to the final calculations was applied. It was noted that the

addition of this acid caused an apparent release of gas, and care had to be taken during the heating step to ensure that material was not lost as the result of frothing and leakage from the tubes. The use of polyethylene centrifuge tubes with fitted screw-cap lids helped reduce these losses and reduce the chances of breakage. With the exception of only one sample, moonsnail viscera from samples collected in April 1995 had normal expected pH values (i.e., pH 6–7) that did not produce gas when acidified. Nevertheless, sample 950413-msv-3 had an initial pH of 8.1 and produced negligible amounts of gas when acidified. It is presumed that this gas is carbon dioxide, because it was both odorless and colorless. Because it is released on acidification, it is probably present as carbonate ion.

The high pH encountered suggests that carbonate would be associated with some typical biological alkaline cation, such as calcium, sodium, or magnesium. Accordingly, we expected higher concentrations of these cations in the viscera, which included the digestive gland/gonadal tissues. Calcium was considered to be the likely cation because of its high concentration in seawater and its involvement in shell synthesis. However, the mineral analyses indicated the opposite of what was expected. That is, the calcium, magnesium, and sodium concentrations on a wet weight basis were approximately one-half of the element concentrations seen in 1995, in which little or no frothing occurred. Thus, mineral analyses alone are not sufficient to explain the presence of apparent excess carbonate ion.

The assessment of health risks posed by the consumption of predatory shellfish and the development of programs for their management will be difficult to implement because our current knowledge, based on this work and other studies in the Puget Sound basin, is limited. To develop an acceptable risk model and program, more information and data concerning the distribution and quantity of these shellfish (seasonally and geographically) will be required. In addition, information about the toxins will be required, for example, levels in specific tissues, total body burden, and seasonal variability. The implementation of a management program will also require detailed information about consumers and their use of these nongame marine invertebrates (NGMI). For example, Carney and Kvitek (1991) found in their survey that over 50% of the collectors harvesting NGMI were Asian, Korean, or Filipino. The usage and consumption rates of NGMI among these ethnic groups are unknown but are suspected to be much higher than that reported for the whole population (6.5 g/day) (Connie Nakano, Project Coordinator, Asian and Pacific Islander Seafood Consumption Study, Seattle, WA, personal communication, 1995).

CONCLUSIONS

The results from this survey indicate that Puget Sound predatory marine snails accumulate PSP toxins to levels above the regulatory level (80 μg of STX equiv./100 g). Further, to increase the likelihood of detection, the viscera of the moonsnails appears to be the tissue of choice in analyzing these animals for PSP toxins. Modification of the sample preparation method may be required. Other parts of the moonsnail either do not accumulate the PSP toxins or accumulate it at levels that are below the current AOAC method's detection limit. In addition, these survey data indicate that other predatory and omnivorous snails accumulate PSP toxins. If the recreational or subsistence collection of these species continues or grows, some form of monitoring may be required for the protection of public health. The management of these health risks will require more information on the seasonality, distribution, uptake, and depuration of toxicity in these molluscan species within the Puget Sound basin. Perhaps most important, however, will be more detailed knowledge of the patterns of human collection, preparation, and consumption of these shellfish.

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TEMPORAL PATTERNS OF REPRODUCTIVE CONDITION IN THE DOUGHBOY SCALLOP, *CHLAMYS (MIMACHLAMYS) ASPERRIMA* LAMARCK, IN JERVIS BAY, AUSTRALIA

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ABSTRACT The broodstock reproductive condition of doughboy scallops, *Chlamys (Mimachlamys) asperrima* Lamarck, in Jervis Bay, Australia, was monitored fortnightly for 2 y. Gonosomatic index (GSI), gonad weight, and macroscopic gonadal appearance were used to assess changes in the reproductive status of the population. In common with *C. asperrima* in Tasmania, a winter-spring peak in reproductive activity was observed, although macroscopically ripe (mature, ready-to-spawn) individuals were present in most collections. Peaks in reproductive indices occurred in June and August 1992 and in September 1993, during which partial spawning was evident. Male and female development was synchronous, although female scallops maintained higher GSIs and macroscopically higher levels of gonadal development and, at their reproductive peak, had higher calorific values for gonadal tissue. Female gonads were also consistently heavier than those of equivalent-sized males, although adductor muscle weight in males was on average 9% heavier than that of females. Observations of reproductive condition indicate that although the optimal times for the harvest of *C. asperrima* in Jervis Bay are likely to be similar to that of southern stocks, the presence of reproductively capable individuals at most times of the year in Jervis Bay could be of advantage in the hatchery production of the species and in the provision of embryos for ecotoxilogical studies.

KEY WORDS: *Chlamys*, scallop, reproductive cycle, gonosomatic index, macroscopic index

INTRODUCTION

The doughboy scallop or fan shell, *Chlamys (Mimachlamys) asperrima* Lamarck 1819 (Pectinidae), is a gonochoristic species found subtidally along the southern Australian coast, from Shark Bay, Western Australia, to New South Wales (NSW) (Wells and Bryce 1988; Fig. 1). Capable of growing to over 100 mm in shell height (Zacharin 1994), *C. asperrima* commonly occurs byssally attached to solid objects in depths of 7–69 m (Young and Martin 1989). *C. asperrima* has been harvested commercially (Young and Martin 1989) and has aquaculture potential (Cropp 1989, O'Connor et al. 1994), and its early ontogenetic stages are used in ecotoxilogical assessments of potential pollutants (Krassoi et al. in press).

To understand the life history of a scallop, such as *C. asperrima*, manage its fishery, or attempt culture, it is essential to gain an understanding of the reproductive behaviour of the species (Barber and Blake 1991). Recruitment, meat yields, quality of "roe on" product, spawning induction, and the duration of broodstock availability are all related to reproductive state, which can vary spatially and seasonally. Little is known of the reproductive biology of *C. asperrima*, and those observations that have been made are largely confined to the southern part of the species distribution (Fig. 1). Rose and Dix (1984; R. Rose pers. comm.) gathered spawnable *C. asperrima* from the D'Entrecasteaux channel, Tasmania, in October/November. Grant (1971) reported individuals from this area in "almost full roe" in early April, and spawning was thought to occur before July (Zacharin 1986). More recently, Zacharin (1994) monitored reproductive changes in *C. asperrima* from D'Entrecasteaux Channel and found peaks in reproductive condition in September 1988 and October 1989. In South Australia, Chernoff (1987) caught newly settled spat between February and May, indicating that spawning had commenced in January and continued until April. To date, no observations of the reproductive condition of populations of *C. asperrima* at the northern part of the species range in either NSW or Western Australia have been reported.

Visual observations have been the most widely used method of assessing gonadal development in scallops (Barber and Blake 1991), incorporating factors such as relative size, shape, turgor, colour, and acini appearance. Similarly, gonadal indices that express gonad weight as a proportion of total body weight or shell height have been used to define gonadogenic cycles in scallops (Thompson 1977, Bricelj et al. 1987). Both methods were used in this study to show that the reproductive cycle of *C. asperrima* in Jervis Bay, although less defined, was similar to that of southern populations.

MATERIALS AND METHODS

C. asperrima were collected from a depth of 15–17 m at a site approximately 500 m northwest of the Murrays Beach boat ramp, Jervis Bay (Fig. 1). This site has a sandy substrate and is well flushed with seawater of salinities in the range of 33–36‰. Water temperatures recorded at the time of scallop collection ranged from 14–23°C.

An initial sample of 73 scallops (shell heights, 34–70 mm) was collected by divers in October 1991. Relationships between shell height (central dorsal to central ventral margin) and gonad weight, muscle weight, soft body weight, and gonosomatic index (GSI) were examined by the use of regression analysis. Linear, multiplicative, exponential, and reciprocal models were fitted to each data set with the "Statgraphics" statistical graphics system (Statistical Graphics Corporation, Rockville, MD), and the model used was chosen on the basis of the highest r^2 "goodness of fit" value. GSI was calculated by use of the method of Latrouite and Claude (cited in Barber and Blake 1991):

$$GSI = (\text{gonad weight}/(\text{soft body weight} - \text{gonad weight})) \times 100$$

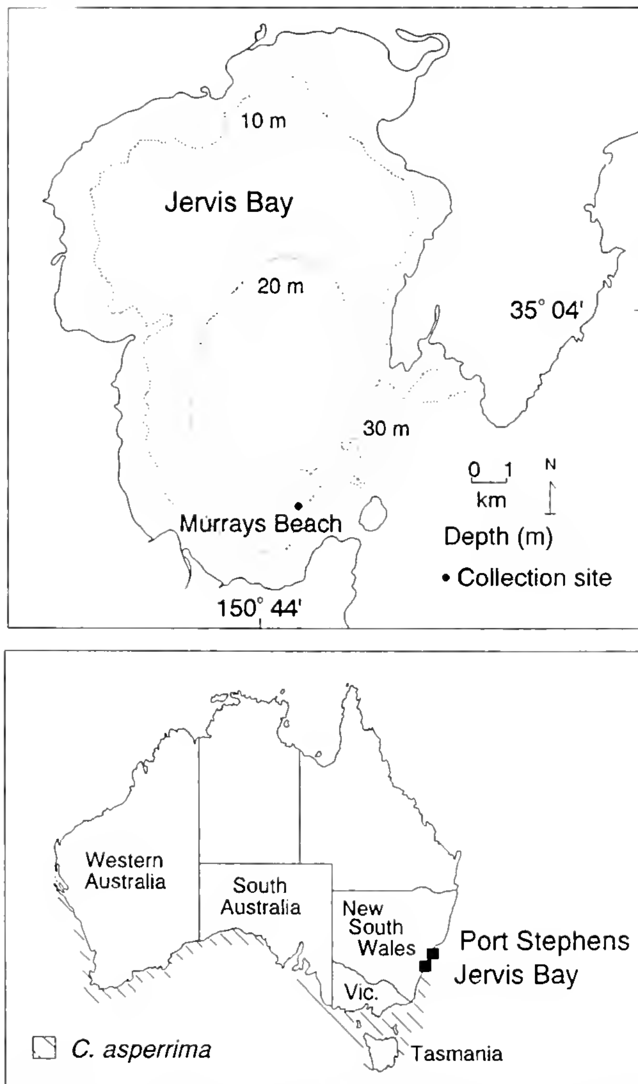

Figure 1. Scallop collection site, Murrays Beach, Jervis Bay, NSW, Australia.

Weather permitting, *C. asperrima* were collected by divers fortnightly from Murrays Beach. Each sample was wrapped in damp jute sacking, packed on ice, and freighted for analysis that evening or the following day. Collections began in October 1991 and continued until October 1993.

Shell heights and genders of approximately 30 *C. asperrima* >50 mm shell height were recorded. The soft body was removed from the shell, and its wet weight was determined to the nearest 0.01 g. The adductor muscle and gonad were then excised and placed in preweighed Petri dishes; any fluid lost after excision was retained within the dish and included in the weight of the tissue. Where sex was indeterminate or infection with bucephalid parasites was suspected, samples of gonadal tissue were examined with a stereomicroscope (40× magnification). Bucephalid-infected gonads were excluded from all analyses reported here.

Although sampling was initiated in October 1991, the macroscopic staging system for *C. asperrima* was not introduced until February 1992. This delay permitted familiarization with changes in gonadal appearance. Each scallop was assigned to a stage in an arbitrary reproductive index (Table 1), similar to those of Hennick (1970) and Dredge (1981). A numerical ranking was assigned to each macroscopic stage, ranging from 1 for spent scallops to 5 for ripe (mature, ready-to-spawn) scallops. Partially spawned scallops were given a ranking of 4, the same as that of well-developed scallops, because many of these scallops still had relatively full, turgid gonads and, on the basis of observations of induced spawnings within hatcheries, were capable of contributing to spawning events.

The gross energy value of ripe male and female gonads was determined by bomb calorimetry. Four batches of five gonads from each sex were compared. Scallops chosen for analysis were collected during peak reproductive periods in late August and September 1993. Each scallop selected was judged to be ripe on the basis of macroscopic criteria and had a GSI >25%. Each gonad was carefully excised to ensure that little or no tissue from the digestive gland was included. The gonad was then dissected, and the intestinal loop was removed. The remaining gonadal tissue was weighed to the nearest milligram, dried at 105°C for 24 h, weighed again, and stored in a freezer until analysed. Dry matter was determined for each pooled sample (five gonads) before gross energy evaluation with a Parr 1241 adiabatic bomb calorimeter (Parr Instrument Co., Moline, IL).

RESULTS

All 73 scallops in the initial collection were judged to be mature on the basis of macroscopic observations. Gonadal tissue samples from the smallest of these scallops (34–45 mm) were examined microscopically (100× magnification) and found to contain either spermatozoa or developing oocytes. *C. asperrima* gonad, muscle, and soft body weights all increased exponentially

TABLE 1.

Macroscopic gonadal staging system for determination of reproductive condition in the doughboy scallop, *C. (Mimachlamys) asperrima*.

Stage	Numerical Ranking Score
Spent/Immature: Sperm or eggs absent. Gonad flaccid and translucent. Both ascending and descending limbs of intestinal loop clearly visible.	1
Developing 1: Gonads are filling; separate acini are apparent, giving a granular appearance; males and females are distinguishable.	2
Developing 2: Gonad less granular in appearance as acini begin to fill. Gonad increasing in turgor.	3
Developing 3: Very little of the intestinal loop visible, usually only a small portion of the ascending limb at the distal extremity of the gonad. Gonad appears uniform in colour and texture as acini fill.	4
Ripe: Gonad uniform in colour, highly turgid, acini not apparent, intestinal loop not visible.	5
Spawning: Gonad uniform in colour; however, flecks or mottling occurs as the result of voided acini. Gonad turgor varies according to the extent of spawning, usually the equivalent of Developing 2 & 3 scallops.	4

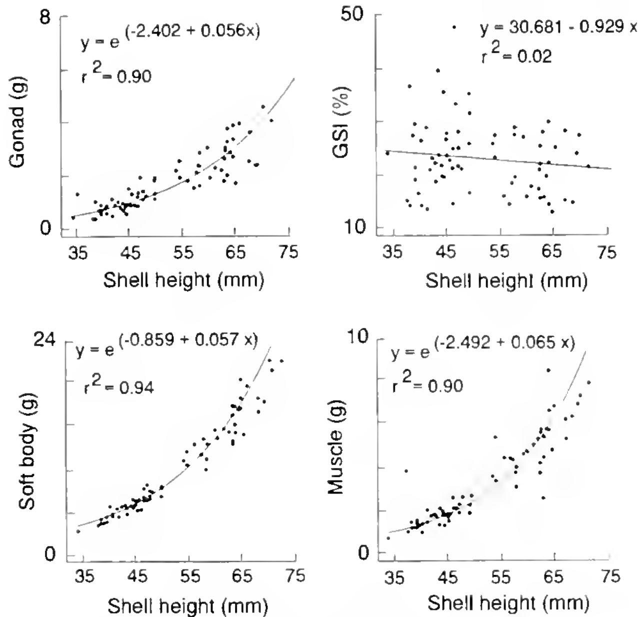

Figure 2. Relationship between shell height and gonad weight, muscle weight, soft body weight, and GSI for the initial collection of *C* (*Mimachlamys*) *asperrima* (October 1991, shell height, 34–70 mm).

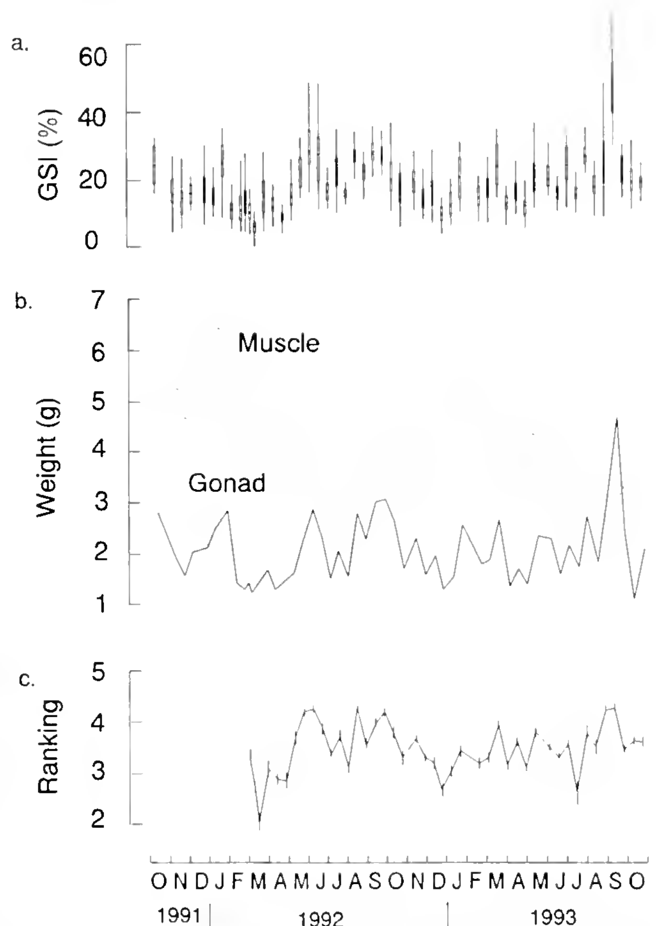

Figure 3. A comparison of (a) a box and a whisker plot of GSI, (b) regression predicted mean gonad and muscle weights for 62.5-mm scallops, and (c) mean ranking for macroscopic stage (±SE) for *C.* (*Mimachlamys*) *asperrima* collected from Jervis Bay from October 1991 until October 1993.

with shell height, and although no significant relationship was found between scallop size and GSI (Fig. 2), subsequent collections were limited to scallops >50 mm shell height. Larger scallops were selected to reduce the possibility of size-dependent variation in GSI (Gonor 1972, Grant and Tyler 1983, West 1990) and to improve the efficiency and precision of dissections. Previous studies with hatchery-produced juveniles indicated the onset of sexual maturity at approximately 30 mm shell height (O'Connor et al. 1994).

During the following 2 y, 1,612 scallops were examined. Of these, 51.2% were female and 44.4% were male. Genders of 4.3% of scallops collected were indeterminate because of parasitic castration by a bucephalid trematode. A comparison of shell height data for each scallop collection found that variances were highly heterogeneous (Cochrans test, C = 0.056; $p < 0.001$) and that shell height varied significantly throughout the sampling period (Kruskall Wallace test, $\chi^2 = 340.2$; $df = 51$; $p < 0.001$), increasing significantly in later samples (slope = 0.18; $t = 10.836$; $p < 0.001$). The largest *C. asperrima* collected from Murrays Beach during this study was 81 mm in shell height. Juveniles (approximately 10 mm) were first observed in December and January each year, often attached to the shells of adults or other bivalves such as flat oysters, *Ostrea angasi*, and razor clams, *Pinna bicolor*.

GSI

A comparison of GSI data between collections found variances to be highly heterogeneous (Cochrans test, C = 0.088; $p < 0.001$), with the greater variability evident when mean GSI was at its peak ($r = 0.52$; Fig. 3). This heterogeneity precluded the use of analysis of covariance to determine the proportion of variance due to changes in shell height, as suggested by Grant and Tyler (1983). In lieu, GSI was regressed with shell height for each collection. Given the high number of collections (52) and the high chance of making type II errors, α was set *a priori* at 0.01. Because only three collections were found to have a significant relationship between GSI and shell height, 13/8/92 (F = 14.65; $df = 28$; $p < 0.001$), 15/2/93 (F = 15.65; $df = 28$; $p < 0.001$) and 24/8/93 (F = 10.7; $df = 29$; $p < 0.01$), we considered GSI to be largely independent of shell height during this study. In each case, a negative relationship was found, that is, GSI decreased with increasing shell height. Significant variation in GSI was evident between collections (Kruskall Wallace text, $\chi^2 = 890.3$, $df = 51$, $p < 0.001$). High mean GSI values (>18%) were generally found from late autumn to early spring (May to September), peaking in the months of June and August/September 1992 and in September 1993 (Fig. 3). Scallop reproductive condition was considered to be poor, that is, smaller, flaccid gonads with much of the intestinal loop visible (GSI < 18%), from early summer to autumn (December to April) in both years.

Macroscopic Staging

Changes in mean ranking for macroscopic stages for each collection showed a pattern similar to that of GSI and gonad weight (Table 2). Ripe scallops were present in most collections (Fig. 4), with the highest occurrence corresponding closely with peaks in GSI. In addition, ripe scallops were uncommon or absent in the collections of March and April 1992, when the lowest mean GSI

TABLE 2.

Correlation matrices for regressed muscle and gonad weights for a 62.5-mm *C. (Mimachlamys) asperrima* (predicted on the basis of regression analysis) for each collection, the mean GSI for each collection, and the mean macroscopic ranking.

Parameter	Regressed Gonad Weight	Mean GSI	Mean Macroscopic Gonad Ranking
Regressed muscle weight	$r = -0.21$ ($p = 0.144$, $n = 52$)	$r = -0.45$ ($p < 0.001$, $n = 52$)	$r = 0.51$ ($p < 0.001$, $n = 43$)
Regressed gonad weight		$r = 0.91$ ($p < 0.001$, $n = 52$)	$r = 0.80$ ($p < 0.001$, $n = 43$)
Mean GSI			$r = 0.84$ ($p < 0.001$, $n = 43$)

figures were recorded. During the study, only 1.5% of scallops were found to be spend and no spent scallops were found between August and December in either year. Spawning scallops were also uncommon, comprising only 1.1% of the scallops ranked. This may have arisen from difficulties in differentiating scallops in the advanced spawning stages from those in Developing 1 & 2 stages. Despite this difficulty, staging offers a fast, useful alternative to GSI and, importantly, is nondestructive. Macroscopic staging at the time of collection was not observed to increase the incidence of unplanned spawnings in scallops maintained for later experimentation.

Tissue Weights

Examination of variation in tissue weights was not made until each weight had been related to shell height by the use of linear least-squares regression (Daniel and Wood 1980, Dredge 1981). For gonad weights, the equation

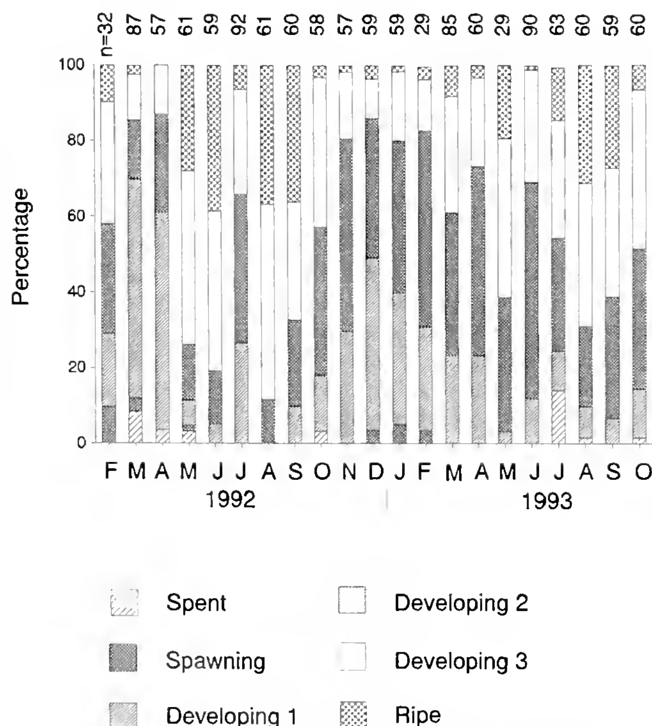

Figure 4. Monthly frequency of macroscopic stages of gonadal development in the scallop *C. (Mimachlamys) asperrima* from February 1992 to October 1993.

where y is gonad weight (in grams) and x is shell height (in millimeters) was fitted to each fortnightly sample, and the predicted gonad weight for a scallop of 62.5 mm shell height was calculated. The exponential equation was chosen instead of linear or multiplicative equations on the basis of higher r^2 percentages when fitted to weight data from the initial collection (Fig. 2). A shell height of 62.5 mm was selected because it approximated both the mean and the modal shell height of scallops collected during the study. The same procedure was repeated for muscle and soft body weights.

Changes in gonad weight with time of year were found to closely reflect changes found in GSI (Fig. 3). Generally, variation in muscle weight reflected the inverse of that observed with condition indices, although no significant negative correlation was found between regressed gonad and muscle weights (Table 2). Peaks in muscle weight occurred in summer in 1992 and 1993, when the average muscle weighed approximately 5.9 g, nearly double the winter low of 3.1 g.

Comparison of Results for Males and Females

The ratio of males to females in each collection did not vary greatly over the sampling period, and the overall ratio of males to females collected did not differ significantly from 1:1 ($\chi^2 = 3.78$; $df = 1$; $p > 0.05$). Observations of bucephalid parasitism, which can prevent the macroscopic determination of sex, indicated that it is not exclusive to either sex. Mean shell heights of males and females collected were 62.3 ± 5.1 and 62.5 ± 4.8 mm (mean ± SD), respectively (Table 2).

Over the 2-y sampling period, the mean muscle weight of males (4.43 g) was significantly ($p < 0.05$) greater than that of females (4.04 g). Conversely, the mean gonad weight in females (2.27 g) was significantly ($p < 0.05$) greater than that of males (1.97 g; Table 2). However, the average soft body weight of males did not differ significantly ($p > 0.05$) from that of females (Table 2). Coefficients of variation for shell height or the various tissue weights did not differ greatly between sexes (Table 2), and changes in each measure over the sampling period were synchronous (Fig. 5). The macroscopic stage of gonad condition for the sexes also varied synchronously; however, females on average achieved significantly higher rankings ($\chi^2 = 261.6$; $df = 5$; $p < 0.001$; Fig. 6). The percentage of females judged to be in ripe or spawning condition over the 2-y period was more than twice that of males, 17.5 and 7.6%, respectively (Fig. 6).

A comparison of gross energy in ripe male and female gonads showed males to have significantly ($t = 6.9$, $p < 0.01$) less energy. Male gonads averaged 17.9% dry matter and 18.66 MJ

TABLE 3.

Comparison of mean shell height and tissue weights of 716 male and 826 female *C. (Mimachlamys) asperrima* collected from Jervis Bay between October 1991 and November 1993.

Parameter	Male			Female				
	Mean ± SD	Range	CV[1]	Mean ± SD	Range	CV[1]	t	p
Shell height (mm)	62.26 ± 5.13	31.30	8.2	62.45 ± 4.78	33.00	7.6	0.73	0.46
Soft body weight (g)	13.76 ± 3.68	21.06	26.8	13.45 ± 3.30	22.98	24.5	1.79	0.07
Gonad weight (g)	1.97 ± 0.87	4.98	44.0	2.27 ± 0.98	6.92	43.1	6.46	<0.01
Muscle weight (g)	4.43 ± 1.60	9.65	36.1	4.04 ± 1.43	10.56	35.3	5.04	<0.01

[1]CV, coefficient of variation (%).

kg^{-1} dry matter, whereas female gonads averaged 19.5% dry matter and 19.65 MJ kg^{-1} dry matter. At the time of collection, the calculated gonad weight for a 62.5-mm female scallop was 5.12 g, and for a 62.5-mm male, it was 4.31 g. On a static basis, this implies that the energy invested in male gonads at that time was 27% less than that in female gonads.

DISCUSSION

Evidence was found to suggest that an annual reproductive cycle occurs in *C. asperrima* from Jervis Bay. Peaks in all reproductive indices occurred in winter and early spring, and on the basis of considerable fluctuations in GSI (Dredge 1981; Sause et al. 1987, West 1990, Zacharin 1994), there were indications that some spawning occurred. The scarcity of spent individuals, even during the winter-spring peak in reproductive activity, indicated that either redevelopment is extremely rapid or that partial or dribble spawning was responsible for these fluctuations. Evidence for partial spawning can be found in the twin peaks in GSI in 1992, in the protracted settlement period for spat in South Australia (Chernoff 1987), and in the stepped decline in the gonadal weight of *C. asperrima* from both D'Entrecasteaux Channel in Tasmania (Zacharin 1994) and Jervis Bay. Peaks in reproductive activity in *C. asperrima* in this study were temporally consistent with reports from Tasmania and with the winter-spring spawning period observed for some other tropical and subtropical pectinids in the southern hemisphere (Sause et al. 1987). Grant's (1971) observation of "almost full roed" *C. asperrima* in April and the October/November collection of spawnable broodstock by Rose and Dix (1984) coincide with the beginning and end of the main period of reproductive activity in Jervis Bay. Further, the peaks in reproductive indices for *C. asperrima* corresponded closely with those recorded by Zacharin (1994) in the D'Entrecasteaux Channel. Recalculating GSI in this study to that used by Zacharin (ratio of gonad weight to soft body weight) found that mean GSI varied in a range from 5.9 to 31.8%, which is similar to the range reported by Zacharin (1994; c. 5–33% for males and females combined).

Observations of spat occurrence were generally consistent with winter/spring spawnings. With the exception of a small number of juveniles less than 7 mm in shell height collected from Jervis Bay in August 1994 (W. O'Connor pers. obs.), recruitment was not observed by divers in 1992 or 1993 until January or February, when juveniles had grown to 8–10 mm in size. Previous experience with hatchery-produced *C. asperrima* has shown that spat could grow to 10-mm shell height in 12–15 wk (O'Connor et al. 1994), and thus, spawnings leading to recruitment during this study were thought to have occurred in early spring of the previous year.

Despite their similarities, two important differences were evi-

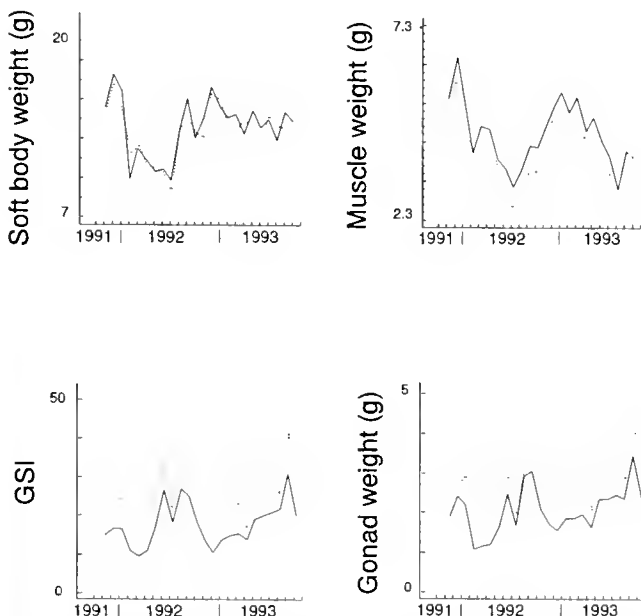

Figure 5. A comparison of monthly mean GSI and regressed tissue weights for male (—) and female (----) *C. (Mimachlamys) asperrima* (shell height, 62.5 mm).

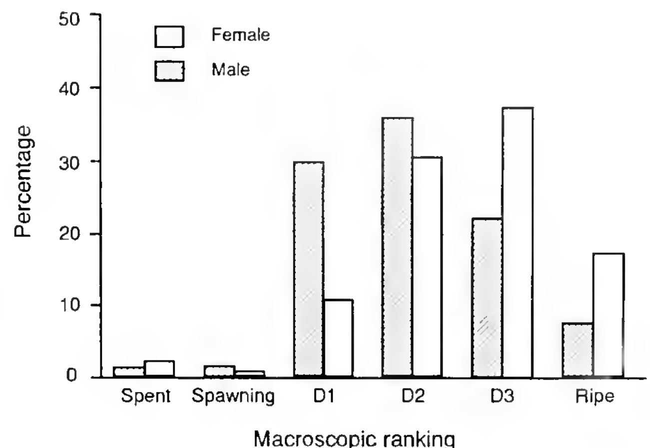

Figure 6. Percent frequency of macroscopic stages for male and female *C. (Mimachlamys) asperrima* collected from February 1992 to October 1993.

der between populations of *C. asperrima* in Jervis Bay and those in D'Entrecasteaux Channel. First, a distinct difference in maximum size was noted. Tasmanian *C. asperrima* have been reported to exceed 100 mm in shell height (Zacharin 1986), whereas those collected from Jervis Bay over the past 3.5 y rarely exceeded 80 mm. Second, Zacharin (1994) observed a "resting phase" from January to March in which gonads were completely spent and the majority of individuals could not be sexed macroscopically. Gonads of *C. asperrima* in Jervis Bay were smaller and macroscopically less developed during this period; however, very few fully spent individuals were found and macroscopically ripe individuals were present in collections from most months. Indeed, *C. asperrima* collected during this time have been induced to spawn (W. O'Connor unpubl. data, R. Krassoi pers. comm.). Similar intraspecific variations in latitudinally differentiated bivalve populations have been reported previously. In general, bivalves from lower latitudes have a smaller maximum size (Newell 1964), as noted here with *C. asperrima*, and their reproductive cycles may become less defined, occasionally exhibiting dual spawning periods or continuous spawning and redevelopment (Hesselman et al. 1989, Barber and Blake 1991, Hoffmann et al. 1992). Among populations of bay scallops, *Argopecten irradians*, the duration of the spawning period increases with decreasing latitude (Sastry 1979), whereas mean gonad index decreases (Sastry 1970). *C. asperrima* are consistent with these observations to the extent that dual peaks in reproductive condition occurred in Jervis Bay in 1992 and the potential for an increase in the period of time over which spawning occurs was observed; however, a comparison with gonad index data for *C. asperrima* from D'Entrecasteaux Channel (Zacharin 1994) does not indicate mean gonad index changes.

Differences in shell height and the availability of spawnable broodstock are of particular interest to aquaculturists and ecotoxicologists. Because *C. asperrima* fecundity generally increases with increasing scallop size (Zacharin 1994, O'Connor pers. obs.), potentially greater numbers of smaller Jervis Bay scallops would be needed to produce large numbers of eggs. However, this should be overcome by the abundance of *C. asperrima* in Jervis Bay and the ease with which they can be induced to spawn (O'Connor and Heasman 1995). More important, the extended availability of spawnable broodstock in Jervis Bay means that embryos are also available for a longer period of time. For hatchery production of the species, this would extend the potential production season without the need for expensive broodstock conditioning. Opportunities to use *C. asperrima* embryos in studies of the effects of toxicants on marine organisms (Krassoi et al. in press) are also greatly enhanced.

Changes in the reproductive condition of scallops have variously been associated with one or more factors including latitude (MacDonald and Thompson 1988), depth (Barber et al. 1988), and genetic predisposition (Cochard and Devauchelle 1993), each of which could explain some of the differences in observations between the Jervis Bay and D'Entrecasteaux Channel scallop populations. In addition, temperature and food availability have been found to be of particular importance (Sastry 1968, Broom and Mason 1978, Shafee 1980) in both interpopulation and seasonal differences in scallop reproductive condition. Increases in Jervis Bay *C. asperrima* reproductive condition were coincident with decreases in water temperature and with annually recurrent phytoplankton blooms off the NSW coast (Hallegraeff and Jeffrey 1993). These blooms can increase algal biomass 10-fold, largely as the result of short-lived diatom blooms (Hallegraeff 1981), potentially providing food for both adults and larvae. Spawning in conjunction with this annual bloom has been reported for several other molluscs on the NSW coast (Hadfield and Anderson 1988). A massive microalgal bloom of the coccolithophorid, *Gephyrocapsa oceanica*, occurred in Jervis Bay from mid-December to mid-January 1993 (Blackburn and Cresswell 1993). The coccolithophorid was readily ingested by both *C. asperrima* and the commercial scallop *Pecten fumatus*, giving the digestive gland a distinctive milky hue. The small rise in GSI seen during this bloom suggested that it was not deleterious to scallops and that reproductive condition changed in response to food availability. The dramatic reduction in soft body weight and the rapid decline in muscle weight in December 1992, relative to 1993 (Fig. 5), may also be indicative of food availability.

Changes in adductor muscle weight have been related to reproductive patterns in several scallop species. Muscle weights were found to decrease as gonad and gonadal indices increased, and *vice versa* (Ansell 1974, Comely 1974, Laurèn 1982). In *C. asperrima*, there was evidence of an inverse relationship between muscle weight and the indices of gonadal development used; however, no significant negative correlations were found. Peaks in muscle weight in December/January in both years corresponded to troughs in the GSI, gonad weight, and the macroscopic index. Conversely, muscle weight was at its lowest shortly before peaks in gonadal indices. In pectinids, the adductor muscle appears to be a primary site of energy storage for later use in gonadogenesis. Storage products, predominantly glycogen and some protein, are accumulated when nutrient availability exceeds net metabolic demand (Barber and Blake 1991). These storage products are then utilised during periods of high demand such as gametogenesis. This process can be site specific and depth dependent (Barber and Blake 1991) and, in the European scallop *Pecten maximus*, is controlled largely by environmental rather than genetic factors (Mackie and Ansell 1993). Although *C. asperrima* GSI in particular showed its greatest variability during winter/spring, presumably as the result of gamete release, the prolonged reduction in muscle weight indicated continuing metabolic demand, consistent with continued gonadogenesis. To this extent, monitoring muscle weight in pectinids is a useful addition to tissue weight indices, particularly where partial or multiple spawnings may occur.

In all weights and indices used, male and female *C. asperrima* varied synchronously and there was no indication of male GSI peaking earlier than that of females, as had been suggested for populations in the D'Entrecasteaux Channel (Zacharin 1994). In most collections, however, female gonads were heavier and muscles were lighter than those of males. Ripe females also had higher gonadal dry matter percentages and higher gross energy levels per kilogram dry weight of gonadal tissue. These weight and energy differences suggest a difference in reproductive effort between the sexes, with a 62.5-mm female having as much as 27% more energy invested in the gonad. This could explain the higher average muscle weight in males, although more frequent or more intense spawning could mean that males expend similar amounts of energy over the reproductive season. In fact, observations made during this and other studies (O'Connor et al. 1994, O'Connor and Heasman 1995) indicate that the difference in energy expended by both sexes may not be great. The scarcity of ripe males collected during this study may indicate more frequent spawning, although laboratory studies have indicated that male *C. asperrima* are more readily induced to spawn using natural cues such as temperature

fluctuations and are capable of partial spawnings. The 9% difference between male and female muscle weights more likely indicates the difference in energy expended in reproduction, rather than a static determination of the relative energy invested in ripe gonads (27%).

Similarities in size frequency data for the sexes, and in the growth of mature scallops in previous studies (W. O'Connor pers. obs.), suggest that there is no great difference in energy expended during the reproductive period. However, in triploid *A. irradians*, where full maturation and spawning were prevented, muscle and soft body weights were 73 and 36% greater, respectively, than those of their diploid siblings, but shell height and length were unaffected (Tabarini 1984). Hence, the effects of differences in reproductive energy expenditure by *C. asperrima* are not likely to be seen in shell growth.

Because *C. asperrima* has been harvested commercially (Young and Martin 1989) and has potential as a candidate for mariculture (Cropp 1989, O'Connor et al. 1994), variation in wet tissue weights has implications for the potential culture or harvest of *C. asperrima*. In markets requiring a whole or "roe on" product (muscle and gonad), scallops would be collected in winter and early spring, when the gonad is larger and more turgid. Conversely, if muscle alone is to be sold, wet weights are greatest in summer. Differences in tissue weights between the sexes are relatively small in comparison to seasonal changes and are not likely to affect "roe on" sales; however, the consistently heavier male muscles would be advantageous for "roe off" markets. If *C. asperrima* were to be cultured, the effects of seasonal changes in reproductive condition on tissue wet weights may be mitigated by the use of triploid induction techniques, which have been found to increase muscle yield in other pectinids (Tabarini 1984).

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MONOAMINES AND PROSTAGLANDIN E_2 AS INDUCERS OF THE SPAWNING OF THE SCALLOP, *ARGOPECTEN PURPURATUS* LAMARCK

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ABSTRACT Intragonadal injections of serotonin (5-HT), dopamine (DA), noradrenaline, and prostaglandin (PG) E_2 (PGE_2) were assayed as inducers of spawning in the hermaphrodite scallop *Argopecten purpuratus*. The three monoamines were effective in inducing the release of sperm but not of oocytes. PGE_2 did not induce the release of either gamete. When a mixture of 5-HT or DA with PGE_2 was injected, gametes of both sexes were released. The injection of DA, followed (within 30 min) by an injection of PGE_2, also induced the release of both gametes. This was not the case for 5-HT. The percentages of gamete fertilization and of larvae survival were much higher for the gametes spawned by an injection of DA combined with PGE_2 than for those gametes spawned as a result of increasing temperature and adding microalgae. These results support the hypothesis that PGs and dopaminergic mechanisms may be implicated in female spawning. This study shows that the injection of DA combined with PGE_2 may be successfully used in hermaphrodite scallops for obtaining viable gametes for fertilization and consequent larval development.

KEY WORDS: Spawning, hermaphrodite scallops, *Argopecten purpuratus*, bivalve reproduction

INTRODUCTION

The spawning of bivalve molluscs is a process controlled by exogenous and endogenous factors (Giese and Kanatani 1987). Among endogenous factors, prostaglandins (PGs) and some amines, produced by nerve cells, are considered to play an important role (Khotimchenko and Deridovich 1991, Deridovich and Reunova 1993). Gonadal dopamine (DA) content has been shown to decrease after the induced spawning of *Patinopecten yessoensis* (Osada et al. 1987). During the spawning of *Chlamys farreri nipponenesis*, Matsutani (1990) detected an increase of the serotonin (5-HT) level in testes and ganglia, at the same time that a decrease in DA content in the ovary was observed. Martínez and Rivera (1994) reported a decrease of DA and 5-HT content in the gonads of *Argopecten purpuratus* during the first hours of a spontaneous spawning.

A great deal of research on this subject in molluscs has referred to the induction of spawning by homogenates of nerve tissue or by monoamines, which are a common secretion product of nerve tissue (Matsutani and Nomura 1982, Matsutani and Nomura 1987, Gibbons et al. 1983, Gibbons and Castagna 1984, Braley 1985, Hirai et al. 1988, Vélez et al. 1990, Ram et al. 1992, Ram et al. 1993, Desrosiers and Dubé 1993).

5-HT has been shown to be one of the most effective spawning inducers, although in the case of gonochoric pectinids, the sensitivity of males to this amine is considerably higher than that of females (Matsutani and Nomura 1982, Matsutani 1990). In the case of hermaphrodite scallops, 5-HT has been effective in inducing the release of sperm but not of oocytes (e.g., *Argopecten irradians*, Gibbons and Castagna 1984, *Pecten ziczac*, Vélez et al. 1990). Among other monoamines assayed, noradrenaline (NA) and adrenaline were capable of inducing spawning only in male *C. farreri nipponensis* (Matsutani 1990).

Matsutani and Nomura (1987) induced egg release from pieces of ovary of *P. yessoensis* and showed that this effect was prevented by the addition of aspirin (inhibitor of PG biosynthesis) and was enhanced by prostaglandin E_2 (PGE_2). The participation of PGs in the release of gametes has been reported by Morse et al. (1977) in experiments where they showed that the ultraviolet (UV) irradiation of seawater or the addition of hydrogen peroxide (activator of PG synthesis) to seawater induced spawning in male and female abalones. It has been reported that the ovarian levels of $PGF_{2\alpha}$ of *Crassostrea gigas* (Ono et al. 1982) and of PGE_2 of *P. yessoensis* (Mori et al. 1984, Osada et al. 1989, Osada and Nomura 1990) increased during the spawning season. It has been suggested that PGs are modulators of the 5-HT action in the induction of spawning in the female *P. yessoensis* (Matsutani and Nomura 1987).

This study describes the successful spawning of *A. purpuratus* using a mixture of DA and PGE_2 as inducers of spawning. It is also shown that the percent gamete fertilization and D-stage larval survival obtained by this method are higher than those obtained by the use of a nonchemical method.

MATERIALS AND METHODS

Experimental Animals

Specimens of *A. purpuratus* were obtained from hanging cultures in La Herradura Bay, Coquimbo, Chile (30°S.) They were maintained in laboratory aquaria with recirculating water and fed with microalgae until they were in condition to be induced for spawning.

Induction of Spawning

Ripe scallops were placed in individual aquaria containing seawater and were injected with 0.4 ml of the testing solution. Half of the solution was injected into the female gonadal portion, and the rest was injected into the male portion. The solutions were prepared by dissolving the amine in filtered seawater (FSW). Control scallops were injected with 0.4 ml of FSW. Three different experiments were done: in the first one, animals were injected with only one compound, in the second experiment, one monoamine and PGE_2 were injected together as a mixture; and in the third

experiment, the amines and the PG were both injected in the same animal, but separately, one 30 min after the first one had been injected. The first and the third experiments were assayed twice each, and the second experiment was assayed three times. Each assay was conducted on a different date, but each time, a set of animals was obtained from the same population and randomly divided into experimental and control groups. The response to the testing solution was recorded as positive when gametes were released. Statistical differences among experimental and control responses were analyzed by the use of the Fisher exact probability test.

Fertilization and Larvae Survival

An experiment was designed to compare percent fertilization and survival of D-stage larvae obtained when spawning was individually induced by a mixture of DA and PGE_2 and when spawning was induced by a method that consisted of adding microalgae and increasing temperature for groups of scallops placed in the same tank (referred to as *usual* method). In both cases, when scallops started to release sperm, they were removed from the aquarium, rinsed with FSW, and placed in another tank containing FSW. Sperm obtained by each of the two methods was mixed separately and maintained for later fertilization. After a few spawning contractions, scallops were individually transferred to a third clean tank, where they remained until oocyte release began. When this occurred, the scallops were rinsed with clean FSW and placed into individual plastic containers with FSW, where they continued to spawn. When spawning seemed to have ended, the scallops were removed from the containers and the number of oocytes in each container was estimated by the counting of replicate 1-ml samples. Fertilization was initiated by the addition of spermatozoa to individual oocyte suspensions. The final ratio of spermatozoa to oocytes was 10:1.

Percent Fertilization

Percent fertilization was calculated as the ratio of the number of dividing oocytes (embryonic state) and the total number of oocytes (fertilized and unfertilized), 2 h after the addition of spermatozoa to oocytes. Samples of 1 ml of suspension from each culture were taken, fixed with I_2-iodine solution, and kept for later counting of dividing embryos and intact oocytes.

D-Stage Larval Survival

The rest of the suspension cultures was combined according to the spawning method and placed in four 50-l tanks containing FSW at final densities of 30 eggs/ml. They were left undisturbed until they developed to D-stage larvae (about 48 h later). The larvae were then carefully filtered, rinsed, and resuspended in FSW. Five 1-ml samples were taken from each suspension culture, fixed with I_2-iodine solution, and kept for later counting of D-stage larvae.

This experiment was replicated in two different periods of the year, once in December (summer time) and again in August (winter time). Results were analyzed and are presented separately. After arc-sin transformations, percent fertilization and D-stage larval survival were compared by a simple analysis of variance.

RESULTS

Induction to Spawning

Assay of Compounds Alone

5-HT, injected at doses of $2 \cdot 10^{-5}$ and $2 \cdot 10^{-3}$ M, induced the release of sperm in 100% of the scallops, but only one animal from each treatment released oocytes (Table 1). At the lowest dose, none of the animals injected with DA released either sperm or oocytes. At the highest dose, 100% of the scallops released sperm, but only one scallop from the first assay released oocytes.

Injections of NA at a dose of $2 \cdot 10^{-5}$ M, except for one individual that released sperm, did not induce the release of gametes of either sex. When NA was injected at a higher dose ($2 \cdot 10^{-3}$ M), 90% of the scallops released sperm and one scallop from each assay released oocytes.

PGE_2, in all doses tested, did not induce the release of gametes in either sex.

Control animals injected with FSW alone did not induce the release of gametes in either sex.

Assays of Monoamines Combined With PGE_2

When a mixture of 5-HT ($2 \cdot 10^{-3}$ M) and PGE_2 ($2 \cdot 10^{-6}$ M) was injected, 100% of the animals responded by ejecting sperm and 41% of them responded by releasing oocytes (Table 2). DA ($2 \cdot 10^{-3}$ M) combined with PGE_2 ($2 \cdot 10^{-6}$ M) induced the release of sperm in 100% of the scallops injected and the release of oocytes in 41% of them. The mixture of NA ($2 \cdot 10^{-3}$M) and PGE_2 ($2 \cdot 10^{-6}$ M) induced the release of sperm in 82% of animals injected and the release of oocytes in 18% of them. The total number of animals (assays 1, 2, and 3) that released oocytes after injections of 5-HT and PGE_2 and injections of DA and PGE_2 was statistically significant ($p < 0.05$, two-tailed Fisher exact probability test).

Assays of Monoamines and PGE_2 Injected Separately

When DA or 5-HT was injected before or 30 min after an injection of PGE_2, 100% of the scallops released sperm (Table 3). Only two animals (out of 10) released oocytes when DA was injected after PGE_2, and 40% of them ($p < 0.05$, one-tailed exact probability test) did so when the order of injection was reversed

TABLE 1.

Induction of gamete release by monoamine or PGE_2 injection in the scallop, *A. purpuratus*.

Testing Solution and Concentration (M)		First Assay		Second Assay	
		Oocytes	Sperm	Oocytes	Sperm
5-HT	2×10^{-5}	0/5	5/5	1/5	5/5
5-HT	2×10^{-3}	1/5	5/5	0/5	5/5
DA	2×10^{-5}	0/5	0/5	0/5	0/5
DA	2×10^{-3}	1/5	5/5	0/5	5/5
NA	2×10^{-5}	0/5	0/5	0/0	1/5
NA	2×10^{-3}	1/5	4/5	1/5	5/5
PGE_2	2×10^{-8}	0/5	0/5	0/5	0/5
PGE_2	2×10^{-6}	0/5	0/5	0/5	0/5
FSW		0/5	0/5	0/5	0/5

Results are expressed as number of animals that released gametes/number of animals tested.

TABLE 2.

Induction of gamete release by an injection of a monoamine combined with PGE_2 in the scallop, *A. purpuratus*.

Mixtures*	Assay 1		Assay 2		Assay 3	
	Oocytes	Sperm	Oocytes	Sperm	Oocytes	Sperm
PGE_2 + 5-HT	3/5	5/5	0/7	7/7	4/5	5/5
PGE_2 + DA	2/5	5/5	2/7	7/7	3/5	5/5
PGE_2 + NA	1/5	2/5	2/7	7/7	0/5	5/5
FSW	0/5	0/5	0/7	0/7	0/5	1/5

Results are expressed as number of animals that released gametes/number of animals tested.
* PGE_2 dose, 2×10^{-6} M; 5-HT, DA, or NA doses, 2×10^{-3} M

(first DA and then PGE_2). In none of the three assays did FSW injections (control) alone induce spawning.

Fertilization and Larvae Survival

A higher proportion of oocytes was fertilized when gametes were obtained by the injection of 0.4 ml of $2 \cdot 10^{-3}$ M DA mixed with $2 \cdot 10^{-6}$ M PGE_2, than when they were obtained by adding microalgae and increasing temperature (Table 4). Less than 50% of D-stage larvae obtained from the fertilization of gametes released by adding microalgae and increasing temperature survived, whereas more than 67% of those obtained by the injection of DA and PGE_2 survived (Table 4). Similar results were obtained for experiments conducted during both summer and winter periods.

DISCUSSION

Results obtained with injections of 5-HT on the spawning of *A. purpuratus* were similar to those obtained by Gibbons and Castagna (1984) and Vélez et al. (1990) for the hermaphrodite species *A. irradians* and *P. ziczac*. In all of these cases, it was shown that injections of 5-HT were effective at inducing the release of sperm but not oocytes. In the case of gonochoric bivalves, most of the studies have shown that the sensitivity of male individuals to 5-HT induction is higher than that of females. This higher sensitivity may be expressed either as a lower dose of the amine necessary to induce the release of gametes (Matsutani and Nomura 1982, Matsutani, 1990) or as a higher percentage of animals that spawn following 5-HT injection (Gibbons et al. 1983, Gibbons and Castagna 1984, Braley 1985, Belda and Del Norte 1988).

Except for a few successful assays on inducing the release of sperm (Hirai et al. 1988, Matsutani 1990), injections of DA or NA have not been demonstrated to be good inducers of spawning. Matsutani and Nomura (1986) have suggested that UV-irradiated seawater and rising temperature induce female spawning by stimulating serotonergic mechanisms via dopaminergic mechanisms. When DA or NA was injected as a single compound (these results), only sperm was released by the scallops. However, the injection of a mixture of any of these amines or 5-HT with PGE_2 did induce oocyte release in *A. purpuratus*. Whenever DA was injected with PGE_2, either in combination or before it, the release of oocytes was shown. This result was not obtained for 5-HT.

These findings are consistent with a suggested (Osada et al. 1989, Matsutani 1990) difference in spawning mechanism between female and male scallops. We have studied (Martinez et al. 1996) changes in the levels of DA, NA, and 5-HT in separate ganglia of *A. purpuratus* associated with non-drug-induced spawning. In those individuals that spawned, DA and NA decreased in the visceral ganglion (innervating mainly the female gonadal portion) and did not change in the cerebropedal ganglion (innervating mainly the male gonadal portion). On the other hand, spawning was associated with a decrease in 5-HT in the cerebropedal ganglion and no change in the visceral one.

Matsutani and Nomura (1987) have suggested that PGs may be modulators of 5-HT action in the female Japanese scallop *P. yessoensis*. They showed that when aspirin was present, 5-HT-induced egg release was inhibited, whereas it was enhanced by PGE_2. Morse et al. (1977) have reported the spawning of *Haliotis rufescens* induced by the addition of PGE_2 $PGF_{2\alpha}$ or by hydrogen

TABLE 3.

Induction of gamete release by monoamine and PGE_2 injected separately in the scallop, *A. purpuratus*.

Testing Solution*	First Experiment		Second Experiment	
	Oocytes	Sperm	Oocytes	Sperm
$PGE_2 \rightarrow$ 5-HT	0/5	5/5	0/5	5/5
$PGE_2 \rightarrow$ DA	0/5	5/5	2/5	5/5
5-HT $\rightarrow PGE_2$	0/5	5/5	0/5	5/5
DA $\rightarrow PGE_2$	3/5	5/5	1/5	5/5
FSW $\rightarrow$ FSW	0/5	0/5	0/5	0/5

Results are expressed as number of animals that released gametes/number of animals tested.
* PGE_2 dose, 2×10^{-6} M; 5-HT, DA, or NA doses, 2×10^{-3} M. Arrows indicate that the second compound was injected 30 min after the first one.

TABLE 4.

Percentage of gamete fertilization and of D-stage larval survival of *A. purpuratus* obtained by two different methods of induction of spawning.

	Usual Induction of Spawning (%)*	*Chemical* Induction of Spawning (%)†
Gamete fertilization		
Summer experiment	52.8 ± 6.4	88.4 ± 2.2**
Winter experiment	62.8 ± 2.9	84.0 ± 0.1**
Larvae D survival		
Summer experiment	30.3 ± 8.0	67.5 ± 10.7**
Winter experiment	29.2 ± 6.7	70.8 ± 5.9**

Results of two experiments are presented as the mean ± SD (n = 5 for summer experiment and n = 3 for winter experiment).
* Addition of microalgae and increasing of temperature (*usual*).
** Significantly different ($p < 0.01$).
† Injection of a mixture of PGE_2 and DA (*chemical*).

peroxide (activator of PG synthesis) to seawater. Matsutani and Nomura (1986) did not find any effect of $PGF_{2\alpha}$ on the induction of spawning in *P. yessoensis*. Osada et al. (1989) showed that the levels of PGE_2 and $PGF_{2\alpha}$ were about four times higher in the ovary than in the testis of *P. yessoensis* and that during spawning, these values increased in the male gonad whereas they decreased in the female one.

Ram et al. (1992) have proposed a model for the regulation of spawning in bivalves. They propose that males and females detect environmental cues by specific chemical sensing receptors and that signals are conducted by the nervous system to the gonad, where they may directly activate it or may induce the release of another hormone that activates the release of gametes. They propose that 5-HT might be the intermediate substance between the nervous system and gonads, but its action is modulated or mediated by PGs.

The use of DA combined with PG may be a very good method to induce spawning, at least for hermaphrodite scallops, because of the high percent fertilization and survival of the resulting larvae, both of which were much higher than results obtained when we used the method of increasing temperature or adding microalgae. As far as we know, this is the first comparative analysis of spawning induction methods using embryos and larvae as the focus rather than the actual yield of spawning. Belda and Del Norte (1988) showed nearly 100% fertilization of oocytes stripped from the ovaries of the scallop *Amusium pleuronectes* when using sperm induced by 5-HT, but the development of fertilized eggs into larvae on the first day was only 0.73%. About 10% survival of veligers from fertilized eggs of the china clam, *Hippopus porcellanus*, was obtained when specimens were induced to spawn by the injection of 5-HT (Alcázar et al. 1987). We think that when spawning is induced by the mixture of DA and PGE_2, most of the gametes that are released are those in the best state of ripeness, and this would not be the case with other induction methods. In this late case, the release of gametes might be the result of a mechanical stimulation (muscular contraction). We have observed that with this mixture (DA and PGE_2) the gonad does not look quite as empty after the release of oocytes as when spawning is induced by other factors. Ram et al. (1993) have reported that in the zebra mussel, *Dreissena polymorpha*, the likehood of female spawning in response to 5-HT was not tightly coupled to the morphological maturity of the gonad, and they conclude that another maturational process in the gonad must be necessary for oocyte release. These results suggest that the release of oocytes in the hermaphrodite scallop *A. purpuratus* is controlled by dopaminergic pathways under the regulation of PGs.

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VELIGERS FROM TWO POPULATIONS OF SCALLOP *PLACOPECTEN MAGELLANICUS* EXHIBIT DIFFERENT VERTICAL DISTRIBUTIONS IN THE SAME MESOCOSM*

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ABSTRACT Veligers of the giant scallop, *Placopecten magellanicus*, spawned from parents that came from two different populations (Georges Bank and Passamaquoddy Bay), were maintained together in large mesocosms 0.6 m in diameter and 9.0 m deep. A new microsatellite probe (PMMS-130) developed by Gjetvaj et al. (unpublished observation) was used to distinguish the population of origin of the veligers. Samples obtained at various depths confirmed that the vertical distribution of the two populations differed. These results confirm population differences in vertical migration behavior seen in a previous study and indicate the value of the microsatellite probe for larvae as well as the efficacy of mesocosm replication techniques.

KEY WORDS: Scallop, larvae, genetic, DNA, migration

INTRODUCTION

Several field studies have found diel changes in the vertical distribution of bivalve veligers (Maru et al. 1972, Harding et al. 1986, Scrope-Howe and Jones 1986, Tremblay and Sinclair 1990, Raby et al. 1994). Mesocosm studies isolate the effect of the behavior of experimental organisms from that of abiotic transport and have demonstrated that veligers of both the great scallop (*Pecten maximus*) and the giant scallop (*Placopecten magellanicus*) do make distinct vertical migrations (Kaartvedt et al. 1987, Silva-Serra and O'Dor 1994, Gallager et al. 1996, Manuel 1996). Although much useful knowledge has been gained through controlled mesocosm experiments, such studies have traditionally been hampered by a reluctance to remove too many animals (which leads to low numbers and a loss of statistical robustness), a lack of replication and control in experimental manipulation, and contamination by other species (Balch et al. 1978).

Recently, Manuel (1996) solved several of these problems by using noninvasive sampling (video profiling) and replicated mesocosms to describe interpopulation differences in the vertical distributions of *P. magellanicus* veligers. The differences in vertical distribution were due to differences in veliger migration behavior and in veliger responses to discontinuities such as the surface and a thermocline placed middepth in the mesocosms. Although the replication of mesocosms allows statistical testing, the number of mesocosms (and thus the power of statistical tests) was constrained by logistics. Variability among mesocosms, as reflected by mean depths (Fig. 1), populations of volunteer organisms, and veliger growth rates, was high (Manuel 1996). This was because replicated mesocosms are not simply replicated physical media, but rather are replicated ecosystems that may differ considerably by the end of a long experiment. Thus, some doubt remains as to whether the differences in vertical distribution observed among populations are the result of different responses to the same stimuli or different responses to different stimuli provided by a different community of organisms. Even if those community differences were caused in some way by the different populations (which would lead to the same statistical result), the results of Manuel (1996) would be biologically insignificant. Concentrations of *P. magellanicus* veligers are unlikely to be high enough, in an open natural system, to make substantial ecosystem changes.

Even with a light microscope, many bivalve species are indistinguishable as veligers (Hurley et al. 1987, Demers et al. 1993). This problem has been overcome by recent advances in scallop molecular biology. Gjetvaj et al. (in prep.) have developed a number of microsatellite probes that can be used (with polymerase chain reaction amplification) for both adult scallops and individual veligers. Microsatellites are permutations of simple repeated sequences of DNA up to a few hundred base pairs in length dispersed throughout the genome. Microsatellite alleles vary in length (i.e., in the number of bases) and exhibit high levels of polymorphism, which make them ideal for use as genetic markers. This experiment is the first to use microsatellite loci to identify the parents of individual scallop veligers. We used the technique to identify the population of origin of scallop veligers at different depths within the same mesocosm. We were able to demonstrate that veligers of *P. magellanicus*, spawned from adults from Passamaquoddy Bay, are found shallower in the same mesocosm than those spawned from adults from Georges Bank. This experiment addresses the potential problems with small numbers of replicates in variable mesocosms, tests the practical potential of the new genetic probe developed by Gjetvaj et al. (in prep.), and confirms the results of a previous experiment (Manuel 1996) with different parents and conditioning methods.

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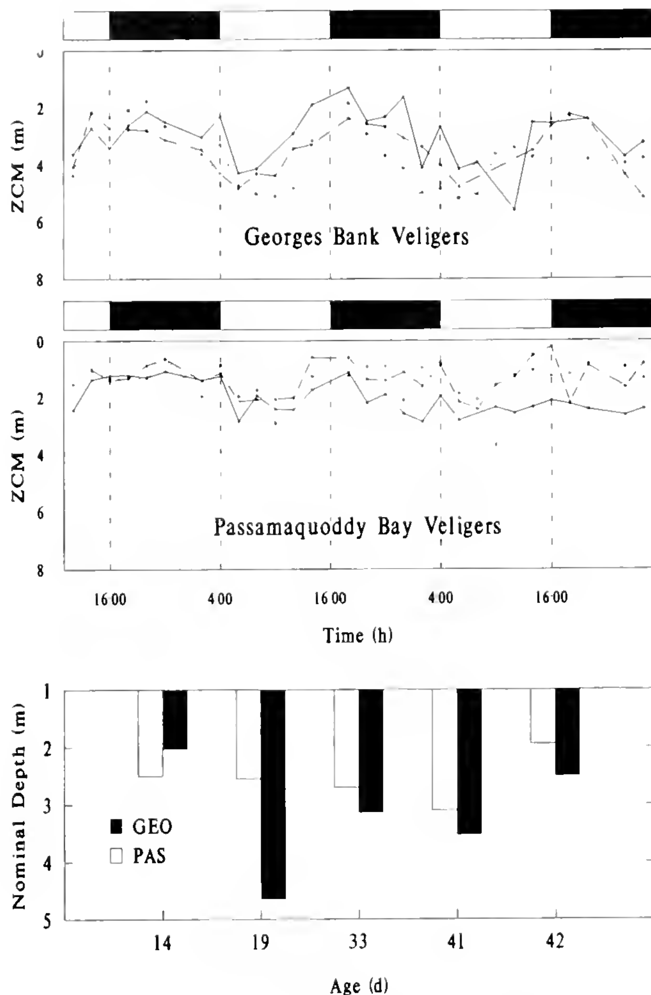

Figure 1. Top two panels after Manuel (1996): ZCM, recorded at 2-h intervals at the age of 28–31 d. Each line plots the ZCM of a single mesocosm over 2.5 d. Clear boxes indicate when lights were on, and black boxes indicate darkness. Bottom panel: comparison of the nominal depth (average of two replicates except at age 14 d) of GEO and PAS veligers in this experiment.

METHODS

Conditioning and Spawning of Adults

Forty-four adult *P. magellanicus* from the Georges Bank (GEO) and Passamaquoddy Bay (PAS) populations were held at a Mahone Bay aquaculture site for 6 months. These were brought to the Aquatron facility at Dalhousie University and stimulated to spawn in September 1993. Scallops were genotyped (see Microsatellite Techniques below) with a microsatellite probe (PMMS-130) developed by Gjetvaj et al. (unpub. obs.). Because some alleles were found in both populations, parents for the experiment were chosen such that each allele appeared in only one or the other population. Unfortunately, misreading of the score of one individual resulted in one allele (154) being represented in both populations (Table 1). However, because each veliger was represented by two alleles, this did not cause any serious difficulties, and we were able to identify the population of origin of individual veligers by determining the genotype of each veliger. We crossed only within the same population for this initial experiment.

After the gonads of the scallops had been emptied, the selected parents were reconditioned by supplying them with ad libitum *Isochrisis galbana* (clone TISO) from October 1, 1993, until spawning on February 27, 1994. Ripe animals were cleaned with a scrubbing brush in clean filtered seawater and induced to spawn in clean, 0.2-μm-pore-size filtered seawater by thermal stimulation and agitation of the water with a submersible pump. Both populations were spawned concurrently, but in separate rooms, with a different person handling each population. Hot fresh water was used frequently to rinse hands and minimize cross-contamination. The eggs from all females in a given population were mixed together and fertilized with the mixed spawn of all of the males from that population. Sperm was added until several sperm could be seen around each egg when a sample was examined under a light microscope. Fertilized embryos were introduced to the surface of 9-m-deep mesocosms filled with 1-μm-pore-size filtered seawater as soon as fertilization success had been assured (i.e., when most of the embryos were in cleavage or blastula stage). Each polyethylene mesocosm in the 10-m tower tank was tied at the bottom with a watertight knot, suspended from the surface with a styrofoam collar, and filled slightly above the level of water in the tower tank to create positive pressure. Each population was held, unfed, in separate mesocosms until 4 d of age (D-stage). The process of filling the experimental mesocosms modified the preexisting temperature regime somewhat, resulting in temperatures above the thermocline between 14 and 16°C and below the thermocline between 6 and 10°C over the 4 d of development from egg to D-stage veligers (Fig. 2A).

Treatment of Veligers

At 4 d, veligers were concentrated on a 53-μm nitex screen, counted, and distributed to two experimental mesocosms. Our objective was to stock each mesocosm with 5.0×10^5 veligers

TABLE 1.

Microsatellite scores of parental alleles from the GEO scallops and number of progeny produced from each of the crosses. One male showed three instead of two bands. The 126 and 162 bands in male G22M segregated together.

Georges Bank Score	Female G11F 144	Female G11F 136	Female G3F 146	Female G3F 140	Total By allele	Total By scallop
Male G22M						
162/126	53	58	37	25	173	361
146	73	56	35	24	188	
Male G37M						
154	24	24	19	15	82	154
150	26	19	14	13	72	
Total (by allele)	176	157	105	77	total (Georges Bank)	
Total (by scallop)	333		182		515	

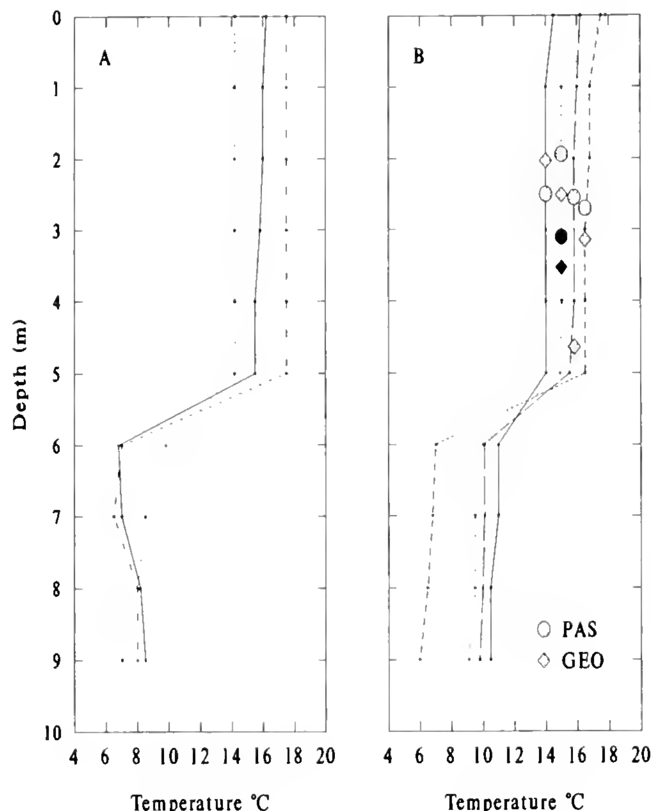

Figure 2. Temperature in the tower tank. (A) Temperature during development to D-stage. Solid line is day 2, broken line is day 3, and dotted line is day 4. (B) Temperature in the tower tank on sampling days. Solid line is day 14, large broken line is day 19, small broken line is day 33, and dotted line is day 41. The nominal depth of veligers from each sample day is represented by circles (PAS) and diamonds (GEO). Shades symbols are day samples; black symbols are from the night sample.

from each population. However, as with a previous experiment (Manuel 1996), we found that GEO veligers developed slightly more slowly than PAS veligers. We did not want to leave PAS veligers unfed for an extra day while we waited for all GEO veligers to get to D-stage and were unsure about the viability of GEO larvae not quite in D-stage after they had been captured on a screen. We decided to redistribute veligers as planned on the fourth day and to exclude individuals not completely in D-stage from sample counts when stocking the mesocosms. Gentle handling apparently allowed most of the trochophores to survive the process, resulting in more GEO than PAS veligers in each mesocosm (about a 60/40 split). Mesocosms were also established that contained veligers from only one of the populations (four mesocosms each for GEO and PAS) to monitor growth rates of the two populations.

Treatment mesocosms were filled with 0.1-μm-pore-size filtered seawater and inoculated with enough cultured TISO to bring the concentration to 1.0×10^4 cells · ml^{-1}. A gravity-fed perforated vinyl sprinkler hose was used to evenly distribute food from the top to the bottom of the mesocosm. At 24 d of age, veligers from both mesocosms were concentrated on a 80-μm nitex screen, sampled, mixed thoroughly, and evenly redistributed in two clean mesocosms. The particle level in the mesocosms was monitored throughout the experiment with a Multisizer Coulter Counter®, and supplemental TISO was added whenever mean concentrations were near or below 5.0×10^3 cells · ml^{-1}. Supplemental TISO was added on nine occasions (days 6, 12, 14, 17, 19, 25, 33, 35, and 39), resulting in particle levels that varied between 4.0×10^3 and 1.40×10^4 cells · ml^{-1} through the experiment.

Stratification

Heating and cooling coils in the tower tank were set at 6-m depth, and we attempted to maintain a 5°C thermocline over 0.5 m. The choice of thermocline strength was relatively arbitrary: because we had a strong thermocline (10°C over 0.5 m) in our first experiment (Gallager et al. 1996, Pearce et al. 1996) and a very weak thermocline (1.5°C over 0.5 m) in our second experiment (Manuel 1996), this experiment was meant to be midway between the two.

Samples for Genotyping of Veligers

Samples of veligers were taken from the surface by dipping with a beaker and at depth by siphoning with a garden hose. The siphon was started in filtered seawater, paused, and then lowered sequentially to the appropriate depths for samples. At each depth, a volume slightly greater than the contents of the siphon hose was drawn before the sample was taken, to prevent contamination by veligers from other depths. Tests of the procedure at the beginning of the experiment using water with and without veligers showed that veligers were flushed from the hose in the parcel of water in which they entered and that there was no apparent mixing in the siphon, even if the siphon was paused for several minutes. Samples were collected from each mesocosm at the surface (0 m), the middle of the layer above the thermocline (2.5 m), just above the thermocline (5 m), and from below the thermocline (8 m). We removed water until we had 30 individuals for each sample, except that if we had not collected enough veligers after 20 l had been removed for any given sample, we stopped (concentrations of veligers were often very low below the thermocline). Water removed in sampling was replaced with fresh filtered seawater. The technique used to collect veligers from the mesocosms for genotyping was much less sensitive for resolving the depth of veligers than the video sampling, so we chose to sample several times and to combine the results, to avoid the chance of accidentally choosing the one time when the two populations did not differ substantially. Samples for genotyping were collected from each mesocosm on six occasions: four times midday (ages 14, 19, 33, and 42 d), once at night (age 41 d), and once when the mesocosms were changed midexperiment (age 24 d). Only the 24-d sample was not depth stratified. That sample was collected after the veligers had been concentrated on a screen to confirm the relative proportions of veligers from each population in the mesocosms (we considered the possibility that the ratio was affected by the differences in distributions). The data from one sample (mesocosm #2 at age 14 d) were discarded because of an obvious recording error. We had data for veligers below the thermocline when records show that none were collected at that depth on that day, and two few veligers were recorded from the first two depths.

Samples thus collected were taken immediately back to the laboratory and narcotized by the addition of an equal volume of 1 M Tris-EDTA to the sample. Thirty individual veligers were extracted with a micropipette in 5 μl of the solution and frozen in separate microcentrifuge tubes at −60°C.

Microsatellite Techniques

DNA was collected with a syringe to extract approximately 1 ml of haemolymph from the adductor muscle sinus of each pro-

sp... ave parent. The haemolymph was then added to an equal ...ume of 95% ethanol. A 200-μl aliquot of each ethanol-preserved blood sample was centrifuged for 2 min at 2,000 rpm. The pellet was resuspended in a TE buffer (10 mM Tris [pH 7.5], 1 mM EDTA) and repelleted by again centrifuging for 2 min at 2,000 rpm. The pellet was then suspended in a high-salt lysis buffer (400 mM NaCl, 10 mM Tris [pH 8.2], 10 mM Na EDTA). Triton 100 (0.75%) and 500 $\mu g \cdot ml^{-1}$ proteinase K were added to the lysate and incubated overnight at 55°C (see Patwary et al. 1994). The protein present in the lysate was precipitated with 400 μl of saturated NaCl solution and vortexed vigorously for 15 min. The tubes were then centrifuged for 30 min at 800 rpm to pellet the precipitated proteinaceous debris. The nucleic acids present in the supernatant were further purified with an equal volume of chloroform. The DNA was then precipitated from the aqueous layer with equal volumes of cold isopropanol. The sample was finally pelleted at 14,000 rpm for 30 min at 4°C, dried, and resuspended in the TE buffer.

DNA was extracted from the veligers by digesting the entire animal in 50 μl of a solution containing 20 $mg \cdot ml^{-1}$ proteinase K, 0.5% Tween 20, 50 mM KCl, 10 mM Tris-HCl (pH 8.5), for 2.5 h at 55°C. The solution was then incubated at 95°C for 5 min and stored at 4°C until used. Polymerase chain reaction (PCR) amplification of the scallop microsatellite locus PMMS-130 was performed in a 20-μl reaction mix containing 1–2 μl of template DNA of unknown concentration, 50 mM KCl, 10 mM Tris-HCl (pH 8.5), 1 mM $MgCl_2$, 0.5 μM each of primer PMMS-130F and PMMS-130R, approximately 1 U of Taq polymerase and 0.2 mM each of ATP, GTP, CTP, and TTP. The reaction mixture was subjected to 25 cycles of 20 s at 94°C, 20 s at 49°C, and 20 s at 72°C to allow the PCR to proceed.

The DNA from 2.5 μl of each PCR amplification mix was size separated on an 8% acrylamide denaturing, sequencing gel according to the directions provided in the Pharmacia T7 sequencing kit. Each gel contained as a size standard several sequencing reactions of the M13 single-strand DNA template provided in the Pharmacia sequencing kit. The sizes of the microsatellite alleles were determined by comparing the migration distances of the PCR products with the migration distances of the M13 standards. Scores indicating uneven numbers of base pairs were taken as the next higher even number. Two independent readers viewed each gel, and where results differed, the gels were reexamined. If a consensus could not be reached, the amplified product was size separated again. Because all alleles except 154 were represented in only one population each, errors in reading, chemistry, two veligers in one sample, or unintentional crosses appeared as impossible combinations from the parents, and those veligers could not be assigned to either population. If this occurred, the datum was rejected.

Statistical Analysis

Differences in distribution were determined by χ^2 analysis with a significance level of $p = 0.05$ and Bonferroni testing where multiple tests were used (Sokal and Rohlf 1981). Because mean depth (ZCM) is affected by several factors other than simply the time of day (Manuel 1996), raw data from different days and times could not be simply combined to test the overall results. The significance of the experiment as a whole was determined by Sokal and Rohlf's (1981) method for combining probabilities from independent tests of significance (pp. 779–782). For the purpose of determining whether GEO veligers were deeper than PAS veligers in the mesocosms, we calculated the nominal depth (d_n) for each population:

$$d_n = \sum \frac{n_i \cdot d_i}{n_p}$$

where n_i is the number if individuals in that population at depth d_i, and n_p is the total number of veligers from that population from all depths on that date. Because we sampled an equal number of veligers, rather than an equal volume of water at each depth, the nominal depth describes the depth of the veligers relative to the other population, rather than the mean depth of the population. In other words, if one population is deeper than the other, its nominal depth calculated in this way will be deeper, but the nominal depth is not the equivalent of mean depth (ZCM) in earlier reports.

RESULTS

Instabilities in the temperature control system made it necessary to manipulate the thermal controls to maintain the desired degree of stratification (5°C). On sampling days, the degree of stratification ranged from 3.7 to 10.3°C, and neither the nominal depth nor the differences between the populations were correlated with the degree of stratification (Fig. 2B). Although we were unable to identify the population of veligers for the purposes of size measurements, veligers raised from the same spawning of these two populations in separate mesocosms (four mesocosms for each population) did not show any significant differences in growth rates between the two populations (Fig. 3). All possible

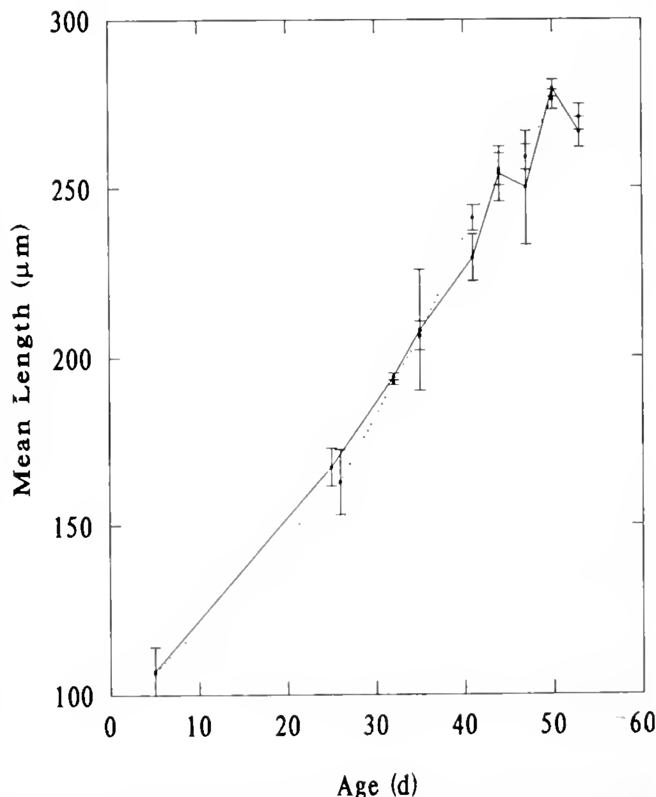

Figure 3. Mean size of veligers in mesocosms containing only PAS (solid line) or GEO (dotted line) veligers. Error bars are SD, n = 4 for each population. Veligers have the same parents and were raised in the tower tank at the same time as mesocosms containing veligers from both populations.

TABLE 2.

Microsatellite scores of parental alleles from the PAS scallops and number of progeny produced from each of the crosses. The 134 × 152 cross (*) could have been produced by three different crosses, and the 152 × 152 cross (**) could have been produced by two crosses, so the total number for each of these is listed only once.

Passamaquoddy Bay Score	Female P4F		Female P10F		Female P1F		Female P8F	
	168	134	138	152	152	142	154	158
Male P18M								
134	11	20	25	87*	*	17	14	12
152	12	*	28	58**	**	29	3	11
Total				327				

genotypes from the parents spawned were sampled during the experiment (Tables 1 and 2). In 43 cases (4.9%), veligers could not be assigned to either population and were rejected. In 57 cases, no visible bands appeared (possibly indicating that only a shell, and not a live veliger, had been sampled). The proportions of progeny sampled from each female parent were consistent with the initial number of eggs spawned, and a comparison of the number of progeny inheriting each of the two alleles from each parent showed no selective mortality at this locus.

A χ^2 analysis of the replicates by depth and age was performed. The majority of the analyses showed little difference between the replicates with the following exceptions: at age 19 d, depths 5 ($p = 0.008$) and 8 ($p = 0.037$) m, which were not significant when Bonferroni testing is applied. The number of veligers is tabulated by population and depth in Table 3. In all but the first replicate at 14 d and the second replicate on day 42 (seven of nine cases), the nominal depth was greater for GEO than for PAS veligers, and when the two replicates are averaged together, the nominal depth was greater on four of five sampling days, which was consistent with the results of a previous experiment (Fig. 1). A χ^2 analysis of the depth distribution of populations by replicate and age was performed. In six of nine cases (all except age 33 d and replicate #2 at age 41 d), the two populations differed significantly or were marginal ($p < 0.10$) (Table 4). If we consider all nine tests together, there are extremely significant ($p < 0.001$) differences in the depth distribution of these two populations of scallop veligers.

TABLE 3.

Number of veligers at each depth and nominal depth for each mesocosm and sampling day.

Age/Depth (m)	Replicate #1 GEO	Replicate #1 PAS	Replicate #2 GEO	Replicate #2 PAS
14 d				
0	20	8		
2.5	11	15		
5	12	8		
8	0	0		
Nominal depth	2.03	2.50		
19 d				
0	6	123	4	16
2.5	11	8	13	14
5	20	3	10	10
8	6	5	20	3
Nominal depth	4.08	2.59	5.16	2.53
24 d	12	7	32	18
33 d				
0	19	11	18	10
2.5	19	10	17	8
5	18	10	20	7
8	4	2	10	2
Nominal depth	2.83	2.76	3.42	2.63
41 d (night)				
0	16	13	15	10
2.5	14	13	12	4
5	24	5	20	7
8	13	9	3	3
Nominal depth	3.87	3.24	3.08	2.88
42 d				
0	12	12	22	7
2.5	7	9	7	19
5	13	2	6	5
8	6	0	1	0
Nominal depth	3.43	1.41	1.54	2.34

DISCUSSION

Although this experiment provided less detail about the vertical distribution of the veligers, it was less time consuming than retrieval of data from the video tapes, and it demonstrated that three mesocosms is a reasonable level of replication for conducting experiments in the tower tank. In particular, it shows that differences in the vertical distribution of veligers are due to differences in veliger responses to the same stimuli, and not to small differences in food, light, water chemistry, etc., or the chaotic amplification of the starting populations in replicated mesocosms. This mesocosm replication technique provides significantly greater flexibility in experimental design for tower tank experiments.

The microsatellite probe developed by Gjetvaj et al. (unpub. obs.) has proved useful in linking genetics and behavior and has the potential to be useful in developing breeding protocols for *P. magellanicus* in hatchery situations. The great genetic variability in microsatellite probes makes it easy to choose parents with dif-

TABLE 4.

Probability (p for χ^2 test) that the vertical distribution of veligers from different populations is the same.

Age (d)	Replicate #1	Replicate #2
14	0.094	—
19	0.004	0.000
33	0.998	0.634
41	0.067	0.489
42	0.010	0.003

ferent genotypes for pedigree analysis. These techniques could be used to determine whether different genotypes or broodstocks have different mortality over time or to allow testing of a number of broodstocks against different rearing techniques at the same time. In our experiments, we have invariably found that growth within a mesocosm was much less variable than growth rates among mesocosms. In hatcheries, where variability among containers is difficult to control, having several lines maintained in the same container would make comparison of lines very much easier and statistically more robust.

In this experiment, the nominal depth of GEO veligers was greater than that of PAS veligers in six of nine samples taken. This agrees with the results of a previous experiment (Manuel 1996) where veligers from the PAS population did not migrate as deeply as did veligers from the GEO population. Interpretation of the significance of such differences requires that we distinguish between genetic and environmental factors and between population and individual variation. In earlier experiments (Manuel 1996), it is conceivable that individual variation in either the genetics or the condition of the parents could have produced differences in the vertical distribution of veligers. The fact that GEO veligers were again found deeper than PAS veligers in this experiment increases the probability that the differences in vertical distribution were the result of genetics at the population level in two ways: the results were repeated in different years (so there is less possibility that something like the microposition of broodstock at the aquaculture site, resulting in perhaps different lipid levels in individual eggs, caused the differences), and we obtained the results from different adults from the same populations. In addition to the above, we used adults conditioned in a different manner (artificial vs. natural) and at a different time of year. It is therefore highly probable that there are real differences in vertical migration behavior between these two populations that are genetically based, and that the differences rest at the population as well as at the individual level.

Manuel (1996) has suggested that scallop veligers are able to gain horizontal transport by migrating in the region of the thermocline. Thermoclines separate large bodies of water that often move in different directions and at different speeds. Even if horizontal transport is not the ultimate cause, it must be the result of vertical migration through such boundaries (Hill 1991). Similarly, in two regions where hydrography differs, the same vertical migration will have different consequences in terms of horizontal movement. If horizontal transport affects the survival of scallop veligers, then it follows that there will be selection for different vertical migration behavior in areas with different hydrography. Passamaquoddy Bay is an estuary with strong tidal influences, whereas Georges Bank is an offshore bank with a large clockwise gyre. Genetic differences in vertical migration behavior are consistent with horizontal transport influencing the vertical migration of scallop veligers.

We also confirmed another phenomenon: GEO larvae are slower than PAS larvae to develop to the veliger stage. Although quantifying the difference was beyond the scope of this experiment, difference in development rate at the same temperature represents another difference in larval ecology among populations. There may be a reason for the shorter nonfeeding trochopore stage of PAS veligers. The bay (PAS) population may be at greater risk of washout than the offshore (GEO) population. If veligers are able to affect horizontal transport by migrating vertically, but trochophores are not, then reducing the time spent in the trochophore stage would reduce the risk of washout.

The differences in the development time of trochophores and the vertical migration behavior of veligers noted here warrant further investigation, both for the purpose of understanding the ecology of wild stocks and for choosing populations for aquaculture purposes. Aquaculturalists should at least be aware of differences in vertical migration behavior among different populations of potential aquaculture species. The movement of animals from one area to another where a local stock already exists may result in crossbred individuals that are not fit enough in the new system to sustain a breeding population. The seriousness of this type of scenario depends on three (at present) unknown factors: (1) the degree of difference among behaviors in the populations, (2) the heritability of those behaviors, and (3) the amount of mortality experienced as a result of "inappropriate" behavior. On the other hand, the knowledge that such differences exist might be an advantage. Chosing animals with appropriate veliger behavior (perhaps by using local parents) may greatly improve the success of stock enhancement programs.

It is also possible that behaviors vary in their effect on survival or growth under the controlled conditions in the hatchery. If that were so, then simply culturing veligers for several generations may alter behavior, and strong selective pressure could severely increase inbreeding by removing all but the progeny that have inherited a particular chromosome from a particular individual. Strong selection for rare behavior within a limited gene pool could result in rapid inbreeding depression and consequent failure to thrive, negating attempts to improve broodstock by selection.

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THE USE OF LIPID EMULSIONS AS CARRIERS FOR ESSENTIAL FATTY ACIDS IN BIVALVES: A TEST CASE WITH JUVENILE *PLACOPECTEN MAGELLANICUS*

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ABSTRACT Although information on bivalve nutrition is still very scarce, several studies have demonstrated the importance of lipids, in particular triglycerides, as a source of energy and essential fatty acids in the early life stages. Experimental diets used so far to study bivalve nutrition either heavily pollute the water or are too complex to prepare in a hatchery. The potential use of lipid emulsions as off-the-shelf supplements was evaluated through the analytical verification of the ingestion and incorporation of n-3 highly unsaturated fatty acids (HUFA) by the juvenile sea scallop *Placopecten magellanicus* fed lipid emulsions of different fatty acid composition as a supplement to *Isochrysis* sp. (clone T-Iso). The average lipid content in the scallops fed the lipid supplements was 20% higher compared with that in the control fed algae only (3.29 ± 0.16 *versus* 2.75% of dry weight, respectively). Changes in the fatty acid composition, in particular of n-3 HUFA, were demonstrated in total lipids, polar lipids, and triglycerides of juvenile sea scallops supplemented with lipid emulsions on the basis of ethyl ester concentrates of n-3 HUFA and were dependent on the level and proportion of 20:5n-3 and 22:6n-3 present in the emulsion. The effective incorporation of essential fatty acids from lipid emulsions indicated that the supplementation of lipid emulsions to live algae may improve and standardize the dietary supply of lipids and fatty acids in hatchery production of bivalves.

KEY WORDS: Bivalve, lipid, fatty acid, algal supplement, *Placopecten magellanicus*

INTRODUCTION

Rearing bivalves in commercial systems has so far relied on the production and use of selected species of unicellular marine algae. Despite the growing knowledge of the effects of environmental conditions, disease, and genetic background, the success of commercial hatchery cultures remains highly unpredictable. Mortalities are often attributed to deficiencies in certain nutritionally important components. Although information on bivalve nutrition is still very scarce, several studies have demonstrated the importance of lipid quantity and quality, particularly triglycerides, in early life stages as a source of energy and essential fatty acids (Helm et al. 1973, Holland and Spencer, 1973, Waldock and Nascimento 1979, Gallager and Mann 1986, Gallager et al. 1986). A requirement for the n-3 highly unsaturated fatty acids (HUFA), eicosapentaenoic acid (EPA; 20:5n-3), and docosahexaenoic acid (DHA; 22:6n-3), has been demonstrated for juvenile oysters (Langdon and Waldock 1981), and recent studies evaluating changes in fatty acid composition during larval development appear to confirm this for larval bivalves (Helm et al. 1991, Marty et al. 1992).

The importance of lipids for larval development has encouraged the development of artificial diets that provide specific lipid supplements during broodstock conditioning and larval rearing. Experimental diets used so far in bivalve nutrition studies, such as mixed diets (Trider and Castell 1980), liposomes (Parker and Selivonchick 1986) and microcapsules (Langdon and Waldock 1981), either heavily pollute the water or are too complex to prepare on a regular basis in a hatchery. Lipid microspheres that are easily prepared by sonication of an oil mixture with lecithin and vitamin E have been proposed as a nutritional supplement for oyster conditioning (Robinson 1992a, Robinson 1992b, Heras et al. 1994). Self-emulsifying concentrates of marine oils, which are widely used in fish hatcheries to enrich filter-feeding prey organisms like *Artemia* and rotifers with n-3 HUFA (Sorgeloos and Léger 1992), are off-the-shelf lipid supplements that may also be acceptable for bivalves. Previous work has demonstrated the potential use of these lipid emulsions as a supplement for the larval bivalves *Mercenaria mercenaria* and *Ostrea edulis* (Coutteau et al. 1994a). This study aimed at the analytical verification of the ingestion and incorporation of the fatty acids supplied through lipid emulsions of different n-3 HUFA content by juvenile *Placopecten magellanicus*.

MATERIALS AND METHODS

Juvenile sea scallops, *P. magellanicus*, were supplied by Fisheries Resource Development Ltd. (Sandy Cove, Nova Scotia) and André Mallet (Mallet Associates, Halifax, Nova Scotia). The seed originated from stocks kept in a field nursery. During a period of 2 d, the animals were acclimated gradually to the experimental temperature (14–15°C) and fed *Isochrysis* sp. (clone T-Iso). Initially, 3.5 g of scallops of approximately the same size (average live weight, 32.7 mg) were stocked per culture unit. The latter consisted of a small lantern net suspended in a bucket containing 25 l of filtered (using cartridge filters with pore sizes of 5 and 1 μm) seawater that was renewed daily. Each bucket was aerated with an airstone to prevent the food from settling. After 17 d of feeding on the experimental diets, scallops were starved overnight

in .iltered seawater, rinsed with distilled water, and stored at -22°C for biochemical analysis.

Isochrysis sp. (clone T-Iso), which was selected as the algal control diet, was grown in 10-l batch culture with F/2 (Guillard) medium and harvested in the exponential phase. The scallops were fed an initial weight-specific daily ration of 0.88% (dry algae per initial wet seed biomass), which was administered over two feedings per day. The latter ration maintained algal concentration in the cultures above 15–20 cells μl^{-1}. Rations were adjusted and the biomass was restocked after 6 d of culture in order to feed approximately constant weight-specific daily rations throughout the experiment (Urban et al. 1983). Algal rations were based on a dry weight of 13.8 ± 0.6 pg $cell^{-1}$ (mean and standard deviation from analysis of four cultures) for *Isochrysis* sp. (clone T-Iso), determined according to the method described by Coutteau et al. (1994b). Lipid emulsions were added simultaneously with the algae to give 0.2% lipid supplementation per initial wet scallop biomass. The experimental lipid emulsions (prepared by INVE Aquaculture N.V.-S.A., Belgium) contained, on a wet weight basis, 50% lipid, liposoluble vitamins (0.013% vitamin D_3, 0.32% vitamin E, 0.08% ascorbyl palmitate, 0.18% vitamin A), emulsifiers, preservatives, antioxidants, and water. Lipid consisted of either coconut oil (Em0, control emulsion lacking HUFA) or ethyl ester concentrates of marine oils (Em50E and Em50D, approximately 50% Σ n-3HUFA, primarily EPA and DHA, respectively).

Lipids were extracted from whole animals (40–50 per extraction), algae (four independent samples in the course of the experiment), and emulsions with a mixture of chloroform:methanol (2:1 v/v) (Folch et al. 1957). Total lipid contents were determined gravimetrically after exhaustive removal of the solvent from the lipid extract. Lipid classes were separated by preparative thin-layer chromatography on silica gel plates with hexane:diethyl ether:acetic acid (85:15:1), and fatty acid methyl esters (FAME) were prepared from total lipids, total polar lipids, and triglycerides by transesterification with 7% BF_3-methanol:benzene (1:1 v/v) (Napolitano and Ackman 1993). Separation of the FAME was carried out on a Perkin-Elmer Model 8420 GC equipped with a flame ionization detector (FID) and an OMEGAWAX-10 flexible fused silica capillary column (30 m × 0.32 mm inner diameter) (Napolitano and Ackman, 1993). Relative areas were converted to weight percent amounts of fatty acids by correcting for the FAME FID responses (Ackman and Eaton 1978). Quantitative data (milligrams of fatty acid per gram of dry weight, mg/g DW) were obtained by adding 10% of 23:0 as internal standard before the transesterification of total lipids and by using the following equation:

$$mg_{FA} \cdot (g\ DW)^{-1} = \frac{area_{FA}}{area_{23:0}} \cdot \frac{mg_{23:0}}{L} \cdot \frac{TL}{CF \cdot 100}$$

with TL, total lipid sample (%DW); L, amount of lipid used for FAME preparation (g); CF, 1.04, correction factor for the conversion of fatty acids in FAME. Subsamples of scallops were dried at 70°C for 36 h to obtain the dry matter and then heated to 450°C for 4 h to obtain the ash weight. Organic matter was calculated from the difference between dry matter and ash.

RESULTS

Isochrysis sp. (clone T-Iso) exhibited an average n-3 HUFA content of 14.0% with a DHA/EPA ratio of 17.8. The emulsions Em50E and Em50D contained approximately 50% n-3 HUFA with a DHA/EPA ratio of, respectively, 0.7 and 5.8. The coconut-oil based emulsion Em0 contained 90% saturated fatty acids of which nearly 55% was 12:0 (Table 1).

Scallops that were starved for 17 d did not show any increase in wet weight and exhibited higher ash content and lower lipid content compared with the fed ones (Table 2). The average lipid content in the lipid-supplemented treatments was 20% higher compared with that in the control fed algae only (3.29 ± 0.16 *versus* 2.75% of dry weight, respectively), whereas dry weight and ash content for all fed treatments were similar (in the range of 44.9–47.8% and 82.2–85.7%, respectively).

The proportions of monoenoic fatty acids increased during starvation, mainly because of an increase of 20:1 (Table 3). A decrease of n-3 fatty acids could mainly be attributed to the decrease of 18:3n-3 and particularly 18:4n-3, which was abundant in the initial scallops. Starved scallops selectively retained n-3 HUFA with C > 20, in particular 22:6n-3, and n-6 HUFA, especially 20:4n-6, whereas the proportion of n-3 HUFA with 20 C, primar-

TABLE 1.

Fatty acid composition (weight % of total fatty acids) of the lipid emulsions and *Isochrysis* sp. (clone T-Iso).

Fatty acid	Em0	Em50E	Em50D	ISO*
12:0	54.5	—	0.2	0.1
TMTD	—	—	—	0.3
14:0	19.3	0.3	0.9	22.8
16:0	13.2	3.1	16.2	8.0
16:1n-9	—	1.3	1.8	0.3
16:1n-7	—	—	—	3.7
18:0	3.0	4.1	1.1	0.2
18:1n-9	7.8	11.2	16.4	9.4
18:1n-7	0.1	3.4	1.4	1.2
18:2n-6	1.9	1.2	2.4	6.4
18:3n-3	—	0.9	0.4	7.0
18:4n-3	—	2.4	0.6	13.4
20:1n-11	—	—	—	3.0
20:1n-9	0.1	3.4	0.3	—
20:1n-5	—	—	—	—
20:2n-6	—	3.7	4.1	0.1
20:4n-6	—	1.8	0.3	0.2
20:4n-3	—	1.5	0.1	—
20:5n-3	—	25.9	6.4	0.7
21:5n-3	—	1.6	0.9	0.5
22:2NMID	—	—	—	1.4
22:1n-11 + n-13	—	1.6	—	—
22:5n-6	—	0.9	1.3	2.9
22:5n-3	—	4.0	2.9	0.7
22:6n-3	—	19.3	37.5	12.1
24:1n-9	—	1.0	2.5	—
Σ saturated	90.0	8.9	18.3	32.9
Σ monoenoic	8.1	24.1	22.2	17.9
Σ polyenoic	1.9	66.8	59.3	47.5
Σ n-3HUFA**	0	52.8	47.9	14.0
DHA/EPA	—	0.7	5.8	17.8

Minor components identified (<1%) and not included in the table are Iso-15:0; Ant-15:0; 15:0; Iso-16:0; Ant-16:0; 7-methyl-hexadecanoic acid; 16:1n-5; 16:2n-6; 16:2n-4; 16:3n-4; 16:3n-3; 16:4n-1; 17:0 + phytanic (3,7,11,14-tetramethylhexadecanoic) acid; 18:2n-4; 18:3n-6; 18:3n-4; 18:4n-1; 20:0; 20:1n-7; 20:2NMID; 20:3n-6; 20:3n-4; 20:3n-3; 22:0; 22:1n-9; 22:1n-7; 22:1n-5; 22:4n-6; 22:4n-3.

* Average values from analysis of four cultures.

** ≥20:3n-3.

TABLE 2.

Average individual wet weight (WW) and composition of juvenile *P. magellanicus* at the start of the experiment and after 17 d of starvation or feeding on various diets.

Treatment	Average WW (mg ind^{-1})	Dry Weight (% WW)	Ash (% DW)	Total Lipid (% DW)
Initial	32.7	45.7	ND	2.01
After 17 d of culture				
Starved	33.0	44.0	88.5	1.23
ISO	47.5	46.1	82.9	2.75
ISO + Em0	54.1	45.7	85.7	3.46
ISO + Em50E	53.9	47.8	82.2	3.14
ISO + Em50D	57.0	44.9	82.7	3.27

DW, dry weight; ND, not determined.

ily 20:5n-3, decreased. Compared with the initial fatty acid composition, scallops fed *Isochrysis* showed an increased proportion of certain monoenoic fatty acids that were also abundant in the alga, *i.e.*, 16:1n-7 and 18:1n-9, whereas the relatively high 20:1n-11 content in the algae did not affect that of the scallops (Table 3). The change of the HUFA content in total lipids after the adaptation to the *Isochrysis* diet reflected the HUFA composition of the alga, *i.e.*, a predominance of 22:6n-3 and a strongly reduced content in 20:5n-3.

The supplementation of the emulsion Em0, despite its high content in 12:0, resulted in the presence of only 1% of 12:0 in the total lipids (Table 3). The proportion of total n-3 HUFA decreased slightly as the result of the Em0 supplementation. The effect of the supplementation of the Em50 emulsions on the fatty acid composition of total lipids was mainly restricted to an increase of the dominant n-3 HUFA, either EPA (from 3.3 to 5.6% for Em50E) or DHA (from 17.9 to 19.4% for Em50D). As a result, the supplementation of lipids with a lower DHA/EPA ratio than that of the algae resulted in a decrease of the DHA/EPA ratio in the scallop lipids from 5.5 in the control diet to 3.0 and 4.7 for the emulsions Em50E and Em50D, respectively (Fig. 1A). In accordance with the increase of the total lipid content and the proportion of n-3 HUFA, the absolute concentration of EPA and DHA was considerably higher in the scallops receiving the Em50 emulsions (respectively, 1.13 and 3.44 mg/g DW for ISO + Em50E, and 0.91 and 4.29 mg/g DW for ISO + Em50D) compared with the control fed algae only (respectively, 0.54 and 3.00 mg/g DW) (Fig. 1A). The supplementation of the Em0 emulsion resulted in an increase of approximately 15% in the concentration of EPA and DHA.

The above changes in the fatty acid composition of the total lipids due to lipid supplementation were amplified in the triglycerides (Fig. 1B) and attenuated in total polar lipids (Fig. 1C). In this way, the DHA/EPA ratio in the various dietary treatments was in the range of 3.2–8.1 and 2.8–3.7 for triglycerides and total polar lipids, respectively. Equivalent values for the total n-3 HUFA content were 15.5–23.0% and 34.7–36.6%, respectively.

DISCUSSION

Research on bivalve nutrition, including the study of lipid requirements, has been seriously hampered by the lack of suitable experimental diets. In this regard, lipid vesicles may have several advantages over other synthetic microparticles, *e.g.*, their near-neutral buoyancy, suitable size range for efficient filtration by bivalves, and composition of nontoxic, digestible materials. Parker and Selivonchick (1986) demonstrated that juvenile *Crassostrea gigas* were able to metabolize the phosphatidylcholine and cholesterol present in the lamellae of liposomes. The protection from leaching of entrapped compounds makes liposomes particularly interesting as carriers for the delivery of water-soluble nutrients to filter-feeding organisms, whereas lipid microspheres consisting of emulsified lipid droplets provide a maximal amount of lipid per particle and may constitute effective carriers for lipid-soluble nutrients. Robinson (1992a) prepared microspheres of a lipid mixture of menhaden oil, egg phosphatidylcholine, and a partially hydrogenated vegetable oil with polyvinyl alcohol as an emulsifier. Although the latter author demonstrated the potential use of the lipid microspheres either as a supplement to or as a substitute for algae in the brookstock conditioning of the Pacific oyster, a similar lipid content and fatty acid composition was reported for nonfed oysters and oysters fed only the lipid microspheres (Robinson 1992b). Recently, Heras et al. (1994) improved the formulation of the emulsion of Robinson (1992a) by adding vitamin E and replacing the menhaden oil with a concentrate of n-3 fatty acid ethyl esters, thereby increasing the proportion of n-3 HUFA in the lipid supplement from 12.6 to 44.3%. Although it has been demonstrated with fluorescent beads that lipid microspheres are ingested and disintegrated by adult *O. edulis* (Heras et al. 1994), the latter does not prove the effective assimilation of nutrients from the lipid supplement. This work showed that essential fatty acids supplied as an emulsion of ethyl esters are assimilated and incorporated into the triglycerides and the polar lipids of juvenile sea scallops fed the lipid emulsion as a supplement to live algae. Similar work with *O. edulis* confirmed this for larval stages (Coutteau et al. 1994a), which may imply that lipid emulsions could be used to provide dietary lipids to the various life stages and species of bivalve molluscs.

The similar growth of scallops receiving a dietary supplement based on coconut oil and those fed a supplement rich in n-3 HUFA indicated that the algal control diet in this study, consisting of the DHA-rich *Isochrysis*, may have satisfied the requirement for n-3 HUFA in juvenile *P. magellanicus*. Nevertheless, the supplementary dietary lipid may have provided additional energy, which was at least partially stored as lipid reserves in the tissues. The very limited accumulation of 12:0 in the total lipids (1% 12:0 in the tissue *versus* 54% in the emulsion), despite the increase of the total lipid content in the scallops fed the emulsion Em0, may indicate a rapid oxidation of a large part of the lipid supplement based on

TABLE 3.

Selected fatty acid composition of total lipids of juvenile *P. magellanicus* at the start of the experiment and after 17 d of starvation or feeding on various diets (weight percent of total fatty acids).

Fatty Acid	Initial	Starved	ISO	ISO + Em0	ISO + Em50E	ISO + Em50D
12:0	0.2	0.2	0.1	1.1	0.1	0.1
TMTD	2.7	—	—	—	—	—
14:0	2.9	1.5	8.3	8.9	8.3	8.0
15:0	0.5	1.1	0.4	0.3	0.3	0.3
16:0	15.2	14.3	11.8	11.8	11.9	11.8
16:1n-7	1.4	1.1	4.8	5.2	4.6	4.8
16:1n-5	0.6	1.0	0.1	0.1	0.1	0.2
16:3n-3	0.3	0.7	0.5	0.3	0.3	1.4
16:4n-1	0.3	1.9	0.9	1.3	1.2	0.1
17:0*	0.6	0.9	0.2	—	0.3	0.2
18:0	3.7	6.6	1.8	2.0	2.1	1.8
18:1n-9	3.4	3.5	8.0	8.4	8.1	8.1
18:1n-7	2.5	2.7	4.1	4.1	4.1	4.0
18:2n-6	2.9	1.3	5.1	5.3	5.0	4.9
18:3n-3	3.5	0.7	5.7	5.8	5.6	5.4
18:4n-3	12.6	1.9	13.5	13.5	13.2	13.2
20:1n-11	1.1	3.4	1.0	1.1	1.0	0.9
20:1n-9	1.1	1.8	0.9	0.9	1.0	1.0
20:1n-7	0.7	1.4	0.4	0.5	0.4	0.3
20:1n-5	0.4	1.2	0.6	0.6	0.5	0.6
20:2n-6	2.0	1.8	1.3	1.5	1.2	1.3
20:3n-3	1.3	0.9	0.8	0.8	0.7	0.7
20:4n-6	1.1	3.4	1.0	1.1	1.0	1.1
20:4n-3	0.8	0.7	0.4	0.4	0.4	0.4
20:5n-3	13.0	10.1	3.3	3.0	5.5	4.1
21:5n-3	1.1	1.3	1.0	0.8	0.9	0.8
22:5n-6	0.6	1.2	2.7	2.2	2.2	2.5
22:5n-3	0.6	0.9	0.6	0.3	0.8	0.6
22:6n-3	19.0	26.3	17.9	16.3	16.7	19.4
Σ saturated	23.7	25.1	23.1	24.1	22.9	22.3
Σ monoenoic	11.7	17.1	20.5	21.6	20.5	20.4
Σ polyenoic	61.5	57.2	56.2	54.0	56.3	57.1
Σ n-3HUFA**	35.8	40.3	23.8	21.6	25.0	26.0
DHA/EPA	1.5	2.6	5.5	5.4	3.0	4.7

Minor components identified (<1%) and not included in the table are Iso-16:0; 7-methyl-hexadecanoic acid; 16:1n-9; 16:2n-6(n-4?); 16:3n-4; 16:4n-3; 20:0; 20:2NMID; 22:2NMID; 22:4n-6; 24:0.

* Includes phytanic (3,7,11,14-tetramethylhexadecanoic) acid.

** ≥20:3n-3.

coconut oil. Similarly, small proportions of 12:0 have been observed in the muscle and liver lipids of sunshine bass fed coconut oil as main dietary lipid (Nematipour and Gatlin 1993). A low accumulation of medium chain triglycerides (MCT) observed in ayu fish fed MCT (mainly 8:0) as a supplement to pollack liver oil has been attributed to the ability of fish to use MCT as a direct source of energy (Nematipour et al. 1989). The use of the coconut oil as a caloric supplement may have had a sparing effect on the catabolism of essential fatty acids provided through the algae, as indicated by the 15% increase of the concentration of EPA and DHA in the scallops fed the emulsion Em0 as a supplement (Fig. 1A).

The conservative nature of the fatty acid composition of the polar lipid fraction compared with the triglycerides is widely accepted for fish (Sargent et al. 1993) and confirmed for various stages of bivalves (Waldock and Nascimento 1979, Langdon and Waldock 1981, Delaunay et al. 1993, Napolitano and Ackman 1993). The conservation of 20:1 fatty acids, in particular 20:1n-11, observed in the starved and *Isochrysis*-fed scallops is in agreement with its possible structural role in the gill membranes (Napolitano and Ackman 1993). The selective retention of 22:6n-3 and 20:4n-6 in the starved scallops, contrary to the decrease of 20:5n-3, indicated the greater importance of the former fatty acids. Similar observations on the relative importance of 20:4n-6 and 22:6n-3 *versus* 20:5n-3 can be deduced from other bivalve studies (Helm et al. 1991, Delaunay et al. 1993), and particularly, DHA may play an important role during larval development and metamorphosis in the scallop *Pecten maximus* (Marty et al. 1992, Delaunay et al. 1993).

For bivalve larvae, it has been suggested that triglycerides may play a double role, first, as a storage of large amounts of saturated and monoethylenic fatty acids for energy purposes and, second, as a temporary reservoir of PUFA that could be transferred to structural polar lipids and specific metabolic pathways during metamorphosis (Napolitano et al. 1988). This is in agreement with several studies showing the importance of the content as well as the fatty acid composition of lipids, particularly triglycerides, for the early life stages of bivalves (Helm et al. 1973, Holland and

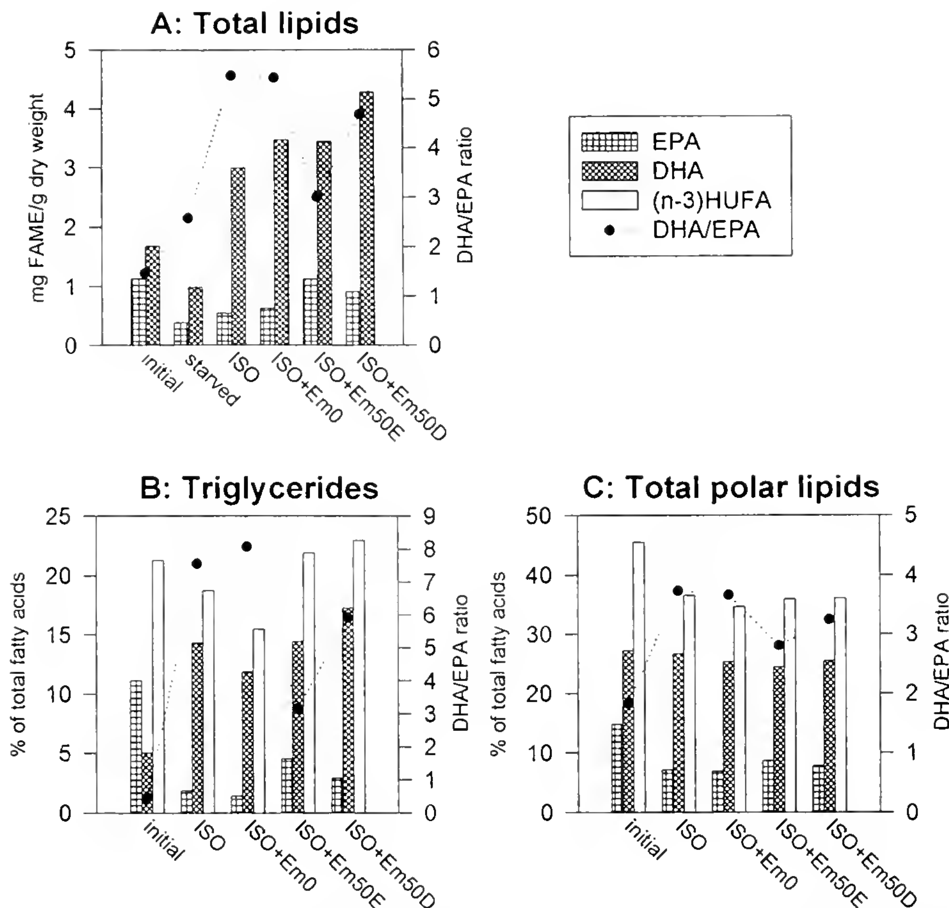

Figure 1. Effect of starvation and supplementation of various lipid emulsions to *Isochrysis* sp. (clone T-Iso) on the content of EPA, DHA, and n-3 HUFA in total lipids [(A) mg fatty acid/g of dry weight] and triglycerides and total polar lipids [(B and C) weight percent of total fatty acids] of juvenile *P. magellanicus*.

Spencer 1973, Gallager and Mann 1986, Gallager et al. 1986, Helm et al. 1991, Utting and Doyou 1992). Lipid emulsions may be a convenient artificial diet to study lipid and fatty acid requirements during critical stages of bivalve culture, *i.e.*, broodstock conditioning and larval rearing. Furthermore, the ability of manipulating both the quantity and the fatty acid composition of bivalve lipids, in particular of triglycerides, through the supplementation of off-the-shelf lipid supplements holds promise for application in hatchery rearing. Hatchery operators currently rely on live algae to supply essential fatty acids, and the fatty acid composition of algae may strongly vary with culture conditions (Pohl and Zurheide 1979). In this study, EPA and DHA concentrations increased by >100 and >40%, respectively, in juveniles fed lipid emulsions as a supplement to *Isochrysis* compared with the milligram/gram dry weight values in the control fed algae only. In particular, this accumulation of essential fatty acids through specific diet supplements could be used to standardize the dietary supply of lipids and essential fatty acids in broodstock conditioning and larval rearing.

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ISOLATION AND CHARACTERIZATION OF A cDNA ENCODING AN ACTIN GENE FROM SEA SCALLOP (*PLACOPECTEN MAGELLANICUS*)*

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ABSTRACT Two full-length complementary DNAs (cDNA) were isolated from a sea scallop adductor muscle–specific cDNA library and sequenced completely. Both clones encode the same open reading frame of 376 amino acid residues. The amino acid sequence is highly homologous to other invertebrate actin sequences, and as in other invertebrates, the N-terminal sequences are much more similar to the nonmuscle actin of higher vertebrates than to their muscle actin. Results suggest that this is the primary actin gene expressed in adductor muscle. The size of the actin gene family in this species is approximately 12–15, determined through Southern hybridization. An actin gene probe may also be useful as a genetic marker because it reveals polymorphisms in sea scallop in at least three loci. Strong signals were obtained when DNA digests from several shellfish and fish were probed with an actin coding region probe, indicating that this clone will be useful in genetic studies of other fish and shellfish species.

KEY WORDS: Actin, cDNA, genetic marker, *Placopecten*

INTRODUCTION

Actins are highly conserved contractile proteins. They are present in eukaryotic cells and play an important role in many diverse cellular process. They are globular proteins that polymerize into filaments for most of their biological functions, such as muscle contraction, cellular motility, and cellular structure (Pollard and Cooper 1986). Actins are divided into two classes, muscle and nonmuscle actins and, in vertebrates, are further grouped into three types: alpha actins, which occur in muscle tissues, and beta and gamma actins, which constitute the cytoskeletal actins present in most cells. Muscle and nonmuscle actins of vertebrates can be distinguished by a set of characteristic differences in amino acids (Vandekerckhoe and Weber 1984). In invertebrates, actins also have both muscular and nonmuscular functions, but these two classes are not readily distinguished on the basis of amino acid sequence.

Actins are usually encoded by multiple genes in multicellular eukaryotes, with the largest number occurring in plants (Hennessey et al. 1993, Shah et al. 1983). These multigene families encode different isoforms that are very similar in their primary structure, yet they fulfill diverse functions in different organisms. They show tissue-specific expression at different stages of development. Promoter regions of the muscle-specific actin gene have been characterized and fused with reporter genes, and the tissue-specific expression of reporter genes has been reported (Macias and Sastre 1990, Pollard 1990). Because actin genes are very conserved at the amino acid level, they have been used to study codon bias and phylogenetic relationships across large phylogenetic distances (He and Haymer 1995, Fang and Brandhorst 1994). The studies on the molecular genetics of actin function have been reviewed by Hennessey et al. (1993). Although actin genes have been studied in a variety of organisms, they have not been studied at the molecular level in bivalves.

The sea scallop (*Placopecten magellanicus*) is an important commercial species that occurs in discrete beds along the coasts of the northern United States and Atlantic Canada (Black et al. 1993). Because of its commercial importance, the sea scallop has been the subject of several genetic studies (Patwary et al. 1994a, Patwary et al. 1994b, Pogson and Zouros 1994, Volckaert and Zouros 1989). As a part of our continued interest in this species, we have constructed an adductor muscle–specific complementary DNA (cDNA) library, primarily to develop cDNA-based markers for population and aquaculture genetic studies of this organism. We have isolated and characterized the first bivalve full-length actin cDNA from our library. We also studied the potential use of this cDNA as a probe in genetic studies of sea scallop.

MATERIALS AND METHODS

DNA Extraction

Adductor muscle samples were ground to powder in liquid nitrogen, immediately mixed with 10 mL/g tissue chilomonas buffer (2% sarcosyl, 0.5 M NaEDTA, 100 mM NaCl, 20 mM Tris [pH 7.6]) to which proteinase K (50–100 μg/mL) was added, and incubated overnight at 55°C. Samples were then stirred briefly and centrifuged in an IEC clinical centrifuge (International Equipment company, Needham Heights, MA) at 1,975 ×g for 10 min; the supernatants were then transferred to a new set of tubes, and the nucleic acids were precipitated by adding two-third volume of cold isopropanol and maintaining for 1 h or more on ice or at

*The sequence data reported in this article will appear in Genbank database under the accession No U55046.

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−..°C. The samples were centrifuged at 3,920 ×g for 15 min, and nucleic acid pellets were dried in a speed vac concentrator (Savant Instruments, Inc., Farmingdale, NY) and dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA) for 30 min at 55°C. Samples were treated with RNAse A (20 μg/mL) for 30 min at 37°C and extracted twice with an equal volume of phenol-chloroform ([phenol:chloroform:isoamyl alcohol = 50:49:1], 0.1 M Tris [pH 7.5], 0.2% β-mercaptoethanol) and once with an equal volume of chloroform-isoamyl alcohol (24:1). The DNA in the aqueous phase was precipitated by the addition of one-third volume of NH_4OAc and 2.5 volume of cold 95% ethanol for at least 1 h at −20°C and centrifuged at 10,000 ×g for 15 min. The pellets were washed in 70% ethanol, dried, and dissolved in TE buffer (10 mM Tris-HCl, 0.1 mM EDTA).

Preparation of RNA

Total RNA was prepared by use of the methods described by Sures and Crippa (1984) and Turpen and Griffith (1986) with modifications. Twenty-one grams of adductor muscle tissue from one male and one female sea scallop was ground together to a powder in liquid nitrogen with a mortar and pestle, mixed with 190 mL of extraction buffer (4.0 M guanidine thiocyanate, 25 mM sodium citrate, 2% sarkosyl, and 1% β-mercaptoethanol), and incubated at room temperature for an hour. An equal volume of chloroform:isoamyl alcohol (24:1) was added to the sample, mixed for 10 min, and centrifuged to separate the two phases. CsCl (0.2 g/mL) was added to the aqueous phase, layered over a CsCl cushion (5.7 M CsCl, 50 mM EDTA), and centrifuged at 184,000 ×g for 6 h in a 70 Ti rotor (Beckman Instruments, Inc., Palo Alto, CA). The pellets were air dried and dissolved in ME buffer [10 mM 3-(N-morpholine) propanesulfonic acid, 4.5 mM EDTA, pH 7.2 0.1% diethyl pyrocarbonate (DEPC)] containing 0.5% sodium dodecyl sulfate (SDS) and 5% phenol, and RNA was extracted with phenol-$CHCl_3$ and $CHCl_3$ and precipitated with ethanol at −70°C for 30 min. The sample was centrifuged, and the RNA pellet was dried in a speed vac concentrator without heat on and dissolved in DEPC-treated water.

Polyadenylated RNA was isolated from total RNA by the use of a Poly(A) Quick mRNA purification kit according to the supplied protocol (Stratagene, La Jolla, CA). A total of 20.0 μg of poly A^+ RNA were obtained from approximately 1,000 μg of total RNA.

The cDNA was synthesized, size fractionated, and ligated into Uni-ZAP vector arms according to the protocol supplied with the ZAP-cDNA synthesis kit (Stratagene). A total of 2.5 μg of messenger RNA yielded a library of 1.9×10^6 pfu.

Library Screening

A few microlitres of the library were plated, and a total of 130 plaques were randomly cored and stored in SM medium (0.58% NaCl, 0.2% $MgSO_4 \cdot 7H_2O$, 50 mM Tris-HCl [pH 7.5], 0.01% gelatin) at 4°C. The Bluescript phagemids from 12 plaques were excised *in vivo* from lambda-ZAP II with ExAssist helper phage. Phagemid DNA was extracted by the use of a Nucleobond AX kit (Macherey-Nagel GmbH & Co., Duren, Germany). The clones containing inserts were partially sequenced in both directions with an ABI 373 sequencer (Applied Biosystems, Foster City, CA), and four were identified as actin clones through Blast P searches (Altschul et al. 1990). Additional actin clones were isolated through subsequent screening with an actin coding region probe, and the two largest clones were completely sequenced in both directions.

Preparation of Probe

A nonradioactive actin probe was prepared by labeling with alkali-labile (Dig-11-dUTP. Labeling was done either by the polymerase chain reaction (PCR) or by the random labeling method. In PCR labeling, the 25-μL reaction contained 1× *Taq* polymerase buffer; 1.5 mM $MgCl_2$; 100 μM each of dATP, dCTP, and dGTP; 86 μM dTTP; 14 μM dig-11-dUTP; 25 ng each of forward and reverse nested primer; 1 U of *Taq* DNA polymerase-; and 5 ng of recombinant plasmid DNA or 1 μL of frozen and thawed recombinant Uni-ZAP XR vector in SM medium. The reactions were performed in a GeneAmp 9600 PCR system (Perkin Elmer, Norwalk, CT) and programmed as follows using the fastest ramp time: 1 cycle at 94°C for 4.5 min, followed by 29 cycles each of 30 s at 94°C, 30 s at 60°C, and 4 min at 72°C, and a final cycle of 30 s at 94°C, 30 s at 60°C, and 10 min at 72°C. Successful labeling is indicated by decreased mobility of the reaction product in an agarose gel compared with an unlabeled control. Random primed labeling of cDNAs was done with a Boehringer Mannheim (Boehringer Mannheim, Laval, PQ, Canada) kit. To avoid common flanking M13 sequences from the probe, a pair of nested primers for each clone was synthesized and used for insert amplification. These amplified inserts were purified with a QIAEX Gel Extraction kit (Qiagen) and used as a template for random labeling. The probe was cleaned either by using Nick columns (Pharmacia Biotech, Uppsala, Sweden) or by using the QIAquick Spin PCR Purification Kit (Qiagen, Chatsworth, CA) and quantified by comparing with known Dig-labeled DNA on a dot blot.

Preparation of Genomic Blots

DNA samples (10 μg each) were digested overnight with 50 U of appropriate restriction enzymes. To aid in DNA digestion, spermidine was added to a final concentration of 5 mM in the reaction. The digested DNAs together with digoxigenin-labeled molecular-weight marker III (Boehringer Mannheim) were fractionated in 0.8% agarose gels at 20 V/cm in Tris-acetate (0.04 M Tris-acetate, 0.001 M EDTA) buffer and were then transferred to positively charged nylon membranes (Boehringer Mannheim) with a Pharmacia vacuGene XL unit and following the manufacturer's protocol no. 1.

Hybridization

Prehybridization was done in a hybridization oven at 39°C for 4 h in a buffer containing 50% deionized formamide, 5× sodium chloride, sodium citrate (SSC), 0.1% *N*-lauroylsarcosine, 0.02% SDS, and 2% blocking reagent (Boehringer) and 100 μg/mL boiled and chilled yeast RNA (Boehringer). Hybridization was done under the same conditions for 18 h in fresh buffer to which 10 ng/mL denatured probe was added. Blots were washed at room temperature twice in 2× SSC–0.2% SDS for 10 min, once in 0.5× SSC–0.1% SDS for 15 min, and once in 0.2× SSC–0.1% SDS for 30 min. To detect Dig-labeled DNA by chemiluminescence with Lumigen PPD, the Boehringer Mannheim protocol was followed, except that the membrane was incubated in 1.25% buffer 2 for 90 min, instead of 30 min in 1% buffer 2, and anti-Dig-alkaline phosphatase was diluted 1:12,500 instead of 1:10,000. The membranes were sealed in polythene bags and exposed to X-ray film.

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   1 gagcacccataccaatagtctttagatttactttggaactaactccaacaaccaacaaacaaac ATG TGT GAC GAC 76
   1                                                                  M   C   D   D   4

  77 GAG GTA GCA GCT TTA GTA GTA GAC AAT GGC TCC GGT ATG TGC AAG GCC GGG TTC GCC GGA  136
   5 E   V   A   A   L   V   V   D   N   G   S   G   M   C   K   A   G   F   A   G    24

 137 GAC GAT GCT CCA CGC GCT GTG TTC CCC TCC ATT GTT GGA AGG CCC CGT CAC CAG GGT GTC  196
  25 D   D   A   P   R   A   V   F   P   S   I   V   G   R   P   R   H   Q   G   V    44

 197 ATG GTT GGT ATG GGT CAG AAA GAC AGC TAC GTA GGA GAT GAA GCT CAG AGC AAG AGA GGT  256
  45 M   V   G   M   G   Q   K   D   S   Y   V   G   D   E   A   Q   S   K   R   G    64

 257 ATC CTC ACC CTC AAG TAC CCC ATT GAG CAC GGT ATC GTC ACA AAC TGG GAT GAT ATG GAG  316
  65 I   L   T   L   K   Y   P   I   E   H   G   I   V   T   N   W   D   D   M   E    84

 317 AAG ATC TGG CAT CAC ACC TTC TAC AAC GAG CTC CGT GTC GCC CCT GAG GAG CAC CCC GTC  376
  85 K   I   W   H   H   T   F   Y   N   E   L   R   V   A   P   E   E   H   P   V   104

 377 CTC CTG ACA GAG GCT CCC CTC AAC CCC AAG GCC AAC AGG GAA AAG ATG ACC CAG ATC ATG  436
 105 L   L   T   E   A   P   L   N   P   K   A   N   R   E   K   M   T   Q   I   M   124

 437 TTC GAG ACC TTC AAC GCC CCC GCT ATG TAC GTC GCC ATC CAG GCT GTC CTC TCC CTG TAC  496
 125 F   E   T   F   N   A   P   A   M   Y   V   A   I   Q   A   V   L   S   L   Y   144

 497 GCT TCC GGT CGT ACC ACC GGT ATC GTC CTC GAC TCC GGA GAT GGT GTC ACC CAC ACC GTC  556
 145 A   S   G   R   T   T   G   I   V   L   D   S   G   D   G   V   T   H   T   V   164

 557 CCC ATC TAT GAA GGT TAC GCT CTT CCC CAC GCC ATC CTC CGT CTC GAC TTG GCT GGC CGT  616
 165 P   I   Y   E   G   Y   A   L   P   H   A   I   L   R   L   D   L   A   G   R   184

 617 GAC TTG ACC GAT TAC CTC ATG AAG ATC CTC ACC GAG CGT GGT TAC TCA TTC ACC ACC ACC  676
 185 D   L   T   D   Y   L   M   K   I   L   T   E   R   G   Y   S   F   T   T   T   204

 677 GCC GAG AGA GAA ATC GTC AGG GAC ATC AAG GAG AAA CTC TGC TAT GTT GCC CTC GAC TTC  736
 205 A   E   R   E   I   V   R   D   I   K   E   K   L   C   Y   V   A   L   D   F   224

 737 GAG AAC GAG ATG GCC ACC GCC GCC TCA TCC TCA TCC CTC GAG AAG AGC TAC GAG CTT CCC  796
 225 E   N   E   M   A   T   A   A   S   S   S   S   L   E   K   S   Y   E   L   P   244

 797 GAC GGT CAG GTC ATC ACC ATC GGA AAC GAG CGT TTC AGG TGT CCC GAA TCC CTC TTC CAG  856
 245 D   G   Q   V   I   T   I   G   N   E   R   F   R   C   P   E   S   L   F   Q   264

 857 CCA TCC TTC TTG GGT ATG GAA TCT GCC GGT ATC CAC GAG ACC ACA TAC AAC TCC ATC ATG  916
 265 P   S   F   L   G   M   E   S   A   G   I   H   E   T   T   Y   N   S   I   M   284

 917 AAG TGC GAC GTC GAC ATC CGT AAG GAT CTG TAC GCC AAC ACT GTC CTG TCC GGA GGC ACC  976
 285 K   C   D   V   D   I   R   K   D   L   Y   A   N   T   V   L   S   G   G   T   304

 977 ACC ATG TTC CCA GGT ATT GCC GAT CGT ATG CAG AAG GAA ATC ACC GCC TTG GCT CCC AGC  1036
 305 T   M   F   P   G   I   A   D   R   M   Q   K   E   I   T   A   L   A   P   S   324

1037 ACA ATG AAG ATC AAG ATC ATT GCT CCA CCA GAG AGG AAA TAC TCC GTC TGG ATC GGT GGC  1096
 325 T   M   K   I   K   I   I   A   P   P   E   R   K   Y   S   V   W   I   G   G   344

1097 TCC ATC TTG GCT TCT CTG TCC ACC TTC CAA CAG ATG TGG ATC AGC AAA CAG GAA TAC GAT  1156
 345 S   I   L   A   S   L   S   T   F   Q   Q   M   W   I   S   K   Q   E   Y   D   364

1157 GAG TCC GGC CCA TCC ATT GTC CAC AGG AAA TGC TTC TAA attattcttcaaattattaggacttcaa 1223
 365 E   S   G   P   S   I   V   H   R   K   C   F   *                                377

1224 attatttttacattttcggtactgtgaagaatggactccacctcgtgctttattgaacaggataagctgacagacagaat 1303

1304 ctcgttttgtccgataaagtgcgatacctaagtgtttttaaaaagacaccagtgacattccgtcagcagcctgggtaagg 1383

1384 caatgtttcgaaggtttttactaaccaaagtatatggacagctatgtatactaaggacacttgccgtctttttatttaat 1463

1464 ataatttaatttaaataaatctatgcaaatctatagtttgatggaagttgatttctcaggacgggaagttatattttgta 1543

1544 caggaagttgagagtatgacgggacgtactgtgtacatctgtgtaactatggtgatgtaatgtatatagcattcacacac 1623

1624 tgtaagaaaaactggcatcatttttcaaagaatattgtgataatgcacccacgtttgtatacatgtcgtatggatgtagg 1703

1704 tccaccatccagagcaaattaaaaatgttaaagtttaaaaaaaaaaaaaaaaaaa                          1759
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Figure 1. Nucleotide sequence and deduced amino acid sequence of the sea scallop actin gene. The nucleotide residues are numbered from the 5′ end of clone *Pm*C-11, and the amino acid residues are numbered from the first in-frame methionine. The nucleotide sequence shown was determined on both strands of the entire cDNA insert of *Pm*C-11 and *Pm*C-4. The potential polyadenylation signal is underlined. The nested primers that were used to amplify this clone are identified by broken underlines.

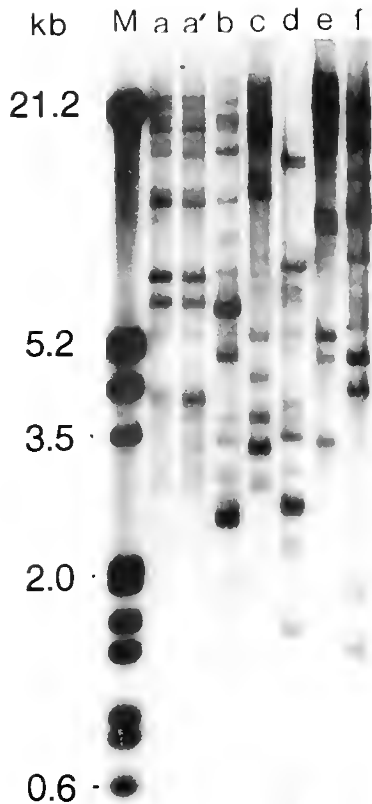

Figure 2. Detection of actin genes in the sea scallop. Sea scallop genomic DNA blot hybridized with an actin coding region probe. DNA from a single animal was digested with the following restriction enzymes: lanes a and a', *Eco*RI (no spermidine added in a'); lane b, *Eco*RV; lane c, *Hin*dIII; lane d, *Nde*I; lane e, *Pst*I; and lane f, *Xba*I. M is digoxigenin-labeled DNA molecular-weight marker III (Boehringer Mannheim).

RESULTS AND DISCUSSION

From 130 randomly chosen Uni-Zap XR recombinant lambda plaques, 12 inserts were partially sequenced. Four of these inserts were identified through BlastP searching as encoding actin. By the use of one of these inserts (*Pm*C-11) as a probe, an additional 30 actin clones were identified from among the 118 remaining plaques. Actin cDNAs were found to be the most common (29%) in our library. The other cDNAs that occurred frequently were myosin (15%), arginine kinase (6%), and tropomyosin (4%). The hybridization of a probe prepared from the *Pm*C-11 noncoding region to the actin clones indicated that they all represent the same gene. This observation was also supported by the complete sequence homology in the 3′ noncoding regions of several clones. These data indicate that, as in sea urchin (Lee et al. 1984), only a single actin gene is expressed in scallop adductor muscle.

Complete sequencing of the two largest clones showed that they were 1,750 and 1,759 base pairs (bp) long. The shorter clone was missing 9 bp at the 5′ end. The largest clone had 64-bp and [illegible]-bp (excluding poly As) noncoding regions at the 5′ and 3′ ends, respectively. Both clones encoded the same open reading frame of 376 amino acid residues (Fig. 1).

Actin sequences are highly conserved at the amino acid level in evolution. The sea scallop sequence differs by only 6 amino acids from the California sea hare (sp/P17304); by 7 amino acids from *Artemia* (sp/P18603); by 8 amino acids from *Caenorhabditis* (Pir/s27135); by 10 amino acids from *Drosophila* (sp/P10987) and silkworm (sp/PO4829); by 11 amino acids from common carp (sp/P12714), chicken (gp/M10279), mouse (pir/A31900), and starfish (sp/P12716); and by 13 amino acids from sea urchin (sp/P02573). Owing to this level of similarity, actin amino acid sequences may not be useful to study phylogenetic relationship among invertebrates. The predicted N-terminal sequences are similar to those of other invertebrates. As in other invertebrates, the first five amino acids in sea scallop are methionine-cysteine and three acidic amino acids—a valine at position 11, methionine at position 17, and a cysteine at position 18. As with other invertebrates, the N-terminal sequences are much more similar to nonmuscle actin of higher vertebrates than to their muscle actin (Rubenstein 1990).

Southern blot analysis was done to estimate the number of genes encoding actin in the sea scallop. Ten micrograms of DNA from a single sea scallop was digested overnight with each of the six enzymes *Eco*RI, *Eco*RV, *Hin*dIII, *Nde*I, *Pst*I, and *Xba*I, electrophoresed, and blotted. Hybridization of the blot with an actin coding region probe produced signals in all lanes (Fig. 2). The same signal pattern was obtained with DNA from a different an-

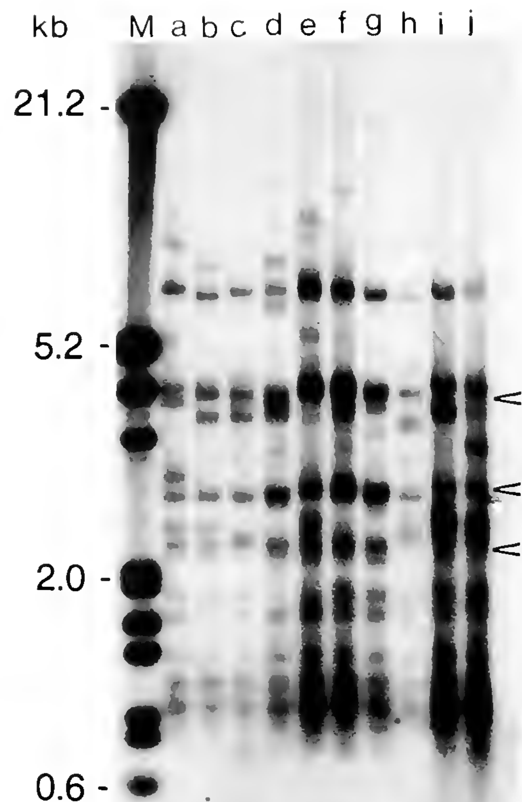

Figure 3. *Hin*cII-digested genomic DNA blot hybridized with an actin coding region probe. Each lane contains DNA from a different animal. The animals are: lanes a and b from Yarmouth, Nova Scotia; lanes c, d, g, and h from Sable Island; lanes e and f from an unknown location; and lanes i and j from Annapolis Basin, Nova Scotia. M is a DNA marker as mentioned in the legend to Figure 2. Arrows indicate polymorphic loci. The apparent polymorphic high-molecular-weight faint bands are possibly the results of incomplete digestion.

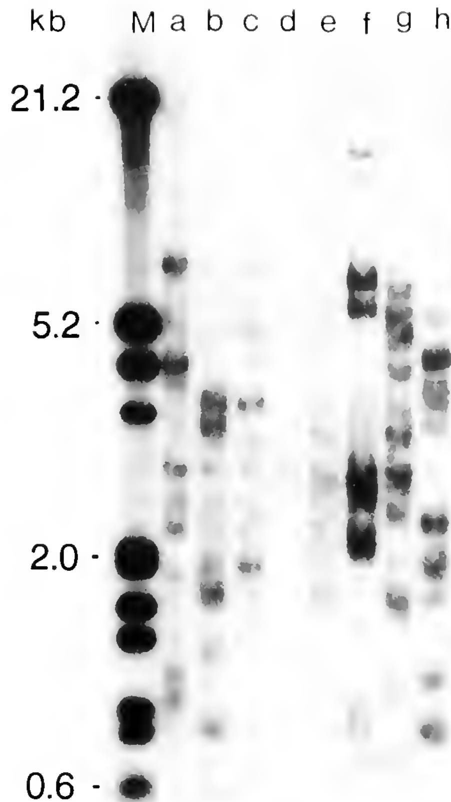

Figure 4. *HincII*-digested genomic DNAs from different species of mollusc and fishes hybridized with actin probe. DNAs are from: lane a, *P. magellanicus* (sea scallop); lane b, *Chlamys hastata* (Icelandic scallop); lane c, *Mytilus edulis* (blue mussel); lane d, *Spisula solidissima* (surf clam); lane e, *Homarus americanus* (American lobster); lane f, *Gadus morhua* (cod); lane g, *Tilapia nilotica* (tilapia); and lane h, *Crassadoma gigantea* (rock scallop). M is a DNA marker as mentioned in the legend to Figure 2. Note that signals in lane d are very weak because only a very small quantity of DNA was available to load.

imal. The Southern blot data suggest the presence of approximately 12 to 15 actin genes in the sea scallop. The sea scallop appears to have a slightly larger family size than other invertebrates such as sea urchin, *Artemia*, and *Drosophila*, which have about 8, 8–10, and 6 genes, respectively (Singer and Berg 1991).

To determine the potential use of actin as a genetic probe, genomic DNAs from 10 sea scallops, 8 from three different beds and 2 from an unknown location, were digested with *HincII*, blotted, and hybridized with an actin cDNA coding region probe (Fig. 3). Although most of the actin-containing DNA fragments revealed by this blot are constant among individuals tested, there are at least three polymorphic loci at 4.3, 3.0, and 2.5 kilobases (kb). The 4.3-kb locus is highly heterozygous, with apparently five different alleles occurring at this site. The 3.0-kb locus is highly homozygous in these samples with only one (lane a) having a heterozygous allele. The 2.5-kb locus is equally homozygous and heterozygous. An additional polymorphic locus may occur at approximately 6.5 kb, although analysis of additional individuals is necessary to confirm this. Although these results are too limited to make any genetic inference about sea scallop populations, they demonstrate that the actin probe may be useful for a variety of genetic studies.

The sea scallop actin gene probe was hybridized with *HincII*-digested sea scallop, Icelandic scallop, rock scallop, blue mussel, surf clam, American lobster, cod, and tilapia adductor muscle on a genomic DNA blot (Fig. 4). In all cases, signals of good intensity were obtained. These results reflect the conserved nature of the actin gene and suggest that it can be used as a reference in gene expression studies and as a heterologous probe to isolate actin genes, as well as to conduct genetic studies on these organisms.

The sea scallop actin cDNA characterized here appears to represent the primary, and perhaps only, actin gene expressed in the adductor muscle. This cDNA will be a useful tool for isolating sea scallop cytoskeletal actin genes and cDNAs, as well as actins from other marine organisms. Interestingly, sea scallop actin genes also appear to be useful genetic markers, although it is not yet clear at what level (individual or population) they will be most useful.

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FOOD-LIMITED GROWTH AND CONDITION INDEX IN THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA* (GMELIN 1791), AND THE BAY SCALLOP, *ARGOPECTEN IRRADIANS IRRADIANS* (LAMARCK 1819)

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ABSTRACT The growth response of the eastern oyster, *Crassostrea virginica*, and the bay scallop, *Argopecten irradians irradians*, to varying degrees of food limitation was evaluated. Under conditions of low current speed, dense assemblages of shellfish can rapidly deplete ambient food concentrations, resulting in measurable effects on growth and condition index. A flume study demonstrated significant growth and condition index responses to resource competition after reductions as small as 27% in relatively high ambient food concentrations (≈4.6 μg/l chlorophyll). Growth rates and condition index are linearly correlated with the average chlorophyll ration consumed. A field study demonstrated similar growth responses when the shellfish were cultured over a range of densities in a commercial aquaculture setting. By comparing the growth and condition index responses in the two experiments, we infer the degree of resource depletion occurring in the field from the correlations constructed in the flume study. Although physiological responses to food limitation will necessarily be site specific to varying combinations of temperature, current speed, and food concentration or quality, this work provides a unique opportunity to compare the growth response of oysters and scallops under a wide range of food availability in both laboratory and commercial aquaculture settings. Doubling the stocking density from 2.5 to 5.0 kg of oysters per bag resulted in a 20% decrease in both the condition index and the growth rate (percent increase in weight). These observations may assist commercial growers determine optimal stocking density for their aquaculture grow-out systems. Natural food availability in Point Judith Pond, a classic salt wedge estuary, is highly variable on a daily basis and is related to the tidal exchange. The variation in food concentration superimposed on the tidal current oscillation leads to massive changes in food flux and the degree of local resource competition. Scallop and oyster clearance rates (milliliters per minute) were constant over a wide range of chlorophyll concentrations, suggesting that these species will filter natural seston at a near-constant rate despite fourfold tidal variations in food concentrations. Scallop clearance rates were reduced when chlorophyll concentrations were depleted to below 12% of the natural levels, suggesting a threshold feeding response.

KEY WORDS: Bivalve, growth, condition index, culture, seston flux, flume

INTRODUCTION

In the marine environment, two basic factors influence the availability of food to benthic suspension feeders. The primary factor is the concentration of phytoplankton and particulate organic matter in the water column. For sparse populations, or where populations are exposed to strong currents, the concentration of food is the only factor controlling food availability. However, when populations of suspension feeders occur at great densities, or when currents are insufficient to replenish the food, local resource competition can deplete the concentration of locally available food (Dame et al. 1984, Frechette et al. 1989, Peterson and Black 1991). Food availability for these populations is determined by a product of both the food concentration and the current speed, or horizontal seston flux (Muschenheim 1987, Grizzle and Lutz 1989, Muschenheim and Newell 1992).

There is evidence that local food depletion can result in food-limited growth in natural bivalve assemblages (Wildish and Kristmanson 1985, Frechette and Bourget 1985, Rice et al. 1989); however, the subject has been the focus of considerable debate (Reiswig 1971, Powell et al. 1987). Although local food depletion in natural assemblages may be the exception rather than the rule, there is little doubt that food depletion occurs at population densities common to commercial shellfish aquaculture. Several studies have described intensive aquaculture systems that suffer from food-limited growth (Duggan 1973, Mason 1976, Rhodes et al. 1981, Malinowski and Siddall 1989, Newell 1990). The worldwide economic importance of shellfish aquaculture is increasing because many wild-harvest fisheries have peaked or are in decline (FAO 1992). Critical to the success of shellfish aquaculture operations is an understanding of the effects of stocking density on growth rate (Incze et al. 1981, Newell et al. 1989). Optimal stocking density in commercial aquaculture is determined by site-specific physical factors (horizontal seston flux), biological factors (species-specific filtration rate), and economic factors such as gear and labor costs. Lower stocking densities will almost always result in less competition for food and faster growth rates, but these increases come at the expense of more investment in gear, more labor to maintain it, and larger lease requirements.

This article describes the results of experiments designed to elucidate the effects of stocking density on food limitation in a modified rack-and-bag shellfish aquaculture operation (Rheault and Rice 1995). We used tightly controlled flume studies to quantify the effects of downstream food depletion on growth and condition index in eastern oysters *Crassostrea virginica* (Gmelin 1791) and northern bay scallops *Argopecten irradians irradians* (Lamarck 1819). The results of the flume study were then compared with growth and condition index data from a concurrent field experiment that used commercial aquaculture techniques over a range of initial stocking densities.

Other researchers have used flumes to examine food depletion in filter-feeding bivalves (Asmus and Asmus 1991, Butman et al.

19‹ + and references therein). Many of these experiments were conducted with cultured phytoplankton (Kirby-Smith and Barber 1974, Eckman et al. 1989, Cahalan et al. 1989) or at population densities insufficient to cause food depletion (Grizzle et al. 1992, Wildish et al. 1992, Judge et al. 1992). Our work attempts to precisely describe the effects of food depletion on shellfish growth and condition index using natural phytoplankton at densities and current speeds that were directly comparable to field conditions. These results were then compared with growth and condition index measurements from animals deployed in the field that were exposed to a wide range of resource competition. By comparing the two groups, we were able to make inferences about the food availability conditions experienced by the shellfish in commercial culture conditions over a range of initial stocking densities. These types of measurements are nearly impossible to make directly on a field population because of the challenge of making long-term, continuous measurements of food concentration and current speed.

MATERIALS AND METHODS

All experiments were conducted in Point Judith Pond in Narragansett, RI (41°24'N; 71°31'W), an 8-km estuary with a 30% semidiurnal tidal exchange with Block Island Sound (Licata 1981) (Fig. 1). The flume study was conducted in a temporary structure built at Camp Fuller, located on Turner Cove, approximately two-thirds of the way up the estuary on the west shore, adjacent to the main dredged navigational channel into the pond. The field study was conducted concurrently, with experimental cages placed 300 and 500 m to the east. The experiment lasted for 6 wk, from August 24 to October 4, 1992.

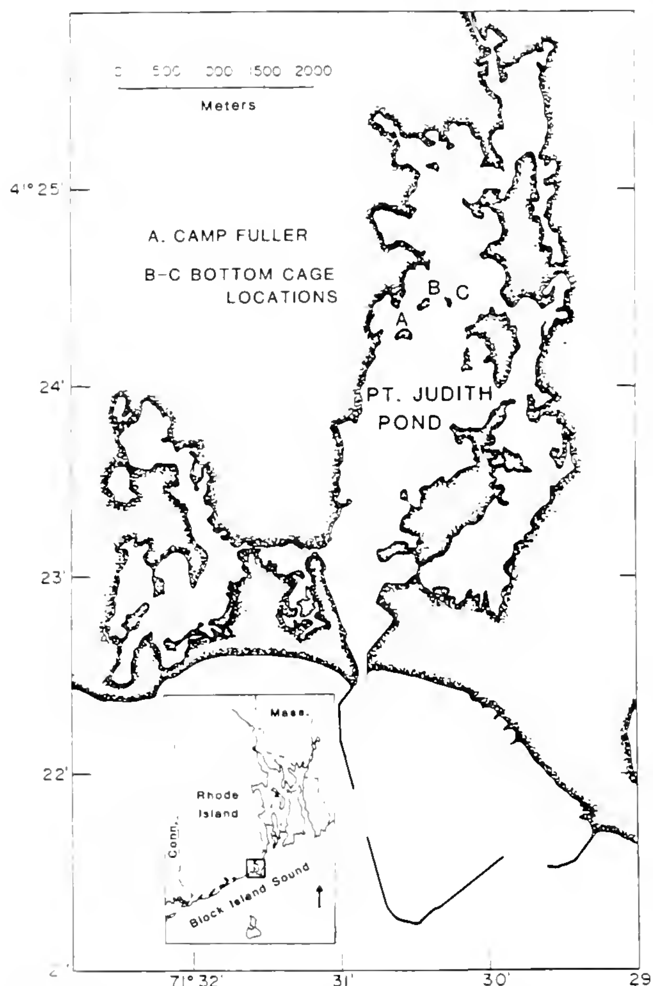


Figure 1. Location map of Point Judith Pond, Narragansett, RI, showing (A) the location of Camp Fuller where the flume was constructed, and the locations of the bottom cages (B and C), 300 and 500 m to the east.

Water for the flume study was pumped continuously to a head tank with a small submersible pump placed on the bottom (with the intake 15 cm above the bottom) at a depth of 1.3 m mean low water (MLW) under a floating dock. The pump intake was protected with a 3-mm mesh, and coarse particles and zooplankton were removed with a 200-μm mesh bag filter. The chlorophyll concentration of the natural seston was monitored continuously over the 6-wk trial with a Turner Designs (Sunnyvale, CA) Model 10AU flow-through fluorometer connected to a peristaltic pump sampling the head tank. Discrete subsamples were filtered and analyzed for seston concentration by total dry weight and organic content by combustion (6 h at 450°C). Water temperature was monitored continuously with a Temp-Mentor recorder (Ryan Instruments, Kirkland, WA) in the head tank.

Juvenile bay scallops, *A. irradians,* and eastern oysters, *C. virginica,* were obtained from Moonstone Oysters, Narragansett, RI, a commercial shellfish aquaculture firm. Hatchery-reared seedstock had been in culture throughout the summer at a nearby location in Point Judith Pond. The starting height (longest axis) of the oysters averaged 45 ± 5.7 mm (SD), whereas scallops averaged 43 ± 3.2 mm (SD). One week before the start of the experiment, individual oysters and scallops were marked with numbered plastic tags attached with quick-setting marine epoxy. All experimental animals were scrubbed to remove epiphytes, toweled dry, weighed to the nearest 0.01 g, and measured (longest axis) to the nearest 0.1 mm with calipers at the start and end of the 6-wk trial. Flume animals were measured again 2 and 4 wk into the experiment. A random sample of 25 animals of each species was sacrificed at the beginning of the experiment to determine tissue dry weight, shell weight, and starting condition index. Scallop tissues were further dissected to separate gonad, adductor muscle, and viscera, as well as visually examined for gonad color, a subjective measure of sexual competence. At the conclusion of the 6-wk trial, all experimental animals were similarly sacrificed and measured.

Flume Experiments

Three experimental plexiglass flumes were constructed with seven experimental compartments each, separated by 13-mm-mesh plastic screens to keep the animals in place. Each flume compartment measured 12 × 13 cm, and the depth was maintained with a standpipe at 6–7 cm. Each compartment was initially stocked with six oysters (mean length, 42.9 mm; mean wet weight, 7.0 g) or three scallops (mean length, 44.7 mm; wet weight, 23.6 g). Daily flume maintenance consisted of quickly draining the flume so that silt and fecal material could be rinsed out. The seawater system was designed to deliver a constant and identical flow of water to each flume. Current speed was measured midstream in the flume with a thermistor probe flowmeter (LaBarbera and Vogel 1976).

Every 4–8 d, the readings from the Turner Designs (model 10-AU) flow-through fluorometer were correlated with discrete chlorophyll samples that were filtered, digested in acetone, and read on another Turner Designs (model 10) fluorometer, followed by acidification (Parsons et al. 1984). Changes in the phytoplank-

ton species composition, as well as fouling on the photocell itself, can cause gradual shifts in the relationship between the fluorescence readings from the flow-through fluorometer and chlorophyll-a concentrations (Chl-a) determined by acetone digestion. The calibration of in vivo fluorescence readings to Chl-a concentrations was achieved by drawing samples from representative downstream flume compartments to obtain a range of Chl-a concentrations. These samples were read with the continuous fluorometer, and subsamples were filtered for Chl-a analysis. Calibration curves were constructed by correlating the fluorescence readings from the Turner 10-AU fluorometer to Chl-a determinations of the discrete filtered samples (>0.35 μm). Fouling on the flow-through photocell of the fluorometer was cleaned daily by 10 min of acetone immersion followed by a freshwater rinse and weekly by disassembling and scrubbing the photocell. Additional subsamples were filtered (Whatman GF/C) for the determination of seston dry weight and percent organic content (by combustion at 450°C for 6 h).

Over the course of the 6-wk trial, 17 feeding experiments were conducted to monitor downstream food depletion in each flume. This was accomplished by setting the flow-through fluorometer to record 20-s average fluorescence data and by placing the peristaltic pump intake into a downstream corner of each flume compartment until the readings stabilized for at least 1 min. The return flow from the peristaltic pump was always directed into the same flume as the intake, but at a different depth. The sequence of sampling was always from the end of the flume to the beginning so that if the sampling disturbed the filtering activity of animals in a compartment, the upstream animals would not be affected.

Two experimental trials were designed to examine the possibility that natural particle sinking and settlement might have an effect on downstream food depletion in the absence of filter feeders. One flume was filled with the empty shells of sacrificed experimental oysters, and a second was left empty. No downstream reduction in Chl-a was noted in either flume. Assimilation efficiency was calculated by use of the ash ratio method described by Conover (1966). Samples of seston and feces were collected on preweighed, precombusted (450°C) Whatman GF/C filters. Filters were rinsed with isotonic ammonium formate, dried at 60°C for 48 h, then weighed to the nearest 0.0001 g with a Mettler AE 200, combusted at 450°C, and weighed again. Assimilation efficiency (AE%) and calculated as:

$$AE\% = [100 \times (F - E)]/[(1 - E) \times F]$$

where F is the organic content (percent) of the food and E is the organic content (percent) of the feces.

Field Graw-Out Experiments

Concurrent with the flume study, field experiments were conducted using the facilities of a nearby commercial aquaculture firm, Moonstone Oysters, in Narragansett, RI. Moonstone uses a modified rack-and-bag shellfish culture method fully described in Rheault and Rice (1995). Shellfish were held in 13-mm-mesh plastic bags (0.6 × 0.6 m) on shelves in cages. The cages rest on the sediment at a depth of 2–3 m, holding the bags 10–60 cm above the pond bottom. Two experimental cages were placed 300 and 500 m to the east of the flume study site (see Fig. 1).

Ten mesh bags were stocked with 20 or more tagged, experimental oysters, whereas additional untagged oysters of a similar size were added to make up the desired initial stocking density. Ten bags were stocked with oysters over a range of initial stocking volumes 1–6 l/bag). For comparison, an additional 10 bags of oysters from the 1991 year-class were stocked over a similar range of starting weights; however, these animals were not measured for individual growth rates or condition index. The total weight of each bag was recorded at the start and end of the experiment.

The growth of individual tagged oysters in experimental bags was monitored by noting each individual's total wet weight and length at the start and end of the 6-wk study. At the end of the study, all tagged individuals were sacrificed for the determination of tissue dry weight, shell weight, and condition index. Tidal current speed was measured with a thermistor probe (LaBarbera and Vogel 1976) both inside and outside the grow-out bags to determine the effects of the cage on the local small-scale hydrodynamics.

Data Analysis

A Turner 10-AU fluorometer was set to collect 20-min average fluorescence readings throughout the 6-wk trial. There were several short periods where the fluorometer was offline for downloading data or cleaning the photocell. Missing data were interpolated from corresponding tidal phases of the previous or following day. Tidal current velocity was modeled by fitting a sine function with an amplitude equal to the maximum measured current speed and a period of 12.4 h. Seston flux for the field study was calculated by multiplying the modeled average tidal velocity by the measured chlorophyll concentration for each 20-min period for the 6-wk study. Predicted tidal heights published by NOAA (Rockville, MD) were compared with locally observed times of tidal maxima or minima to determine the lag time for our location in the pond. The seston flux values for all of the 20-min periods each day were averaged to obtain daily seston flux estimates for the field site.

The results of the 17 feeding trials were used to calculate the percentage of available food removed by each individual in the flume. Because this percentage was observed to be relatively constant over a wide range of concentrations (see Results), it was possible to estimate the ration available to each downstream flume chamber and, conversely, to estimate the ration consumed by each animal in the flume, every 20 min for the 6-wk trial. Individual daily ratios were calculated by multiplying the percent consumed by the average of 72 20-min average chlorophyll concentration measurements made each day.

Condition index (CI) was calculated by use of the methods described by Lucas and Beninger (1985):

$$\text{Oyster CI} = (100 \times TDW)/(WW - SW)$$

and,

$$\text{Scallop CI} = (100 \times TDW)/(SH)$$

when TDW is tissue dry weight in grams, WW is whole wet weight in grams, SW is weight of shell in grams, and SH is shell height in centimeters. Instantaneous growth, expressed as a percent increase in length or weight per day, was calculated as:

$$\%\text{ incr per day} = \ln[(W_t/W_o)/t] \times 100$$

when W_o is the initial wet weight in grams (or shell length in centimeters), and W_t is the weight (or length) at time t in days.

Approximately 10% of the oysters were found to have unidentified juvenile oyster disease (Davis and Barber 1994) or were severely distressed by infestation of *Polydora websteri* (mud blister) (Wargo and Ford 1993). These animals would continue to feed

n ,mally but had abnormal shell growth and condition indices that were usually statistical outliers. Three oysters in the field trial suffered shell deformations when their shell grew into the mesh of the bag. A similar number of scallops in the flume ceased growing when their hinge ligament became disarticulated. Animals in the flume experiment that were not growing after the first 2 wk were replaced by healthy individuals. Distressed or obviously infected animals were eliminated from the statistical analysis.

Shellfish growth responses to downstream food depletion in the flume study were broken down into three 2-wk subsets and after tests for normality of error terms and equality of variance were analyzed with a multiple analysis of variance (SPSS, Chicago, IL) for the effect of flume position and date. The effect of flume position on condition index (determined only at the end of the experiment) was analyzed with procNPAR1WAY (SAS; SAS Institute, Cary, NC), and differences in growth and condition index between pairs of ration levels were examined with the Bonferroni (Dunn) T-test (SAS).

In the field study, individual responses in growth or condition index were compared over the range of initial stocking densities by linear regression analysis (Statgraphics; Manugistics Inc., Rockville, MD). The effect of initial stocking density on the growth rates of entire bags (as percent increase in weight per day) were also examined with linear regression.

RESULTS

There was a strong effect of the tide on both chlorophyll concentration and temperature (Fig. 2). As the flood tide brought colder, more oligotrophic, ocean waters into the estuary, temperature would decline by 2–6°C and chlorophyll concentrations would drop by as much as 80%. Although the temperature varied greatly over a tidal cycle, there was only a 0.6°C difference between the average temperatures over the first two 2-wk sample periods (8/24–9/8 mean, 20.7°C, 9/8–9/21 mean, 20.1°C). The third 2-wk sample period, 9/21–10/2, showed a three-degree decrease in the average temperature to 16.7°C.

Chl-a concentrations were similarly variable with the tides (Fig. 2; Appendix III in Rheault 1995), but daily averages of chlorophyll concentrations were relatively stable, between 4 and 7 $\mu g\ l^{-1}$ for most of the experiment. Two large rain storms on 9/2–3 and 9/27 caused Chl-a concentrations to decrease sharply (Fig. 3b). Chl-a concentrations averaged 5.7 μg/l for the first 2 wk, 4.6 $\mu g\ l^{-1}$ for the second 2 wk, and 4.5 $\mu g\ l^{-1}$ for the last 2 wk. Size fractionation of the seston revealed that the majority of the seston was smaller than 20 μm: 78 ± 11% of the total seston (SD; n = 17), 70 ± 15% of the organic component of the seston (SD; n = 17) and 78 ± 8% of the chlorophyll (SD; n = 8). Seston concentrations were highest and had a greater percentage of organic matter in the first 2 wk of the experiment (Fig. 3a).

Flow rates to each flume were checked daily for the first week and twice a week thereafter. For the first 4 d, the flow rates were 1.7 to 1.9 l min^{-1}. To increase the degree of downstream food depletion, we decided to slow flow rates to 1.0–1.1 l min^{-1} for the next 4 wk. In the last week of the experiment, ambient Chl-a concentrations were depressed after a 7-cm rainstorm, so flow rates were increased to 1.5 l min^{-1}. Seston flux to each flume was

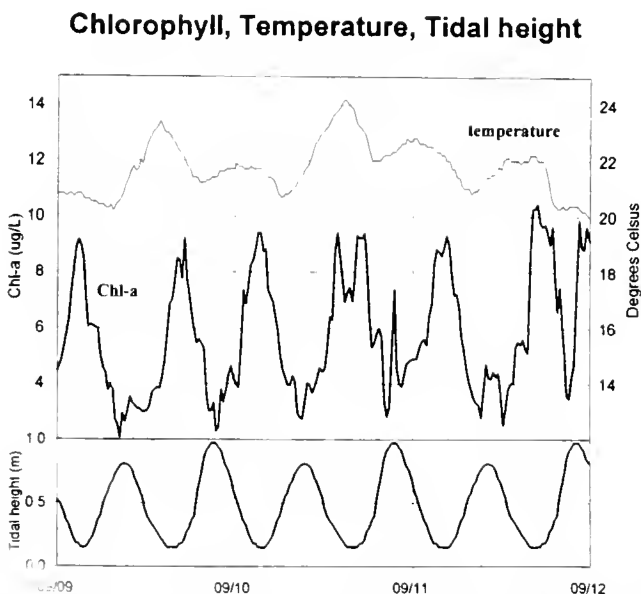


Figure 2. A representative sample of continuous data collected during the 6-wk flume experiment. Chl-a ($\mu g\ l^{-1}$) and temperature (°C) are plotted with tidal height (m).

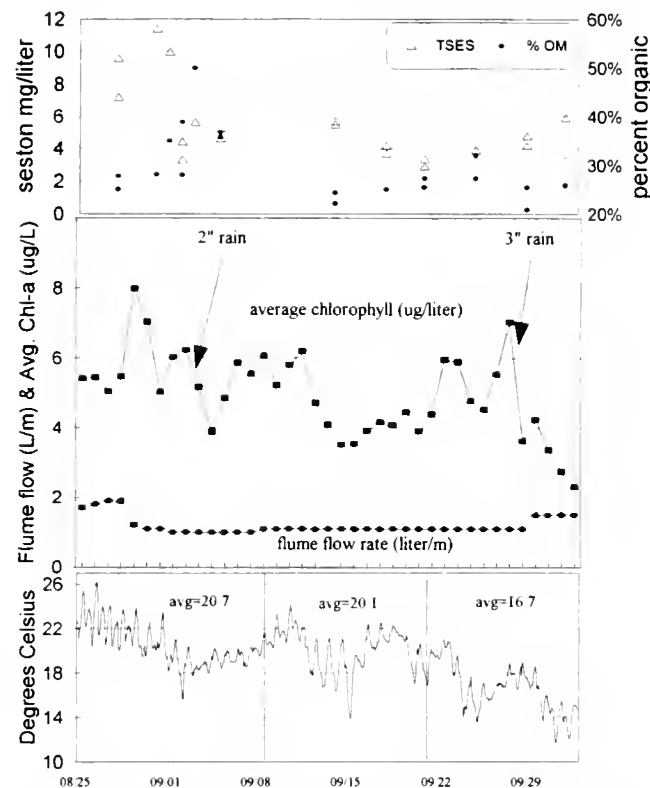
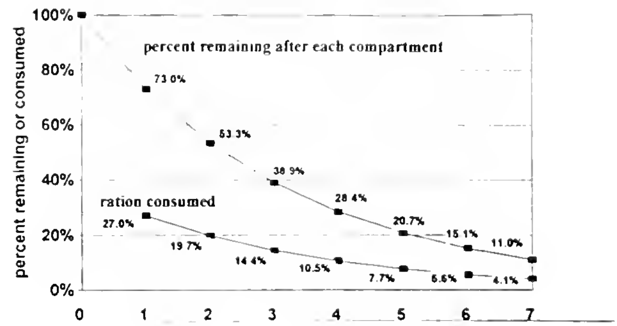


Figure 3. The graphs describe food, flow, and temperature conditions in the flumes over the course of the 6-wk experiment (8/24/92 to 10/2/92). (a) Discrete samples of total seston concentration are indicated by triangles, and percent organic matter (%OM) in those seston samples is indicated by circles. (b) Daily average Chl-a concentration calculated from a continuous record of 20-min means. Arrows indicate two rainstorms that noticeably diluted the Chl-a concentration. Flow rate of water in the flumes is given. (c) The temperature record (°C) is 20-min averaged data recorded continuously in the head tank feeding the flumes. Average temperature values for each 2-wk period are indicated.

calculated as the product of the average Chl-a concentration and the flow rate to each flume (Fig. 3b). Current speed at the center of the flume was measured at 0.38 cm/s at a flow rate of 1.1 l min^{-1}.

Downstream food depletion was calculated 17 times over the 6-wk trial by measuring the decline in Chl-a concentration in each compartment and was expressed as a percentage of the incoming concentration. Food depletion at each stage of the flume remained stable despite fivefold differences in incoming Chl-a concentration, although higher flow rates at the beginning and end of the experiment did reduce depletion percentages. Clearance rates were depressed in the last week of the experiment when temperatures dropped below 16°C (Fig. 3c). The feeding rate, or ration consumed (as percentage of incoming concentration), for each flume compartment was calculated from the difference in Chl-a concentrations between compartments. The downstream Chl-a depletion percentages from 17 feeding experiments were averaged for each flume compartment, and an exponential depletion function was fit to the data (Fig. 4). The best-fit downstream depletion curves for the oysters show that each flume compartment removed 27% of the food flowing in from the chamber above whereas scallops removed 35%.

The daily ration (Fig. 5) was calculated by multiplying the daily average chlorophyll concentration (Fig. 3b) times the flow rate (Fig. 3b) times the percentage consumed by each compartment (Fig. 4). The feeding studies indicate that oysters in the first flume compartment consumed an average of 0.38 mg of Chl d^{-1} per oyster, and scallops in the first compartment consumed an average of 0.99 mg of Chl d^{-1} per scallop. Downstream animals consumed proportionally smaller amounts. For comparison purposes, it is convenient to express these rations on a per gram of tissue dry weight basis. Using average starting tissue dry weights of 0.37 g per oyster and 1.33 g per scallop, an oyster in the first flume compartment consumed a ration of 1.03 mg of Chl d^{-1} per g dry wt, and a scallop consumed 0.74 mg of Chl d^{-1} per g dry wt. There was no consistent trend in the percent organic matter in either the downstream seston or feces samples for either scallops or oysters (Table 1). Scallops had a consistently higher assimilation efficiency (AE%) than oysters, whereas AE% declined for both species as the organic fraction of the seston declined later in the experiment.



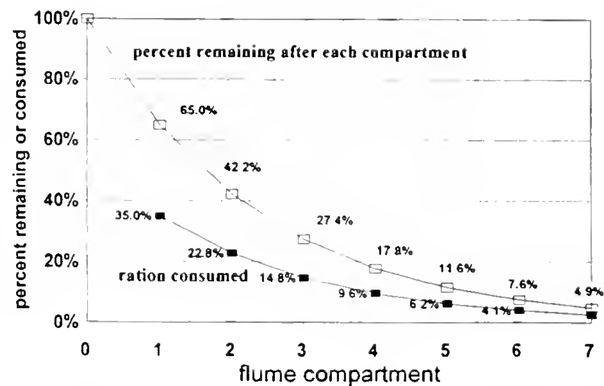


Figure 4. These plots show downstream chlorophyll depletion in the flume as the percentage of the incoming concentration remaining at each flume compartment for oysters (a) and scallops (b). Abscissa numbers refer to flume compartment numbers. Each point is the average of 17 feeding experiments. Semilogarithmic transformation of these Chl-a depletion data showed that scallops removed a greater percentage of available food (35% at each compartment) than did oysters (27% at each compartment). The ration consumed is the difference in concentration between compartments.

Flume Growth

Both oysters and scallops responded to decreasing downstream food availability with similar declines in incremental growth (percent per day increase in both length and weight) (Figs. 6 and 7) as well as condition index (Fig. 8). Incremental growth rates also declined with time over the course of the 6-wk experiment.

Growth rate (both length and weight increases over the entire 6-wk trial) and condition index (determined at the end of the experiment) were significantly correlated with the calculated average ration for each flume compartment (Tables 2 and 3). Pairwise analysis of the condition indices of oysters from each compartment suggests that oysters can respond measurably to reductions in ambient food concentration as low as 27% of the initial concentration. The condition index was the only measure that detected significant differences between the first two compartments ($p = 0.0025$). Percent increases in length or weight were more variable and did not show significant differences between adjacent chambers, even though the correlations with ration were highly significant (Tables 2 and 3).

Field Grow-out Study

The field grow-out study showed the growth responses of oysters to variations in initial stocking density measured three different ways: change in weight of the entire bag, individual growth in total wet weight, and individual condition index. Bags were initially stocked at 1–5 kg/bag (equivalent to 2.7–13.5 kg/m^{-2}). The low-density bags more than doubled in weight in both the 1991 and 1992 year-classes, but smaller 1992 year-class oysters grew faster than the 2-yr-old 1991 year-class (Fig. 9). Individual whole wet weight increase (percent per day) was negatively correlated with starting bag weight ($r^2 = 0.128$, $p < 0.0001$). Oysters in the lightly stocked bags increased weight at an average 2.4% per day, compared with only 1.9% per day in the heavier bags (Fig. 10a). The condition index for tagged individuals was also negatively

TABLE 1.

Percent organic matter in seston and feces and assimilation efficiency. (AE%)

Date	Flume Chamber Number	Seston, % Organic	Feces, % Organic	
			Oysters	Scallops
Sept. 1	1	35.0	15.6	18.5
	4	37.4	ND*	12.7
	7	32.6	19.4	15.4
	Mean	35.0	17.5	15.3
	AE%		**60.7**	**66.5**
Sept. 3	1	49.9	25.5	21.7
	4	48.5	24.0	ND
	7	52.6	28.2	23.1
	Mean	50.0	25.8	22.4
	AE%		**65.3**	**71.2**
Sept. 5	1	35.7	19.3	23.8
	4	32.1	30.7	22.5
	7	38.6	23.8	25.0
	Mean	36.2	24.6	23.2
	AE%		**42.4**	**46.8**
Sept. 18	1	25.5	ND	ND
	4	28.0	ND	ND
	7	21.4	18.3	18.9
	Mean	25.0	18.3	18.9
	AE%		**32.7**	**30.2**

* ND, not done.

correlated with starting bag weight ($R^2 = 0.321$, $p < 0.0001$) (Fig. 10b). The average condition index varied from a high of 7.6 in the lightly stocked bags to 5.4 in the heavily stocked bags.

Maximum tidal velocities (measured at peak flood tide) were 5–8 cm/s, and the calculated average current speed was 4.1 cm/s. Current speeds measured inside the mesh bags averaged 10% of the bulk flow measured near the experimental cages. The horizontal chlorophyll flux in the field had greater short-term variability than did the flux to the flume because the field flux had the tidal velocity variations superimposed on the variation in concentration, whereas the flume experienced a constant flow rate. However, daily averages of the 20-min flux estimates were nearly identical to the flux estimates in the flume (Fig. 11).

DISCUSSION

Several workers have studied feeding in scallops using raceways and flume. Kirby-Smith (1972) recommended that to maintain maximal growth of bay scallops in a raceway system, the effluent water should contain at least 60% of the incoming phytoplankton concentration. Rhodes et al. (1981) calculated that chlorophyll concentrations above 1.0 μg l^{-1} are enough to maintain maximal growth in bay scallops. The data presented in our study suggest that growth rate and condition index will decline after even smaller decreases in food concentrations. Significant reductions in oyster condition index were detected in the second flume chamber where chlorophyll concentration was reduced by only 27%.

Cahalan et al. (1989) used flume experiments to try to separate the effects of current speed and food concentration on growth in bay scallops, but their study used cultured algae and low population densities and did not result in food depletion. Similar studies with scallops feeding on natural particulate by Kirby-Smith (1972) and Kirby-Smith and Barber (1974) showed food-limited growth at slow current speeds but failed to describe the relationship between available ration and growth. Rhodes et al. (1981) studied the carrying capacity for bay scallop seed in raceways and projected a minimum ration for maximal growth of 74 mg of Chl d^{-1} per liter of biomass. Scallops of the size used in our study would pack 41 to the liter and collectively consume an average of 40 mg of Chl per day. Rhodes' scallop seed were considerably smaller (<10 mm) than those used in this study (mean, 44 mm). Smaller scallops will pack tighter (more dry weight per unit volume), and smaller shellfish in general will consume more on a per gram dry weight basis (Dame 1972).

Seston Flux

Weekly phytoplankton counts over the summer and fall of 1990 in Point Judith Pond showed that *Skeletonema costatum* was the numerically dominant species and that chlorophyll concentrations were consistently 2–10 times greater at the head of the pond than at the mouth (Rheault 1995), suggesting that the pond is a net source, rather than a net sink, of primary productivity. In this study, the tidal exchange resulted in two- to fivefold variations in chlorophyll concentrations, peaking at low tide and declining until high tide (Fig. 2).

This observation has important implications for studies that rely on single daily or weekly seston sampling to estimate food availability. One can minimize the sampling error by consistently sampling at the same phase of the tide, but errors of a few hours either way could have drastic effects on estimates of average food concentrations. This phenomenon will be most pronounced in tidal estuaries (such as Point Judith Pond) where rich eutrophic waters are mixed with a salt wedge of oligotrophic ocean waters. Likewise, simply sampling surface waters may grossly overestimate

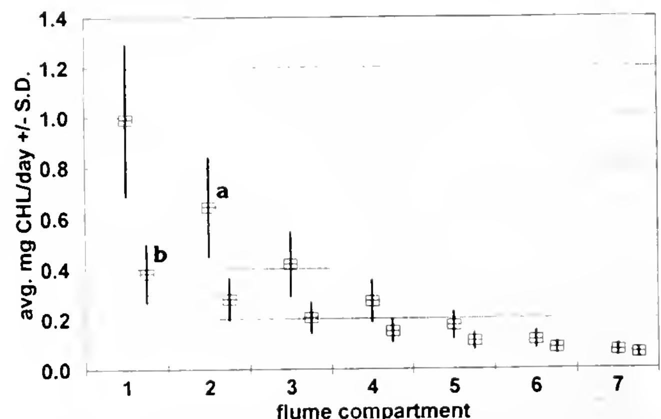


Figure 5. The average daily ration consumed is the product of Chl-a concentration (μg l^{-1}; Fig. 3b), the flow rate (l min^{-1}; Fig. 3b), and the percentage of food removed by each compartment (Fig. 4), divided by the number of animals in each compartment (six oysters or three scallops). Daily chlorophyll ration is expressed in units of mg d^{-1} per scallop (a) or per oyster (b). Data are presented as the mean value plus or minus 1 SD for the 6-wk flume trial.

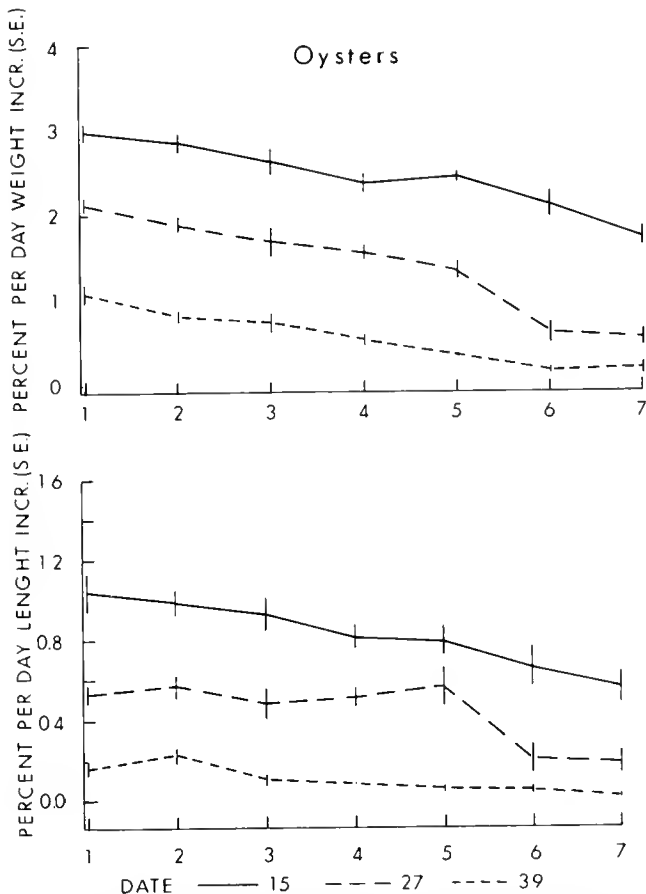


Figure 6. Instantaneous oyster growth is presented as percent increase per day in weight (a) or length (b) for each of the flume compartments. The mean growth rates (n = 10 or 12) for each 2-wk period are plotted separately, with standard error (S.E.) bars. The top curve represents growth in the first 2 wk, the middle curve is growth in the second 2 wk, and the bottom curve represents growth during the last 2 wk.

food conditions experienced by bottom-dwelling suspension feeders. When the water column is stratified the benthos will be bathed in the cool, saline, dense, oligotrophic ocean waters, whereas the surface waters are relatively warm and food rich. Furthermore, these shifts in food concentration and water temperature are at odds with the description of Point Judith Pond as a typical "well-mixed" estuary (Licata 1981).

The semidiurnal fluctuations in both tidal currents and chlorophyll concentrations make the estimation of field seston flux very difficult. Maximum and minimum chlorophyll concentrations were observed at low and high tide, respectively, periods when the tidal current speed drops to zero. An additional confounding factor was the fact that current speeds in the shallow (<2-m) pond can be greatly influenced by wind speed and direction. We have observed a number of occasions when moderate breezes would cause local water currents to move in the opposite direction of the tidal flow. The only way to get an accurate estimate of seston flux in these field conditions would be to place a continuously recording current meter on site with the continuously recording fluorometer.

Lacking these data, we estimated field fluxes from a rudimentary sine wave model of the current speed (Rheault 1995, Appendix III). We measured maximum current speeds of 5–8 cm s^{-1} and calculated an average current speed of 4.1 cm s^{-1}. Current speeds inside the mesh bags were measured directly at 10% of the bulk flow, or 0.41 cm s^{-1}. For comparison, the current speed in the center of the flume measured 0.38 cm s^{-1} when the flow rate was 1.1 l min^{-1}, suggesting that (on average) the flume animals were experiencing conditions very similar to those of the field animals. However, the flume animals were exposed to a constant current speed, whereas the field animals experienced an oscillating current regime. The variation in current speed caused increased short-term variability to the flux estimates; however, daily averages of the flux to the flume and the flux to the field were not significantly different.

Feeding

Loosanoff (1958) found no effect of temperature on pumping rate in adult oysters taken from Long Island Sound at temperatures between 16.0 and 28.0°C; however, below 16.0°C, he noted a 50% decrease in pumping rate. Other researchers have subsequently modeled bivalve clearance rate response to changes in temperature (Doering and Oviatt 1986) and found a nearly linear response. Bayne et al. (1977) also reported a direct effect of tem-

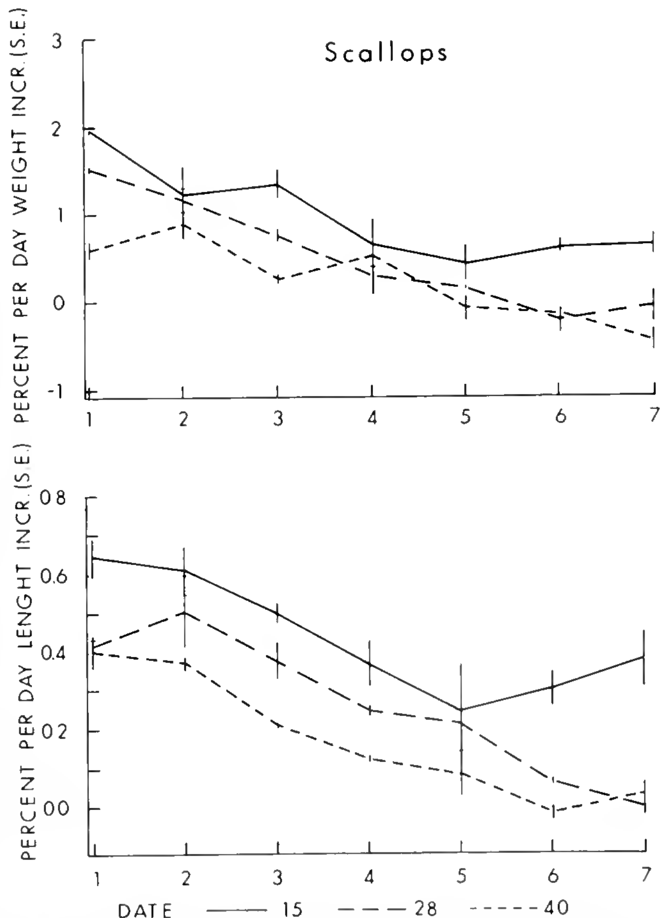


Figure 7. Instantaneous scallop growth is presented as percent increase per day in weight (a) or length (b) for each of the flume compartments. The mean growth rates (n = 3) for each 2-wk period are plotted separately, with standard error (S.E.) bars. The top curve represents growth in the first 2 wk, the middle curve is growth in the second 2 wk, and the bottom curve represents growth during the last 2 wk.

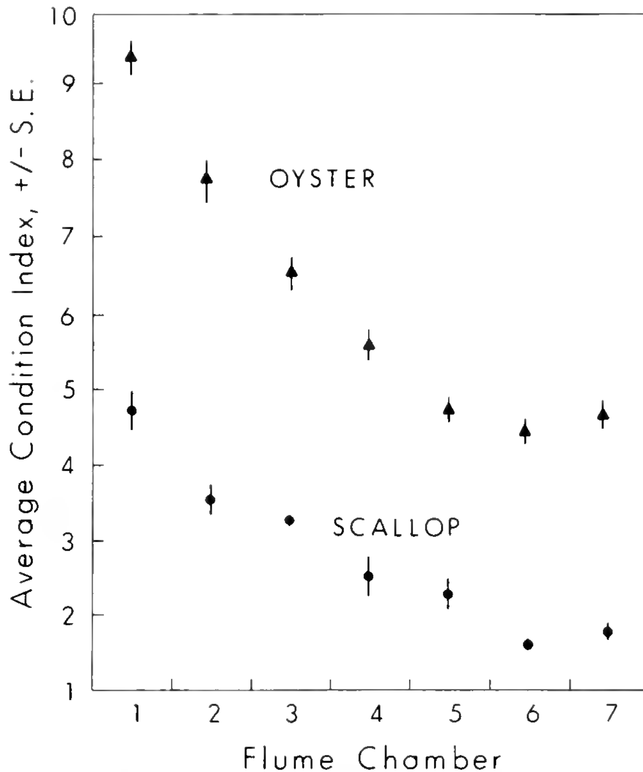


Figure 8. Mean oyster (curve A) condition index (± standard error, S.E.) at the end of the 6-wk flume experiment is plotted for each flume compartment (n = 10 or 12). Mean scallop condition index (±S.E.) after the trials is plotted in curve B (n = 3).

perature on both filtration rate and assimilation efficiency in mussels. Temperatures for the first 4 wk of our study remained well above Loosanoff's 16.0°C threshold but averaged only 16.7°C for the last 2-wk period (Fig. 3c). Thus, it is likely that temperature declines in the last 2 wk accounted for much of the observed decline in growth rates for both the scallops and the oysters over this time.

We observed that the percentage of food removed at each stage of the flume remained stable despite fivefold differences in incoming chlorophyll concentration. At the beginning of the experiment, when flow rates were slightly elevated, the percentage removed at each stage was correspondingly smaller. These two findings imply that the shellfish were clearing a fixed volume of water regardless of the food concentration. This conclusion is supported by Haven and Morales-Alamo (1970), who also described a "well defined pattern of particle removal [by oysters] when results are expressed in terms of percent removal." Tenore and Dunstan (1973) also reported that oysters removed a constant percentage of the food from the water when the ambient food concentration was within the range of 0.26–0.76 mg of C per liter, a "food concentration typical of natural environments." Below these levels, they reported sharply lower clearance rates. Similarly, *Mytilus edulis* has been observed to cease feeding if food concentrations drop below a certain concentration, a so-called "threshold feeding response" (Thompson and Bayne 1972, Wilson and Seed 1974, Bayne and Scullard 1976, Butman et al. 1994). This concentration may correspond to the level where the energy required to pump water past the gills exceeds the energy gained by the food captured.

During our experiment, the incoming seston concentrations were always in Tenore and Dunstan's (1973) "typical" range; however, downstream concentrations dropped off rapidly. Head tank seston concentration averaged 1.36 mg l^{-1} ash free dry weight (AFDW), corresponding to 0.45 mg of C per liter (assuming a ratio of 0.33:1 carbon:AFDW, calculated from Prosser 1973). Seston concentrations in the flumes declined rapidly downstream and may have regularly dropped below the concentration where animals could reap an energetic benefit by active feeding. This would explain why scallops in the last three compartments consistently removed less chlorophyll than was predicted by the curve fit to the data from the feeding rates of the scallops in the first four compartments (Fig. 4). To our knowledge, this would be the first report of a "threshold feeding response" in *A. irradians irradians*. An alternative explanation for this could be that any chlorophyll remaining in the flume after the fourth compartment was composed of particles that were too small to be effectively retained by the scallops but were still detected by the fluorometer and the individually filtered samples.

Palmer (1980) reported that bay scallops adjust their clearance rate according to ambient food concentrations to ingest algae at a near-constant rate. This conflicts with our observations that scallops cleared a near-constant percentage of the available food over a fourfold range of Chl-a concentrations. In our flume experiments, downstream scallops filtered a slightly smaller percentage of the available ration in contrast to Palmer's predicted response of increased clearance rate at lower food concentrations.

There was no consistent trend in the percent organic matter in either the downstream seston or the feces samples for either scallops or oysters (Table 1). Any decline in the percentage of organic matter in the remaining downstream seston would be an indication that the shellfish were either selectively filtering organic particles and leaving inorganic silt in suspension or perhaps feeding on resuspended fecal material (Loosanoff 1949, Newell and Jordan 1983).

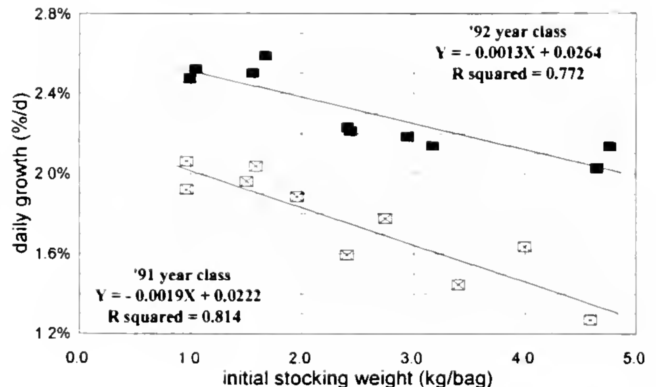


Figure 9. The effect of varying initial stocking density (kg per bag) on oyster growth in the field study. Weight increases of entire bags of oysters are presented as percent increase per day. Six-month-old oysters from the 1992 year-class (filled squares) grew faster than larger 18-month-old oysters from the 1991 year-class (hatched squares). Calculated linear regressions are plotted and formulae are given for each year-class. Both regressions were significant ($p < 0.0005$), and the slopes were significantly different from zero ($p < 0.0001$).

TABLE 2.

Statistical analyses of growth and condition index response.

	Vs. Condition Index	Vs. % Incr/d Weight	Vs. % Incr/d Length
Oysters			
Linear regressions of ration (mg of Chl-a d/oyster)			
y-intercept	3.29	0.93%	0.32%
Standard error (SE) of y estimate	0.754	0.31%	0.14%
r^2	0.839	0.607	0.361
No. of observations	75	75	75
Degrees of freedom (n − 2)	73	73	73
x coefficient	15.98	3.59%	1.01%
SE of coefficient	0.8201	0.0034%	0.0016%
Probability > F	0.0001	0.004	0.0001
Scallops			
Linear regressions of ration (mg of Chl-a/d/scallop)			
y-intercept	1.57	1.43%	5.29%
SE of y estimate	0.353	6.06%	3.04%
r^2	0.889	0.942	0.817
No. of observations	18	18	18
Degrees of freedom (n − 2)	16	16	16
x coefficient	3.26	79.50%	20.93%
SE of coefficient	0.2878	0.0494%	0.0248%
Probability > F	0.0001	0.0001	0.0001

Clearance Rate

The flume study data can also be used to estimate individual clearance rates if one assumes 100% retention efficiency on the gill and no refiltering within each compartment. Six oysters cleared 27% of the food available, with average flow rates of 1.1 l m^{-1}. This is equivalent to a 50 ml/min clearance rate for a 45-mm oyster with an average tissue dry weight of 0.40 g (or 124 ml min^{-1} per g dry wt). This agrees well with Jörgensen's (1966) estimate of 129–208 ml min^{-1} per g dry wt but is four times the rate reported by Palmer (1980) for much larger (60- to 100-mm) oysters. Dame et al. (1984) reported ingestion rates of oysters feeding on natural particulate of 0.39–2.02 mg of Chl d^{-1} per g dry weight, which agrees well with the average value of 0.74 mg of Chl d^{-1} per g dry weight reported here. Three scallops cleared an average 35% or 128 ml min^{-1} (equivalent to 97 ml min^{-1} per g dry weight for an average 43-mm scallop with a tissue dry weight of 1.3 g). This agrees well with Palmer's (1980) average clearance rate of 95 ml min^{-1} per g dry weight reported for 40-mm scallops fed <1.5 mg l^{-1} wet algal weight of cultured algae.

TABLE 3.

For oysters, Bonferroni (Dunn) pairwise *T*-tests for significant difference between mean growth and condition index from each flume compartment. Different letters denote significant differences between means ($\alpha = 0.05$).

Flume Position	Condition Index	Weight % incr/d	Length % incr/d
1	a	a	a
2	b	a	a
3	c	ab	ab
4	cd	b	b
5	de	b	b
6	de	c	c
7	de	c	c

Growth

Overall, the growth rates reported here are as fast or faster than some of the most rapid rates reported in the literature, pointing to the excellent growing conditions at this location (Ingle and Dawson 1952, Kirby-Smith and Barber 1974). Two trends are immediately obvious from the growth rate data (Figs. 6 and 7): growth rates are decreasing over time, and rates are also lower in downstream flume compartments. The depression of growth rate from the first 2 wk to the second 2 wk was probably related to the observed declines in average chlorophyll concentration (Fig. 3b), in total seston, and in seston organic content (Fig. 3a). The further decline in growth rate in the last 2 wk is probably related to temperature (Fig. 3c). A small proportion of this decline is also related to the fact that larger bivalves have an intrinsically slower rate of growth (Rheault 1995).

The downstream declines in growth and condition index (Figs. 7–10) all mimic the curves describing the percentage of incoming food remaining and the ration consumed by each compartment (Figs. 5 and 6). Plotting either growth or condition index against the calculated ration consumed reveals that these regressions are linear and the correlations are highly significant (Table 2). The correlations of length increase with ration were slightly weaker than the weight or condition index correlations, especially for oysters. This is to be expected because there are several examples in the literature where length is decoupled from other indices of growth (Dame 1972, Lawrence and Scott 1982, Hilbish 1986). It is important to note that these regressions are constructed from the final length and weight measurements and, therefore, represent an average growth rate for the entire 6-wk period. These average growth rates are obviously slower than the peak rates measured during the first 2-wk period and faster than those measured in the

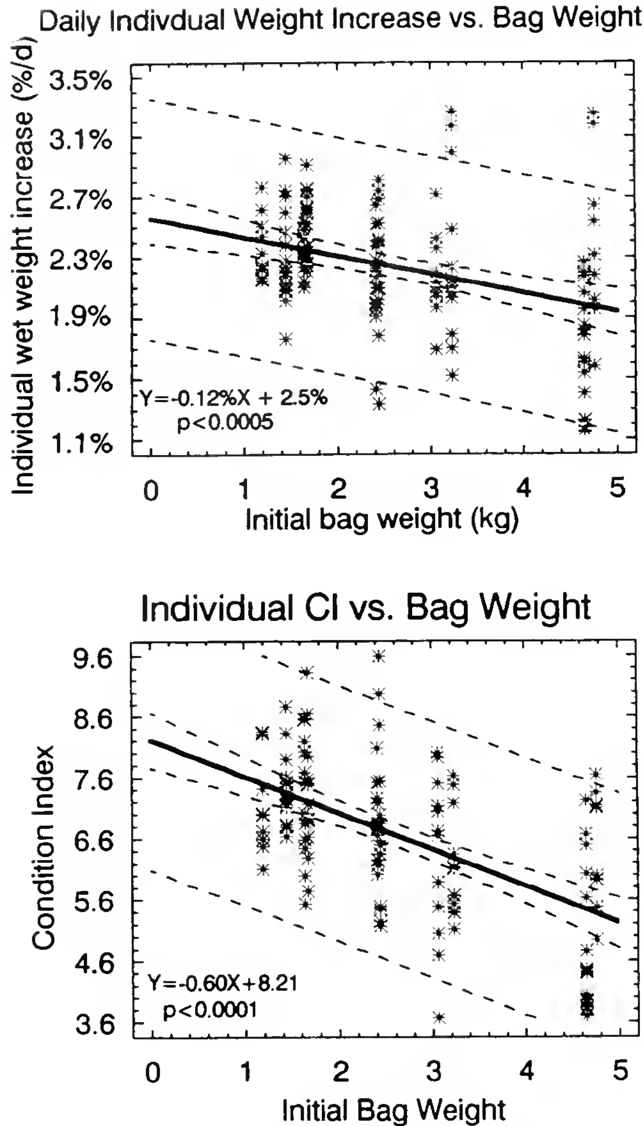


Figure 10. Individually tagged oysters from bags (Fig. 9) were measured after the 6-wk field study. Growth rates (percent increase in weight per day [a]) and condition index (b) are plotted against the initial stocking density of the bag. Linear regression formulae are shown with each line plotted with 95% confidence limits (inner pair of dashed lines) and 95% prediction limits (outer dashed lines).

last 2-wk period. Unfortunately, because both temperature and chlorophyll concentrations were declining over this period, our experimental design does not allow us to separate out the effects of these two variables.

Field Study Extrapolations

The calculated average current speed in the field grow-out bags (0.41 cm s^{-1}) was nearly identical to that measured in the flume (0.38 cm s^{-1}); however, the field animals experienced an oscillating tidal current, whereas the flow to the flumes was constant. These oscillations in flow resulted in large fluctuations in flux, such that four times a day, flux decreased to zero and maximum flux rates would be 1.7 times that experienced in the flume. It is unclear what the effect of this variability in food availability and flow has on the organism's ability to feed and assimilate food; however, there is some evidence that an intermittent feeding regime may enhance growth (Langton and McKay 1976).

In spite of the cyclic nature of the current speed, the daily average of all the 20-min flux estimates yielded a daily flux rate similar to that calculated for the flumes (Fig. 11) (see also Rheault 1995, Appendix III). Therefore, by comparing growth rates and condition indexes between the two studies, it is reasonable to infer the degree of seston depletion at various stocking densities in the field. Although there may be differences in the individual animal's perception of local food-depletion effects (depending on whether that individual is located in the middle of the grow-out bag or near the edge), the average growth response and condition index data from individuals in the grow-out bags should be comparable to those from the flume study.

Weight increases by individual oysters in the high-density bags ranged from 1.7 to 2.2% d^{-1} (Fig. 10a) and are comparable to those recorded in the second and third flume compartments (Fig. 6a). Oysters in low-density bags (1–2 kg per bag) appeared to grow slightly faster, at 2.4% d^{-1}, than did those in the first flume compartment, at 2.2% d^{-1}. Oyster condition index in the field study ranged from 5 to 8 (Fig. 10b), corresponding to condition indexes in the fifth and second flume compartments, respectively (Fig. 8). Extrapolating the growth response of the field animals to the flume study, we project that the low-density bags were experiencing a 27% local seston depletion, whereas the high-density bags were experiencing a 72% reduction in the ambient seston concentration.

Seven bags containing tagged scallops were stocked at 1.8 kg per bag. The average condition index was 4.2 ± 0.57 (SD; n = 68), similar to the condition index recorded in the first or second flume compartments. The average length increase was 0.5 ± 0.06% per d^{-1} (SD), which was similar to the second flume compartment, and the average total wet weight increase was 0.75 ± 0.23% d^{-1} (SD), comparable with the third or fourth flume compartments. Overall, scallops stocked at 1.8 kg per bag had a growth response similar to those in the second flume compartment; thus, we can infer that on average they were experiencing local seston concentrations approximately 42% of the ambient seston concentration outside of the bags.

Food Quality

Several researchers have remarked that chlorophyll concentration is a poor estimate of food availability or food quality for

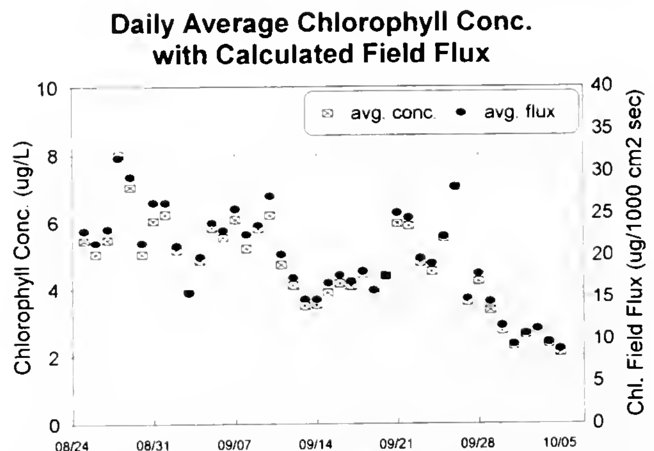


Figure 11. Daily average Chl-a concentration in flume (squares) is plotted with calculated daily Chl-a flux for the field site (circles).

suspension-feeding bivalves (Soniat et al. 1984, Wikfors personal communication). The in vivo method of measuring chlorophyll has its own limitations because changes in species composition, light adaptation, or nutrition can influence the phytoplankton excitation-emission response (Loftus and Seliger 1975). Nonetheless, earlier work in Point Judith Pond (Rheault and Rice, accepted) examining the food-limited growth of three species of juvenile shellfish demonstrates that the fluxes of both chlorophyll and particulate organic matter (POM) were significantly correlated with shellfish growth. Stepwise multiple regressions of chlorophyll or POM flux and temperature accounted for over 85% of the variance in growth.

The continuous recording fluorometer permitted us to accurately chronicle the temporal variability in food availability characteristic of estuarine environments. It is questionable whether any discrete sampling regime could adequately characterize the food concentration under these conditions. Whereas the measurement of carbon, nitrogen, lipid, protein, carbohydrate, or caloric content might have provided more physiologically meaningful estimates of food, these measurements can only be made on discrete samples and do not readily give one a picture of the true variability of these features in a dynamic estuarine environment.

Indices of Growth

This study used several approaches to monitoring the growth and condition index responses to varying conditions of food limitation. For oysters, the condition index was determined using the ratio of dry tissue weight to the estimated shell cavity volume (total wet weight to shell weight) (Lawrence and Scott 1982). This approach avoids some of the problems associated with varied shell thickness and morphology in oysters. We also monitored growth in shell height (longest axis) and total wet weight. Shell height showed the most variability and had the poorest correlation with ration, flume position, or bag stocking density. Condition index proved to be the most sensitive of the indices to changes in ration downstream and is the preferred method of assessing the health of a population (Lucas and Beninger 1985, Rainer and Mann 1992).

For scallops, we used a simpler condition index, based on the ratio of tissue dry weight to shell height (longest axis) because scallop shell morphology is more regular than that of oysters. The variability in shell growth rate and condition index was much less than that seen in wet weight growth, possibly because a small percentage of the scallops were visibly gravid. It was not anticipated that 6-month-old animals should be capable of developing ripe gonads (Barber and Blake 1981).

For oysters, condition index was the most consistent static index of physiological state (Lucas and Beninger 1985), showing the lowest coefficient of variation and the best correlations with ration or bag-stocking density. Condition index provides a reliable measurement for comparing similar-sized animals; however, condition index varies with size, season, and reproductive output, as well as with physiological state. For condition index to become useful as a spot check index of physiological state would require data on well-fed and starved animals over a range of sizes and seasons. With these data, one would have a reference point with which to instantly gauge the health of the sample in question (Lawrence and Scott 1982).

CONCLUSIONS

In conclusion, the downstream depletion of natural seston in a flow-through flume resulted in marked reductions in growth and condition index. Chlorophyll concentration was monitored continuously in the incoming flow, and downstream food depletion was characterized several times over the 6-wk trial. The bivalves in each stage of the flume removed a constant percentage of the available food over a wide range of concentrations, resulting in smaller rations downstream. Scallop and oyster clearance rates (milliliters per minute) were constant over a wide range of chlorophyll concentrations, suggesting that these species will filter natural seston at a near-constant rate, despite fourfold tidal variations in food concentrations. Scallop clearance rates were reduced when chlorophyll concentrations were depleted to below 12% of the natural levels, suggesting a threshold feeding response similar to that reported for mussels (Thompson and Bayne 1972, Wilson and Seed 1974). Concurrent field grow-out studies conducted nearby compared the effect of varying initial stocking densities in a commercial shellfish aquaculture operation. Comparable changes in growth and condition index were observed in the field trials, allowing estimates of in situ horizontal seston flux. Although physiological responses to food limitation will necessarily be site specific to varying combinations of temperature, current speed, and food concentration or quality (Newell and Shumway 1993), this work provides an opportunity to compare the growth response of oysters and scallops under a wide range of food availability in both laboratory and commercial aquaculture settings. In these experiments, doubling the stocking density from 2.5 to 5.0 kg of oysters per bag resulted in a 20% decrease in both the condition index and the growth rate (percent increase in weight). These observations may provide valuable insights to the commercial grower by assisting in decisions pertaining to optimal stocking density for aquaculture grow-out systems.

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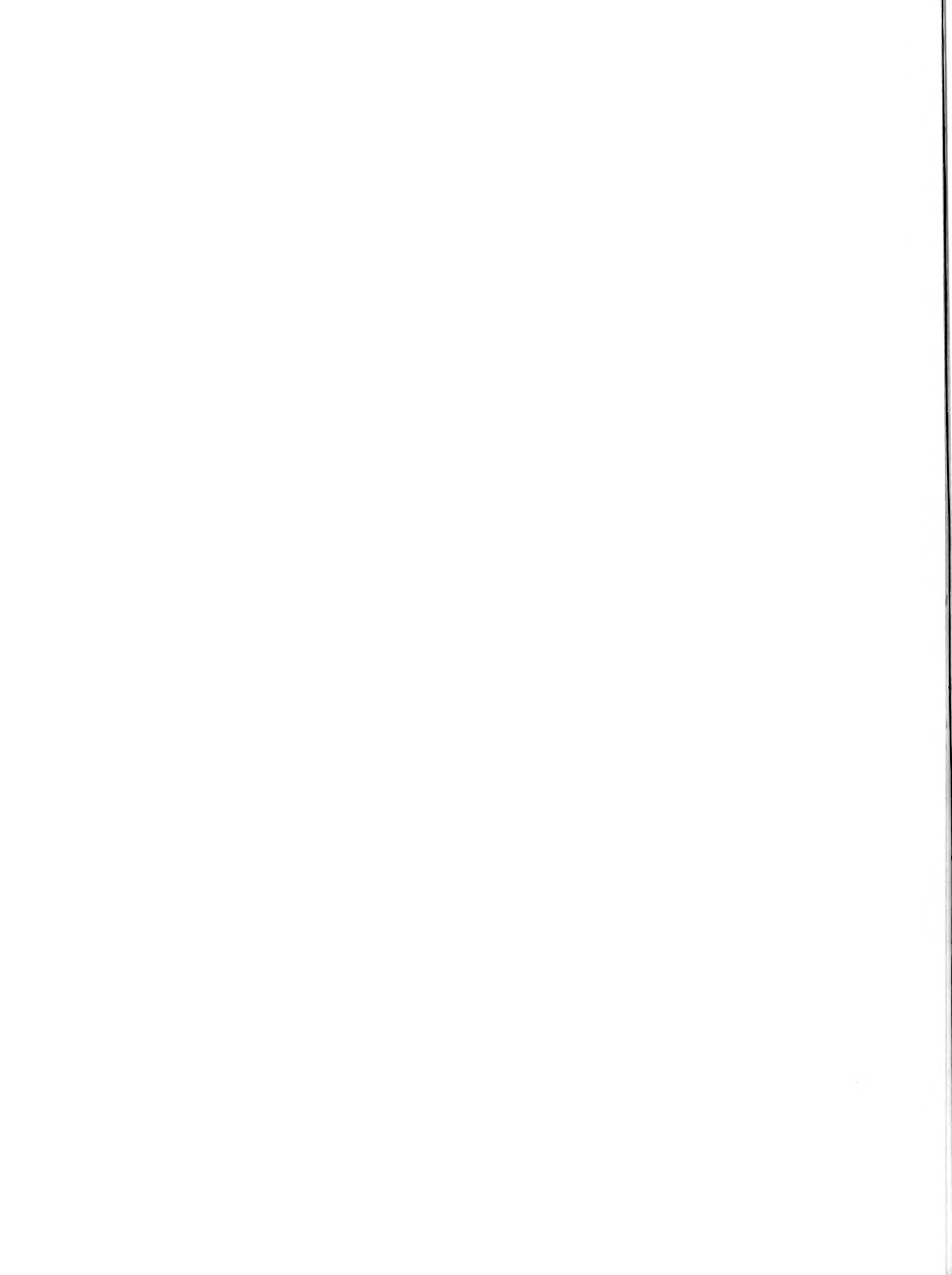

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GAMETOGENESIS OF EASTERN OYSTERS, *CRASSOSTREA VIRGINICA* (GMELIN, 1791), AND PACIFIC OYSTERS, *CRASSOSTREA GIGAS* (THUNBERG, 1793) IN DISEASE-ENDEMIC LOWER CHESAPEAKE BAY

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ABSTRACT Gametogenic cycles were compared for oysters, *Crassostrea virginica* and *C. gigas*, held in flumes receiving water from the York River, VA, where two protozoan parasites, *Haplosporidium nelsoni* and *Perkinsus marinus*, are endemic. Gametogenesis in *C. virginica* was characterized by a general lack of development, maturation, and spawning. Only two *C. virginica* developed mature gametes, and none showed evidence of spawning. From July onward, most individuals exhibited gamete resorption. In contrast, gamete development, maturation, and spawning were well defined and synchronous in *C. gigas*. Mature individuals predominated in June, and spawned individuals predominated in July. Mean gonadal area indices (GAI) were significantly different ($P \leq 0.001$) between months and species. Mean oocyte areas were significantly different ($P \leq 0.001$) between months. Significant month × species interactions ($P \leq 0.001$) for both GAI and oocyte areas supported the differences in gametogenic cycles observed between species by the use of subjective staging. Among mature females, *C. gigas* had both a significantly greater ($P \leq 0.001$) GAI and mean oocyte area than *C. virginica*. Combined prevalence of the parasites *H. nelsoni* and *P. marinus* increased from 0 to 86.7% in *C. virginica* between May and August. Infection intensity increased from epithelial infections to systemic infections from June through September. These parasites were never detected in *C. gigas*. The difference in gametogenic cycles observed between oyster species could be related to differences in susceptibility to the parasites *H. nelsoni* and *P. marinus*, genetic differences in gametogenic cycles, or a combination of both factors. This study establishes the ability of *C. gigas* to produce gametes and spawn in the environment prevailing in lower Chesapeake Bay.

KEY WORDS: *Crassostrea virginica, Crassostrea gigas*, gametogenesis, *Haplosporidium nelsoni, Perkinsus marinus*, disease

INTRODUCTION

The fact that once-abundant populations of Eastern oysters, *Crassostrea virginica*, in the mid-Atlantic region of the eastern United States have been severely depleted by the protozoan parasites *Perkinsus marinus* (Dermo) and *Haplosporidium nelsoni* (MSX) is well documented (Andrews 1988, Haskin and Andrews 1988). *H. nelsoni* first appeared in Delaware Bay in 1957 and was responsible for oyster mortalities of 60% in seed areas and 90–95% in planting grounds in New Jersey within 3 y (Haskin et al. 1966, Haskin and Ford 1979, Ford and Haskin 1982). *H. nelsoni* was seen in Chesapeake Bay (Virginia) in 1959 and, within 2–3 y, caused the mortality of 90–95% of oysters in the lower bay (Andrews and Wood 1967). As a result of these two pathogens, annual harvests that in Virginia averaged 3.5 million bushels before 1960, are now less than 50,000 bushels (Mann et al. 1991).

To augment oyster production in the Chesapeake Bay, the introduction of the Pacific oyster, *Crassostrea gigas*, has been suggested (see Mann et al. 1991). Knowledge pertaining to the ability of *C. gigas* to survive, grow, and reproduce in the Chesapeake Bay environment is essential to this debate. Survival ability relates primarily to tolerance of the endemic pathogens *H. nelsoni* and *P. marinus*. In exposure experiments, Meyers et al. (1991) and Barber and Mann (1994) found that *C. gigas* was highly resistant to *P. marinus* compared with *C. virginica* controls. When exposed to infective stages in a quarantined flume system, a high initial prevalence of *P. marinus* infection occurred, but in general, intensity remained low and the disease never developed. The mortality of *C. gigas* that did occur was not related to disease (Barber and Mann 1994). Similarly, when triploid (to minimize the chance of gamete release) *C. gigas* were exposed to *H. nelsoni* in Chesapeake Bay (Virginia), they were found to be highly resistant to this parasite; within 4 mo, *H. nelsoni* prevalence was 84–92% in *C. virginica* control groups and 0% in *C. gigas* (Burreson et al. 1994). Clearly, *C. gigas* is capable of surviving exposure to both Chesapeake Bay pathogens.

The growth of *C. gigas* in the Chesapeake Bay environment was evaluated by Barber and Mann (1994), who found that in quarantined flumes, the mean shell height of *C. gigas* was significantly ($P \leq 0.05$) greater than that of *C. virginica* through age 19 mo. In addition, there was no difference in mean shell height between groups of oysters exposed to light and heavy doses of *P. marinus*.

Knowledge of the reproductive capability of *C. gigas* in Chesapeake Bay is needed to determine its potential for establishing viable natural populations and possibly outcompeting the native *C. virginica*. The object of this study was to compare the gametogenic cycles of *C. virginica* and *C. gigas* in lower Chesapeake Bay (Virginia), where both *H. nelsoni* and *P. marinus* are endemic.

MATERIALS AND METHODS

In March 1992, approximately 300 *C. virginica* (mean shell height, 68.3 mm) were collected from Horsehead Reef in the James River, VA, and placed in a flume located on the Virginia Institute of Marine Science (VIMS) campus. A similar number of *C. gigas* (mean shell height, 73.6 mm) were held in a separate flume (see Barber and Mann 1994). Both flumes received a continual supply of raw seawater from the York River, VA. Flow rates of about 20 l/min were maintained in both flumes. Flumes

*This work was initiated while the author was employed by the Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA, 23062.

were drained and flushed as needed to remove fouling organisms and biodeposits. Water temperature and salinity were continuously recorded from the VIMS pier in the York River.

Beginning in March and continuing each month until October 1992, 15 individuals of each species (n = 13 *C. virginica* in June) were processed for histological examination (Howard and Smith 1983). Shucked oysters were fixed in Davidson's AFA. A standard transverse section of the visceral mass taken at the intersection of the gills and labial palps (including gill, mantle, stomach, intestine, digestive diverticula, and gonad) was dehydrated, cleared, and embedded in Paraplast. Sections (6 μm) were mounted on slides and stained with Harris' Hematoxylin and Eosin Y.

Slides were examined for gametogenic activity with a compound microscope (Nikon Labophot, 100×). Each individual was assigned a stage of gonadal development on the basis of descriptions made by Kennedy and Battle (1964), as follows.

Stage 0 (Inactive)

In this stage, follicles are nonexistent or elongated, with walls consisting of undifferentiated germinal epithelium.

Stage 1 (Early Active)

Follicles contain oogonia or spermatogonia and primary oocytes or spermatocytes but no free oocytes or spermatozoa.

Stage 2 (Late Active)

Secondary (free) oocytes and spermatocytes predominate the follicles; there are some spermatozoa.

Stage 3 (Mature)

Mature gametes (ova or spermatozoa) completely fill the follicles: ova with distinct nucleus and nucleolus are present, as are spermatozoa oriented with tails toward the follicle lumen.

Stage 4 (Spawned)

Follicles contain spaces devoid of gametes, although numerous gametes may still remain; follicle walls may be broken. Redevelopment, as evidenced by an increased number of primary oocytes or spermatocytes, may be occurring.

Stage 5 (Resorbing)

Follicles have a shrunken appearance and contain numerous phagocytes and products of resorption. Gametes are refractory, and redevelopment is not evident.

In addition to the subjective staging of all individuals, gametogenesis was quantitatively assessed with an image analysis system consisting of a video camera mounted on a microscope and connected to a videographics adapter (Truevision Targa+). Images from the prepared slides were enhanced, and data were collected by the use of image analysis software (Image Pro Plus). For all individuals, a gonadal area index (GAI), defined as the ratio of the gonadal area to the area of the entire visceral mass multiplied by 100, was calculated. This technique is a sensitive indicator of both gametogenic cycles (i.e., timing) as well as relative fecundity (amount of gametogenic material produced) (Barber et al. 1988a, Barber et al. 1991). In addition, for each female, the cross-sectional areas of 25 gametes (oogonia, oocytes, and ova) sectioned through the nucleus were measured (600×). Measuring areas reduces the variability associated with measuring the diameters of nonspherical objects (e.g., Barber and Blake 1983, Barber et al. 1988b). Both mean GAI and mean oocyte areas were compared for both oyster species over time with two-way analysis of variance (ANOVA) (Wilkinson et al. 1992).

Slides of each oyster were also examined (100×) to determine the prevalence and intensity of parasite infection. For *H. nelsoni*, infections were classified as either "epithelial" (confined to the gills) or "systemic" (found throughout the tissues). For *P. marinus*, "epithelial" infections were those confined to gut epithelium, whereas "systemic" infections involved other tissues.

RESULTS

The pattern of gametogenesis, as revealed by the number of individuals in each gametogenic stage, differed between the two species. For *C. virginica*, sexual differentiation (Stage 1) was evident in both March and April (Fig. 1). In May and June, continued development to Stage 2 was seen, but five individuals were inactive (Stage 0) in June. From July through September, most individuals either were undergoing the resorption of gametes (Stage 5) or were inactive (Stage 0). In October, all but one individual were inactive (Stage 0). Only two mature (Stage 3) individuals and no spawned (Stage 4) individuals were seen at any time, and there were inactive (Stage 0) oysters present in all months. Thus, a typical cycle of gamete growth, maturation, and spawning was not observed in *C. virginica* (Fig. 1).

In contrast, gametogenesis in *C. gigas* was complete and synchronous (Fig. 2). From March through May, most individuals were sexually differentiated (Stage 1). Further development occurred rapidly after May, and most individuals were mature (Stage 3) in June. In July and August, spawned (Stage 4) individuals predominated, and in September and October, the vast majority of individuals were inactive (Stage 0). Thus, *C. gigas* exhibited an obvious, complete gametogenic cycle, having clearly defined periods of maturation, spawning, and resorption (Fig. 2).

Trends in the quantitative measures of gametogenesis differed between the two oyster species and supported the general patterns of gametogenesis indicated by the staging process. For *C. virginica*, the mean GAI was 0 in March and April and reached a maximum of 9.8% in June (Fig. 3). Slight declines in mean GAI after June reflected the general loss of gametes due to resorption (rather than spawning) noted above. GAI continued to decline in October, as gametes in all but one individual were resorbed. For *C. gigas*, the mean GAI was 0 in March and May, but in June, a

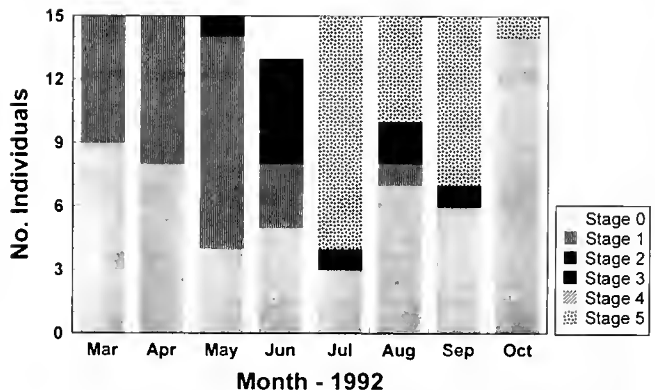

Figure 1. The number of individuals (*C. virginica*) of each gametogenic stage from March through October 1992.

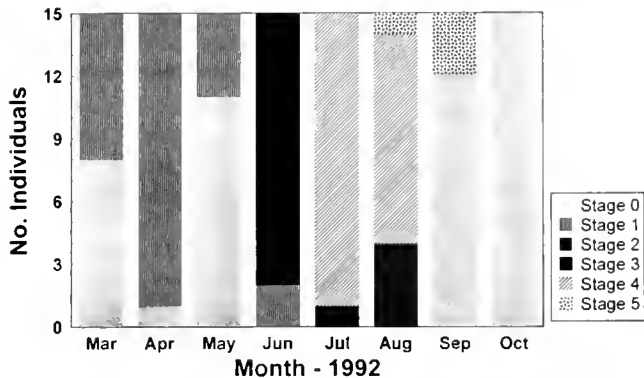

Figure 2. The number of individuals (*C. gigas*) of each gametogenic stage from March through October 1992.

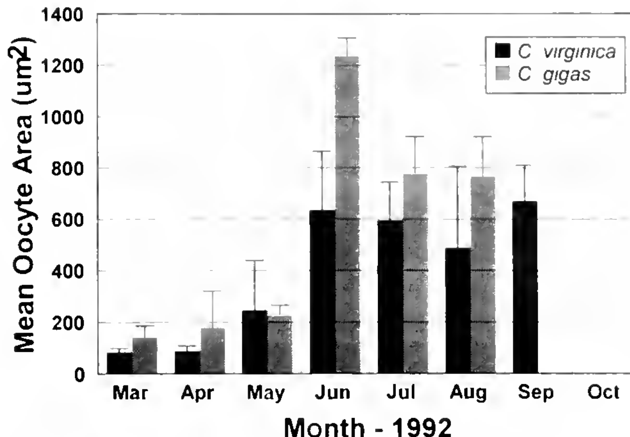

Figure 4. Mean (+1 SD) oocyte areas of *C. virginica* and *C. gigas* from March through October 1992.

maximal value of 41.5% occurred in conjunction with a predominance of mature individuals (Fig. 3). Subsequent reductions in mean GAI in July and August supported the observation of spawned individuals of this species. The GAI in October was 0, reflecting the fact that the resorption of gametes was complete.

Two-way ANOVA revealed significant differences ($P \leq 0.001$) in GAI both between months and between species. There was also a significant month × species interaction ($P \leq 0.001$), indicating that the cycle of gametogenesis, as indicated by GAI, differed between the two species. The difference in mean GAI between species was considerable (Fig. 3). For mature (Stage 3) individuals, the mean GAI of 43% for *C. gigas* was significantly greater (t-test, $P \leq 0.001$) than that of 13% for *C. virginica*.

Considerable differences in mean oocyte areas were also seen between the two oyster species. For *C. virginica*, little oogenic activity occurred from March through May, as mean oocyte areas were well below 300 μm^2 (Fig. 4). The mean oocyte area increased to 635 μm^2 in June and then fluctuated between about 490 and 670 μm^2 from July through September, reflecting a combination of oocyte resorption (most individuals) and oocyte development (five individuals). There were no female oysters with measurable oocytes in October. For *C. gigas*, the mean oocyte area was also below 300 μm^2 from March through May (Fig. 4). In June, however, the mean oocyte area increased to a maximum of over 1,200 μm^2. This was followed in July and August by a decrease in mean oocyte area of 700–800 μm^2, as the larger, more mature oocytes were released during spawning. There were no female oysters with measurable oocytes in September or October.

As indicated by two-way ANOVA, there was a significant difference in mean oocyte area between months ($P \leq 0.001$) but not between species ($P > 0.122$). There was also a significant month × species interaction ($P \leq 0.001$), again supporting the existence of different patterns of gametogenesis between oyster species, as determined by oocyte area. Even though there was not an overall difference in mean oocyte area between species, the mean oocyte area of mature (Stage 3) females was significantly greater ($P \leq 0.001$) for *C. gigas*, averaging 1,236 μm^2, compared to 820 μm^2 for *C. virginica*.

There was a considerable difference in the prevalence of parasites infecting the two oyster species. Parasites were only found in *C. virginica* (Table 1). The combined prevalence of infection by *H. nelsoni* and *P. marinus* ranged from 0% in March through May to 86.7% in August; prevalence decreased to 26.7% in October. The intensity of infection by both parasites, as indicated by the number of systemic infections, increased from June through September before declining in October (Table 1). Individuals infected by both *H. nelsoni* and *P. marinus* were seen in July (n = 1),

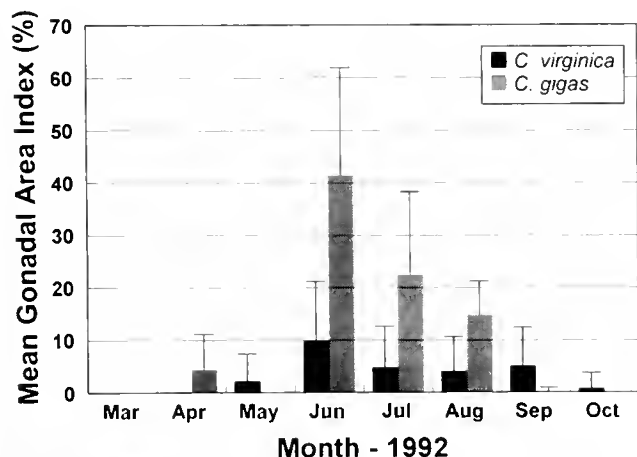

Figure 3. Mean (+1 SD) GAIs of *C. virginica* and *C. gigas* from March through October 1992.

TABLE 1.

Number of oysters, *C. virginica*, infected by each of the parasites *H. nelsoni* and *P. marinus* and overall prevalence (% of oysters infected) on each sampling date.

Date	No. of Oysters	*H. nelsoni* U-E-S	*P. marinus* U-E-S	Prevalence (%)
March 17	15	15-0-0	15-0-0	0/15 = 0
April 17	15	15-0-0	15-0-0	0/15 = 0
May 16	15	15-0-0	15-0-0	0/15 = 0
June 17	13	10-2-1	9-4-0	7/13 = 53.8
July 16	15	9-1-5	10-5-0	10/15 = 66.7*
August 14	15	6-3-6	5-5-5	13/15 = 86.7*
September 18	15	10-0-5	7-3-5	12/15 = 80.0*
October 20	15	13-1-1	13-1-1	4/15 = 26.7

Infection intensity is categorized as: U = uninfected; E = epithelial; S = systemic.

* Includes oysters infected by more than one parasite species.

August (n = 6), and September (n = 2). In addition, two individuals (n = 1 in both June and September) infected with *P. marinus* also contained sporocysts of the trematode *Bucephalus cuculus*.

The weekly mean water temperature and salinity in the York River, VA, in 1992 are shown in Figure 5. Temperature generally increased from a low of 3.8°C in February to 27.5°C in August and then steadily decreased from September through December. Salinity was generally above 20 ppt, fluctuating between a low of 17.6 ppt and a high of 23.4 ppt. Salinity was below 20 ppt from April to July and again in September.

DISCUSSION

There were clear differences between the two oyster species examined in this study in both qualitative and quantitative aspects of gametogenesis, even though both experienced identical environmental conditions. The primary qualitative difference in gametogenesis was the lack of a clearly defined, synchronous, and complete cycle of gamete development and spawning in *C. virginica*. Although sexual differentiation occurred as early as March and gamete development continued until June, only two individuals having mature gametes were seen and at no time was evidence of spawning observed. Instead, beginning in July, a large proportion of oysters began to resorb gametes, as follicles began to shrink and were infiltrated by hemocytes. In addition, inactive oysters were present in all months. The lack of postspawn individuals and the large number of inactive and resorbing individuals suggest that spawning never occurred in *C. virginica*. In contrast, *C. gigas* exhibited a complete, well-defined, and synchronous gametogenic cycle in which a single spawning occurred between 17 June and 16 July. Like *C. virginica*, sexual differentiation began as early as March and continued through April. The greater number of inactive individuals seen in May compared with April could be related to the relatively low salinity (<20 ppt) occurring at that time; the optimal salinity for the development of *C. gigas* larvae at 25°C is between 20 and 26 ppt (Amemiya 1928). Gamete development was rapid after 16 May, however, and most individuals were mature in June. In July, most individuals had spawned. This was followed by minor redevelopment in August and rapid resorption in August and September. Unlike *C. virginica*, no inactive *C. gigas* were seen from June through August. There were significant differences in both GAI and oocyte area over time for both *C. virginica* and *C. gigas*. Overall, the mean GAI was significantly greater for *C. gigas* than for *C. virginica*, but the mean oocyte area was similar for both species. The fact that there was a significant interaction between species and month for both GAI and oocyte area is reflective of the fact that the pattern (or extent) of gametogenesis, as measured by these two parameters, differed for the two oyster species.

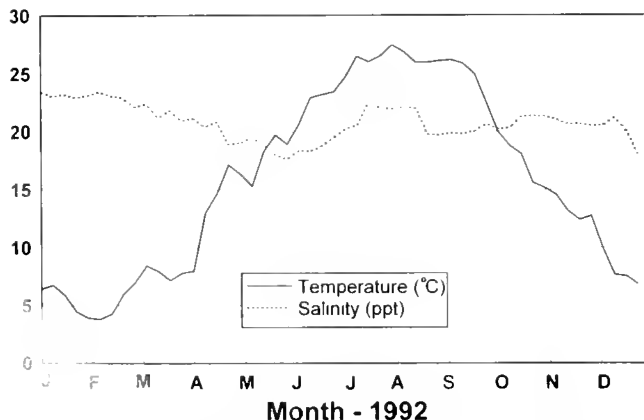

Figure 5. Weekly means of water temperature and salinity in the York River, VA, in 1992.

There were major differences in disease prevalence between *C. virginica* and *C. gigas*. In the case of *C. virginica*, neither *H. nelsoni* nor *P. marinus* was detected in this species until June, 3 mo after being transferred from the James River to the York River, making it likely that oysters were uninfected at the time of transfer. It is possible, however, that some early *P. marinus* infections were missed as a result of relying on histology for diagnosis rather than the standard thioglycollate technique (Ray 1966) or a more sensitive hemolymph assay (Gauthier and Fisher 1990). From June through August, both the prevalence and the intensity of infections increased. In addition to high levels of *H. nelsoni* and *P. marinus*, the trematode *B. cuculus* was seen in 2 oysters, and 11 oysters were infected by more than one parasite species. In contrast, no parasites were seen in *C. gigas* at any time during this study. It is possible, however, that *C. gigas* did become infected with *P. marinus*, as noted previously (Meyers et al. 1991, Barber and Mann 1994), but infections never developed to the point where they were detectable by histological examination. *C. virginica* was thus susceptible and lacked resistance to all three parasite species, whereas *C. gigas* was either not susceptible or was highly resistant to the parasites.

The lack of gametogenesis exhibited by *C. virginica* may in part be related to the source of the oysters used in this study. As described by Cox and Mann (1992), oysters on Horsehead Reef in the James River, VA, tend to have a lower fecundity compared with oysters inhabiting locations further downriver, probably the result of salinity, which averages less than 15 ppt. In spite of producing fewer eggs than oysters from other areas, however, Horsehead oysters showed evidence of spawning in both July and August (Cox and Mann 1992). In addition, there is no reason to suggest that oysters from Horsehead Reef would not be capable of undergoing a normal gametogenic cycle after transfer to the York River (salinity >20 ppt).

A more likely explanation for the failure of *C. virginica* to undergo a complete gametogenic cycle is the combined negative effects of *H. nelsoni* and *P. marinus* and, to a lesser extent, *B. cuculus*, on reproduction. Several studies have noted a quantitative difference in gamete production between infected and uninfected individuals. For example, Barber et al. (1988a) found that compared with uninfected individuals, gamete production in oysters with epithelial and systemic *H. nelsoni* infections was reduced by 35 and 81%, respectively. Similarly, oysters with heavy *P. marinus* infections had significantly smaller gonadal areas than did uninfected oysters (Dittman 1993). Choi et al. (1994) found a negative correlation between the intensity of *P. marinus* infection and the rate of gonadal production. Although not considered pathogenic, sporocysts of the trematode *B. cuculus* typically invade oyster gonad tissue, causing castration (Menzel and Hopkins 1955, Sindermann 1990). Both oysters containing sporocysts of *B. cuculus* in this study were devoid of gametes. Although few *C. virginica* were free of parasites during the months of July through September, when the greatest gametogenic activity should have occured, differences between infected and uninfected individuals were noted. For example, the mean GAI of uninfected individuals was significantly greater (t-test, $P \leq 0.01$) than that of parasitized individuals from June through September. Further, in July, Au-

gust, and September, when parasite prevalence and intensity were greatest, 14, 12, and 14 individuals, respectively, were either inactive or actively resorbing gametes. This, in combination with the fact that parasites were not detected in the only mature individuals encountered in this study, strongly suggests that in the York River, most if not all gamete production in *C. virginica* was overcome by the negative effects of both *H. nelsoni* and *P. marinus*.

Parasites might also have an effect on the timing of gametogenic events of oysters. Ford and Figueras (1988) and Ford et al. (1990) found that in Delaware Bay, gametogenesis in *C. virginica* was inhibited in late spring when *H. nelsoni* levels were high, but as temperature-associated remission occurred in August and September, many oysters developed mature gonads and spawned. In this study, however, only 5 (out of 45) oysters were undergoing gamete development from July to September. Although it is possible that rapid development and spawning occurred between samples and were undetected, it is more likely, given the large number of resorbing and undifferentiated individuals seen during this period, that oysters generally succumbed to the effects of parasites, which maintained a high intensity until October, at which time the temperature had dropped to below 15°C and was too low to support gamete development and spawning. Thus, the combined effects of *H. nelsoni* and *P. marinus* particularly, both in terms of intensity and duration throughout the year, limited the ability of *C. virginica* to produce gametes and spawn.

Observed differences in gametogenesis between *C. virginica* and *C. gigas*, especially those pertaining to the timing of events and rates of development, may have a genetic basis. It is known, for example, that there are genetic differences in the timing of gonadal maturation and spawning between populations of *C. virginica* having different geographic origins (Barber et al. 1991, Ford et al. 1990): As seen in this study, *C. gigas* spawned once between mid-June and mid-July. According to Andrews (1979), *C. virginica* in Chesapeake Bay typically spawns over a 3-mo period (June to August). Differences in the quantitative aspects of gametogenesis such as fecundity and egg size might also differ between species. In this study, the mean GAI of mature *C. virginica* was 13%. Much greater maximum mean GAIs of 30% and 35–45% were found by Barber et al. (1988a) and Barber et al. (1991), respectively, who compared gonadal development among several groups of *C. virginica* in Delaware Bay. It is unclear to what extent the difference between maximal GAI found here and those seen in previous studies is related to local environmental conditions versus levels of parasitism. In contrast, the fecundity of *C. gigas* appears to be much greater than that of *C. virginica*. The GAI of 43% found in this study for mature *C. gigas*, although much greater than that found for mature *C. virginica*, is less than the maximal values of 65–79% reported for *C. gigas* in previous studies (Mori 1979, Perdue et al. 1981, Allen and Downing 1986), perhaps the result of environmental variations between locations. Another difference between species noted in this study was mean oocyte size. Mean oocyte areas of mature females were 1,236 μm^2 for *C. gigas* and 820 μm^2 for *C. virginica*, which correspond to mean diameters of 39.7 and 32.3 μm, respectively. Barber et al. (1988a) reported a maximum oocyte diameter for *C. virginica* from Delaware Bay of 36–37 μm. Even with a greater mean oocyte area, the larger GAI of *C. gigas* gives it a distinct advantage in terms of number of eggs produced. For mature oysters having a total cross-sectional area of 100 mm^2, 34,790 eggs would be bisected in the gonadal area of *C. gigas* compared with 15,850 eggs for *C. virginica*. Thus, in Chesapeake Bay waters, there are differences between oyster species in the timing of maturation and spawning as well as in relative fecundity, egg size, and egg number, which are unrelated to the differential effects of parasitism.

Given the fact that both *H. nelsoni* and *P. marinus* have been endemic in lower Chesapeake Bay since 1959 (Andrews and Wood 1967; Andrews 1988) with no sign of abating, *C. gigas* appears to have a distinct advantage over *C. virginica* in terms of disease resistance (Meyers et al. 1991; Barber and Mann 1994), growth (Barber and Mann 1994), and as seen in this study, reproductive potential. The production of *C. virginica* in Chesapeake Bay is unlikely to increase until both parasites diminish in virulence or resistance to both diseases is developed by the oyster host.

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THE SUITABILITY OF LAND-BASED EVALUATIONS OF *CRASSOSTREA GIGAS* (THUNBERG, 1793) AS AN INDICATOR OF PERFORMANCE IN THE FIELD

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ABSTRACT The introduction of *Crassostrea gigas* to the mid-Atlantic requires prior knowledge of their likely ecological response, according to the International Council for Exploration of the Seas Committee guidelines. Without at least an experimental introduction, however, such knowledge is unattainable. Are comparisons of survival, growth, disease resistance, etc., conducted in land-based tanks suitable for estimating the performance of *C. gigas* in the field? In June 1991, equal numbers of spat from three crosses—MSX-resistant *Crassostrea virginica* (eastern), *C. gigas* form Miyagi, and *C. gigas* form Hiroshima—were split into two replicates and reared in upwellers for the first summer and in a land-based tank for the second. After the first season, *C. virginica* had the highest mortality (65, 36, and 13% for eastern, Miyagi, and Hiroshima, respectively) and average spat size was about 30% greater in both *C. gigas* groups. For the second year, the three crosses were transferred to a 16,000-L tank; two replicates of eastern oyster were also placed in Delaware Bay. Cumulative mortality for the second season (through 11/92) was eastern, 60%; Miyagi, 73%; Hiroshima, 93%; and eastern in Delaware Bay, 37%. In the tank, Miyagi oysters grew fastest, followed by Hiroshima and eastern; however, eastern oysters grown in the field were larger than all tank-reared groups. All oysters in the tank were infested with *Polydora websteri*, *C. gigas* heavily and eastern oysters lightly; eastern oysters grown in the field were virtually free of infestation. These data indicate that tank-based comparisons are unlikely to yield a true measure of performance in the local environment.

KEY WORDS: Non-native species, Pacific oyster, eastern oyster, disease resistance, Miyagi, Hiroshima

INTRODUCTION

Crassostrea gigas (Thunberg, 1793), an oyster native to Japan but widely introduced around the world, has recently generated interest in the mid-Atlantic as a candidate for revitalizing the oyster fisheries of both Delaware and Chesapeake Bays (Mann et al. 1991, Lipton et al. 1992, Gaffney and Allen 1992). There are two principal ways that *C. gigas* might help. The first is by the direct introduction of the species as a replacement or supplemental fishery. This approach engenders the most controversy because it is (probably) irreversible using normal diploid populations. The second is the incorporation of useful genes from *C. gigas*, especially for disease resistance, into the native eastern oyster through hybridization or gene insertion. The idea that gene incorporation is a reasonable approach is indicated by recent evidence of disease resistance in *C. gigas*. *C. gigas* is more tolerant to *Perkinsus marinus* (Dermo) than *Crassostrea virginica* (Meyers et al. 1991) and also seems to be resistant to *Haplosporidium nelsoni* (MSX) (Allen unpublished data, Burreson et al. 1994). So far, however, hybrids between eastern and Pacific oysters have failed (Allen et al. 1993).

Whether direct introduction or gene introduction takes place, the field evaluation of pure Pacific oysters, Pacific-eastern hybrids, or genetically modified eastern oysters will be essential. In addition to the question of disease resistance, there are a host of ecological questions regarding the suitability of the new candidate oyster for culture in the mid-Atlantic. Some have suggested that these tests need to be conducted in confined systems, i.e., land-based and quarantined. This was especially true when proposals to test certified triploid Pacific oysters in the mid-Atlantic were tendered (Allen 1993). Would the same be required of hybrids or genetically modified oysters? If, for example, hybrids were produced, would tests run in land-based tanks yield meaningful data on the likely field performance of candidate oysters? The following study was conducted to answer this question.

MATERIALS AND METHODS

1991 Season

In late May and early June 1991, larvae from three crosses were placed into culture at Rutgers Cape Shore hatchery: eastern oysters (*C. virginica*), a cross between two Rutgers MSX-resistant lines; *C. gigas* form Miyagi, an F2 Washington state import; and *C. gigas* form Hiroshima, an F2 import from southern Japan. Larvae were reared in 211-L polypropylene tanks filled with 1-µm-pore-size filtered seawater at ambient temperature and salinity. During the larval culture period, seawater temperature ranged from 23 to 28°C and salinity ranged from 20 to 24 ppt. Larvae were fed a mixture of *Isochrysis* aff. *galbana*, *Thalassiosira pseudonana*, and *Chaetoceros calcitrans* at densities recommended by Breese and Malouf (1975). Tanks were drained every 48 h, at which time larval growth and survival rates were determined. Miyagi had the highest survival to 48 h, but all groups had similar survival to the eyed stage (Table 1). All groups were set as cultchless oysters with epinephrine, generally following the guidelines recommended by Coon et al. (1986). Recently set spat were held for 7–10 days in hatchery downwellers before rotation to a recirculating, upweller nursery system. Approximately 1 wk after rotation to the upweller, 4,130 animals from each of the three groups were taken from the size class retained by a 1.98-mm screen. Mean shell length (N = 50) was 3.8, 3.2, and 3.4 mm for eastern, Miyagi, and Hiroshima, respectively. The samples were split into two groups of 2,065, and each group was placed into a 30-cm-diameter × 46-cm-tall upweller silo receiving ~4 L/min of Delaware Day seawater, filtered to 100 µm. The relative position

TABLE 1.

Spawning and larval survival data for eastern, Miyagi, and Hiroshima progeny groups used for this study.

Parent Stock	Spawn Date	No. of Spawners		No. of Eggs (millions)	Percent Survival to		Progeny Code
		Female	Male		48 h	Eyed	
DF × BLA	5/28/91	18	12	13	23	9	eastern
XWAA	5/29/91	5	8	17	55	21	Miyagi
ASJPNA	6/4/91	8	15	25	23	10	Hiroshima

DF and BLA are seventh- and fifth-generation lines of MSX disease–resistant strains bred at the Haskin Shellfish Laboratory. XWAA is the second generation of *C. gigas* form Miyagi imported from Washington in 1989. ASJPNA is a first-generation line of *C. gigas* form Hiroshima imported from Japan in 1990.

of each silo within the raceway was rotated on a daily basis to minimize position effects. In July and August, upweller silos were graded every 2 wk by the use of a sieve series (11 sieves: 1.52, 1.98, 2.87, 3.5, 5.54, 7.9, 8.98, 10.9, 12.0, 13.42, and 18.9 mm), at which time the total number of oysters and the total volume were calculated for each size class. In September and October, silos were graded every 4 wk. Incoming seawater temperature during the first season study period reached a maximum of 35.1°C in late July. In late November, groups were enclosed in 2-mm plastic mesh bags and enclosed in wire mesh trays. All test groups were overwintered in a designated broodstock sanctuary. At the sanctuary site, seawater temperatures, ranging from a low of 1.0°C (12/91) to a high of 4.1°C (3/92), were recorded during weekly sampling periods. Salinity was approximately 30 ppt.

1992 Season

In April 1992, the groups were transferred to a 1.5-m-tall × 3.7-m-diameter, 16,000-L holding tank. Oysters were held in 104-cm-long × 48-cm-wide × 11.5-cm-deep wire mesh trays lined with 6-mm plastic mesh. The position of the trays within the tank was rotated on a daily basis to minimize position effects. The tank was drained into an underground line, cleaned, and refilled with raw seawater daily. In late May, after a large-scale mortality event, a 5-cm airline was added as a source of aeration. From April through November, live counts were taken every 2 wk; the shell length (to the nearest 0.1 mm) and whole weight (to the nearest 0.1 g) of 25 oysters in each replicate were measured monthly. This regime was also followed for two replicates of eastern oysters grown in wire mesh trays (as described above) on adjacent tidal flats in Delaware Bay.

Beginning in early September, we noticed that the interior shell surface of all *C. gigas* mortalities was covered with vesicles created by the mud worm *Polydora websteri*. On November 9, 1992, 25 live animals from each of ten groups (N = 250) were sacrificed to quantify *P. websteri* incidence. Oysters were opened, and each valve was scored as having light incidence (presence of isolated blisters only), heavy incidence (multilayer continuous vesicles covering the entire shell surface), or no incidence. Percent incidence was calculated by dividing the number of oysters having light (or heavy) infections by the total number of oysters examined for each group. The maximum incoming seawater temperature during the second season study period was 30.5°C (reached on [illegible] 25 and again on July 16).

Statistical Analyses

All data were analyzed with the computer program SYSTAT (Wilkinson 1990) by analysis of variance (ANOVA), followed by Tukey's HSD Multiple Comparisons. Survival data were arcsine transformed before statistical analysis (Sokal and Rohlf 1981).

RESULTS

1991 Season

Mortality

Most of the mortality for all groups occurred during an approximately 30-d period from Day 15 to Day 42 (Fig. 1). At the end of this period, Hiroshima had significantly higher survival than eastern or Miyagi oysters (Tukey's HSD = 0.033). After 98 d, cumulative percent mortality totaled 65, 36, and 13% for eastern, Miyagi, and Hiroshima, respectively. Cumulative mortality in both *C. gigas* races was significantly lower than in the *C. virginica* cross (Tukey's HSD, $p = 0.005$ with Hiroshima, 0.021 with Miyagi).

Growth

Even though all oyster spat were initially taken from spat retained on a 1.98-mm sieve, the initial starting volume was 30%

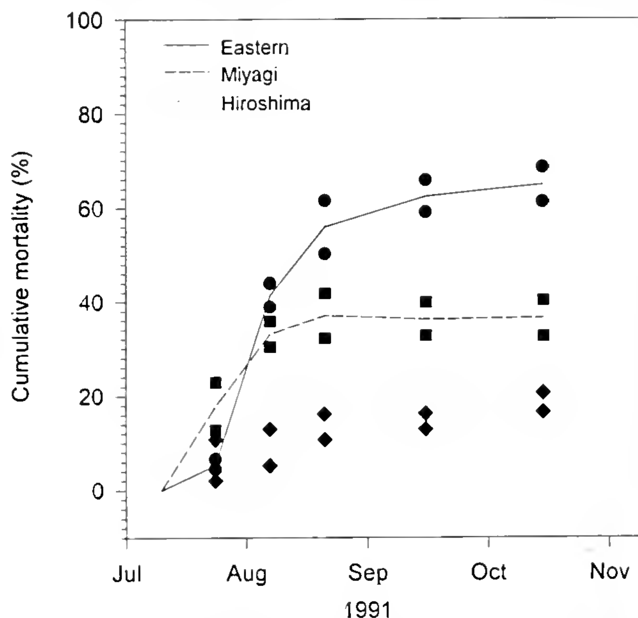

Figure 1. Cumulative percent mortality (symbols) of spat by replication for eastern (*C. virginica*) (circles) and *C. gigas*, forms Miyagi (squares) and Hiroshima (diamonds), from July 9 (Day 0, first deployed as spat) to October 15, 1991 (Day 98), in upweller silos. Mean cumulative percent mortality is depicted by the lines.

less for Miyagi (38 ml) and 19% less for Hiroshima (44 ml) compared with eastern (54.4 ml). This was the result, as mentioned previously, of the somewhat smaller initial mean spat size for Miyagi (3.2 mm) and Hiroshima (3.4 mm) than that for eastern (3.8 mm). The subsequent increase in total volume in the two *C. gigas* groups exceeded that of *C. virginica* during the first season. At Day 98, total pooled volume was approximately 3.2 times greater in Hiroshima (5,265 ml) and 2.5 times greater in Miyagi (4,134 ml) compared with eastern (1,623 ml). This comparison, however, does not take into account that by Day 98, there were about twice as many spat left in the *C. gigas* groups as in the *C. virginica* replicates. We standardized the increases in the volume of the spat by dividing the total volume by the total number of spat, yielding a measure of average spat volume. The increase in spat volume of both *C. gigas* groups exceeded that of *C. virginica* (Fig. 2), despite the twofold difference in density in the upweller silos. ANOVA showed a significant difference among the groups for average spat volume, but a Tukey's test failed to show a significant difference between the *C. gigas* races and eastern oysters (probabilities for pairwise comparisons: Miyagi vs. eastern, $p = 0.055$; Hiroshima vs. eastern, $p = 0.064$). A breakdown of oyster populations by size class showed that 86% of eastern, 98% of Miyagi, and 97% of Hiroshima spat were larger than 9 mm by mid-November (Fig. 3).

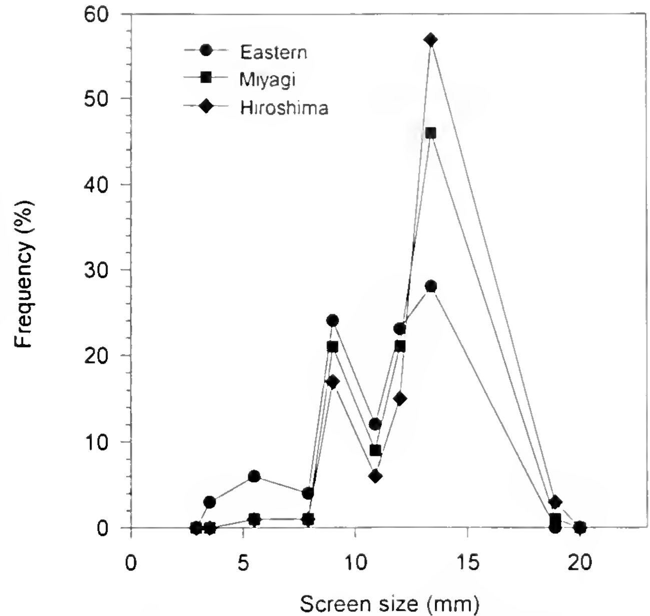

Figure 3. Size class distributions for eastern (*C. virginica*) and *C. gigas*, forms Miyagi and Hiroshima, on October 15, 1991, 98 days after deployment into upweller silos.

Second Season

Mortality

A large-scale mortality event began during the third week of May and continued through June (Fig. 4). Mortality was highest in Hiroshima oysters at nearly every sampling period, significantly higher than both eastern groups in June, July, and August. The survival of Miyagi oysters was intermediate between Hiroshima and tank-held eastern. Cumulative percent mortality from May through June was 43% for eastern, 56% for Miyagi, and 83% for Hiroshima, accounting for the majority of mortality within tanks. The corresponding mortality for the same period of time in the replicates of eastern oysters grown in Delaware Bay was 3%. The cumulative percent mortality from April 1992 to November 1992 was 60% for eastern, 73% for Miyagi, 93% for Hiroshima, and 37% for easterns held in Delaware Bay.

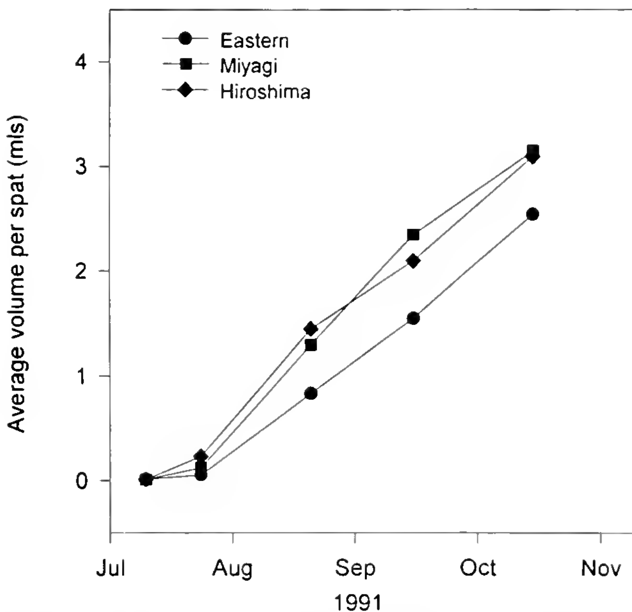

Figure 2. Average spat size (total volume/total number of spat) for eastern (*C. virginica*) and *C. gigas*, forms Miyagi and Hiroshima, on October 15, 1991, 98 d after deployment into upweller silos.

Growth

During the second season, Miyagi grew fastest, followed by Hiroshima and eastern; however, easterns grown on the tidal flats were larger than all tank-reared groups (Fig. 5). Mean shell length (in millimeters) and mean whole weight (in grams) at the end of the study period (11/92) was 44.7 mm and 17.6 g for eastern, 51.9 mm and 22.4 g for Miyagi, 45.7 mm and 16.8 g for Hiroshima, and 56.0 mm and 28.2 g for eastern oysters grown in Delaware Bay. The weight of field-grown easterns was significantly larger than that of the other groups in September, October, and November.

P. websteri Incidence

On November 9, 1992, it was determined that multilayer vesicles covered the entire shell surface of the flat valve in 87% of Miyagi and 94% of Hiroshima oysters (Fig. 6). No vesicles were found in either eastern group. However, isolated blisters were found on the flat valve of 66% of tank-reared eastern and in only 2% of those animals grown in Delaware Bay.

DISCUSSION

The objective of this study was to evaluate whether land-based comparisons of growth and survival in tanks are reasonable estimations of these same measures in the field. Obviously, because the *C. gigas* races could not be deployed in the field, there was no corresponding field group for this species. We can only extrapolate on how *C. gigas* would have performed in the field on the basis of the comparison of tank- and field-grown eastern oysters.

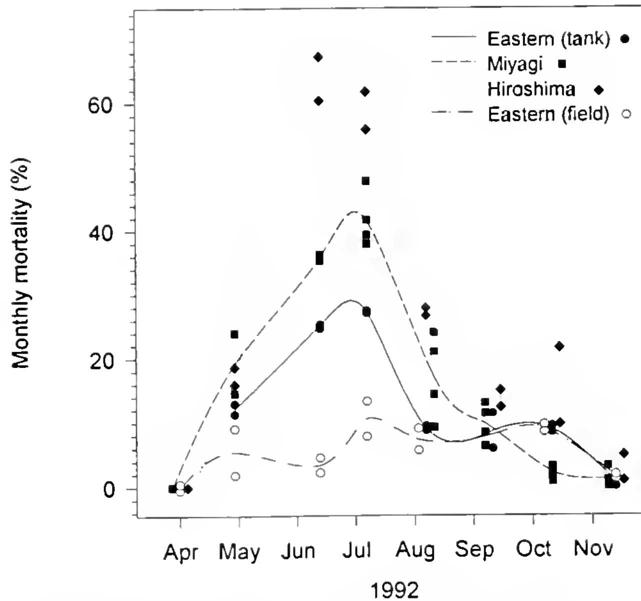

Figure 4. Percent mortality by month (symbols, by replication) from April 29 to November 9, 1992, for eastern (*C. virginica*) and *C. gigas*, forms Miyagi and Hiroshima, juveniles grown in tanks and for eastern oysters grown in the field in Delaware Bay [Eastern (field)]. Mean percent mortality is depicted by the lines.

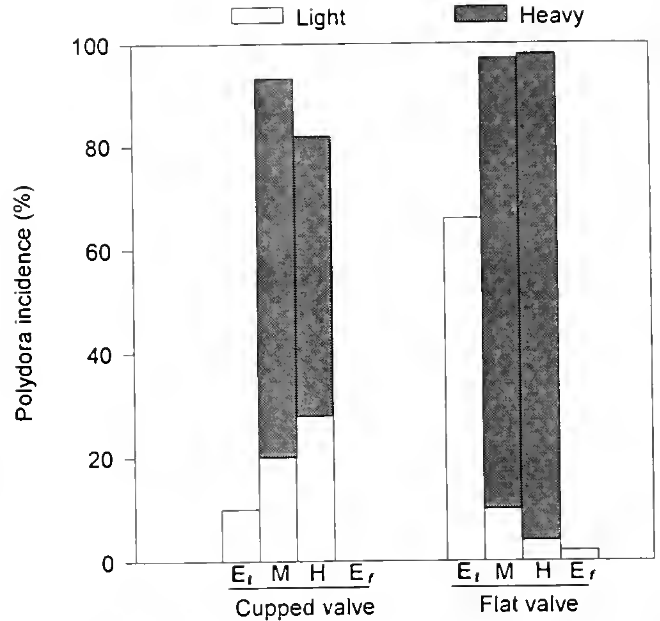

Figure 6. Mean percent incidence of *P. websteri* infections found in cupped and flat shells from eastern (*C. virginica*) (E_t) and *C. gigas*, forms Miyagi (M) and Hiroshima (H), oysters grown in a tank and from eastern oysters grown in the field (E_f). Isolated blisters were scored as light infections; vesicles covering the shell surface were scored as heavy infections.

This study also provided an opportunity to compare the performance of *C. virginica* vs. two races of *C. gigas* "head to head" in conditions mimicking Delaware Bay, albeit in tanks only.

The performance of larvae in the hatchery was similar among the three groups, although cultures were not replicated and statistical comparisons could not be run. The survival of *C. gigas* larvae to 48 h equaled (Hiroshima) or exceeded (Miyagi) that of *C. virginica*, but survival to the eyed stage was similar in all groups. After setting, *C. gigas* were fast starters and seem to thrive in the warmer waters during juvenile periods. After 4 mo in the upweller nursery system, *C. gigas* spat survived better and average spat size was about 30% greater than the *C. virginica* group. It is relevant to note at this point that spat in our upweller system are normally rotated to our field grow-out system when they reach a size of approximately 9.0 mm. This normally occurs within 3–6 wk. Four weeks after rotation to the upweller system, about a quarter of *C. virginica* spat were ≥9.0 mm, whereas about half of the *C. gigas* groups were of appropriate size. Holding all groups in this system for an additional 3 mo subjected them to a food-limiting environment during that period. It is likely that, had we been able to rotate these spat to the field according to our normal grading routine, the size differences between the *C. gigas* and the *C. virginica* would have been even greater than those observed.

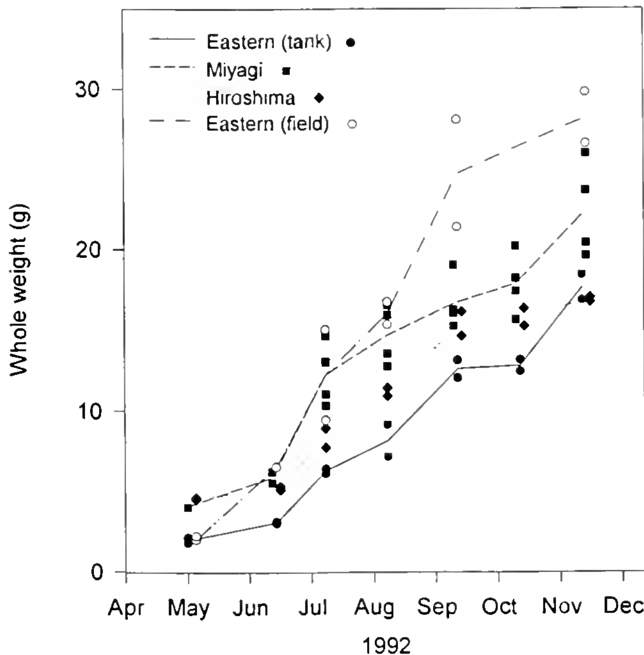

Figure 5. Whole weight (symbols), by replication, of eastern (*C. virginica*) and *C. gigas*, forms Miyagi and Hiroshima, juveniles and eastern oysters grown in the field in Delaware Bay [Eastern (field)] from April to November 1992. Mean whole weight is depicted by the lines.

The results of the second study period presented an entirely different outcome. Large-scale mortality began during the third week of May and continued through June, affecting particularly *C. gigas*, and accounted for the majority of the mortality during the 2-yr period. We were surprised by this early season mortality for a number of reasons. First, study groups were housed in a 16,000-L tank that was drained and refilled every 24 h. There was little chance of food deprivation, and the tank was vigorously aerated beginning 5/22, an extraordinary precaution not used in other years. Second, these groups were stocked at approximately one-third the density at which we normally hold tank-reared groups, discounting the possibility that waste product build-up was problematic. Finally, there was no evidence of disease when nearly dead oysters were examined (S. Ford, HSRL, Rutgers University). We can only speculate that the mortality event was due to the unseasonably warm temperatures during May 1992 (maximum incoming seawater temperature occurred on May 25). We further

speculate that the mortality was associated with gonad production in the Pacific oysters, a phenomenon referred to as "summer mortality" in commercially grown oysters on the West Coast of the United States (Perdue 1983). Whatever the reason, the outcome was to reverse the order of the group standings regarding cumulative percent mortality. Additionally, mortality was preferentially higher in the larger size classes of oysters (personal observation), which greatly reduced the size differences between *C. gigas* and *C. virginica* observed before the mortality event. A final unexpected variable encountered during the second season was the extremely high incidence and severity of *P. websteri* infestation in oysters, especially *C. gigas* held in tanks. The higher incidence of *Polydora* in the tank-grown oysters was most likely the result of the recruitment of late-stage *Polydora* larvae brought into the tank system in the daily delivery of unfiltered seawater. Lunz (1941), in an article examining *Polydora* infestation in South Carolina oysters, indicated that a large number of mud blisters within the shell may restrict the living space of the oyster and that the animal may be forced to spend considerable energy in secreting shell material for covering the mud worms. Heavy infestations may also cause heavy mortality (Roughley 1922). Thus, it is reasonable to assume that the severe *Polydora* infestations found among *C. gigas* in particular had a deleterious effect on both survival and growth.

Differences in growth were apparent among all three groups of oysters held in the tank. Growth and survival differed between Miyagi and Hiroshima oysters, supporting their status as physiological races. Compared with eastern oysters grown in the field, those in the tanks performed poorly for growth, survival, and even *P. websteri* infestation. Extrapolating from how well *C. gigas* did in the tank during the first season to how *C. gigas* might have done in the field might cause us to estimate that they would have done extraordinarily well. On the other hand, the presence of *Polydora* in the tank and not in the field and the extraordinary mortality of *C. gigas* in the tank during the second season of study signify that the two environments are not sufficiently similar to make such extrapolations. Although it may be possible to engineer a system that includes extensive filtration to remove abundant larvae and lowering the temperature of incoming seawater with chillers, at some point, the system no longer models "field" conditions. We can only conclude that tank-based comparisons are not likely to generate a true estimate of growth and survival, and perhaps response to disease, in the local environment.

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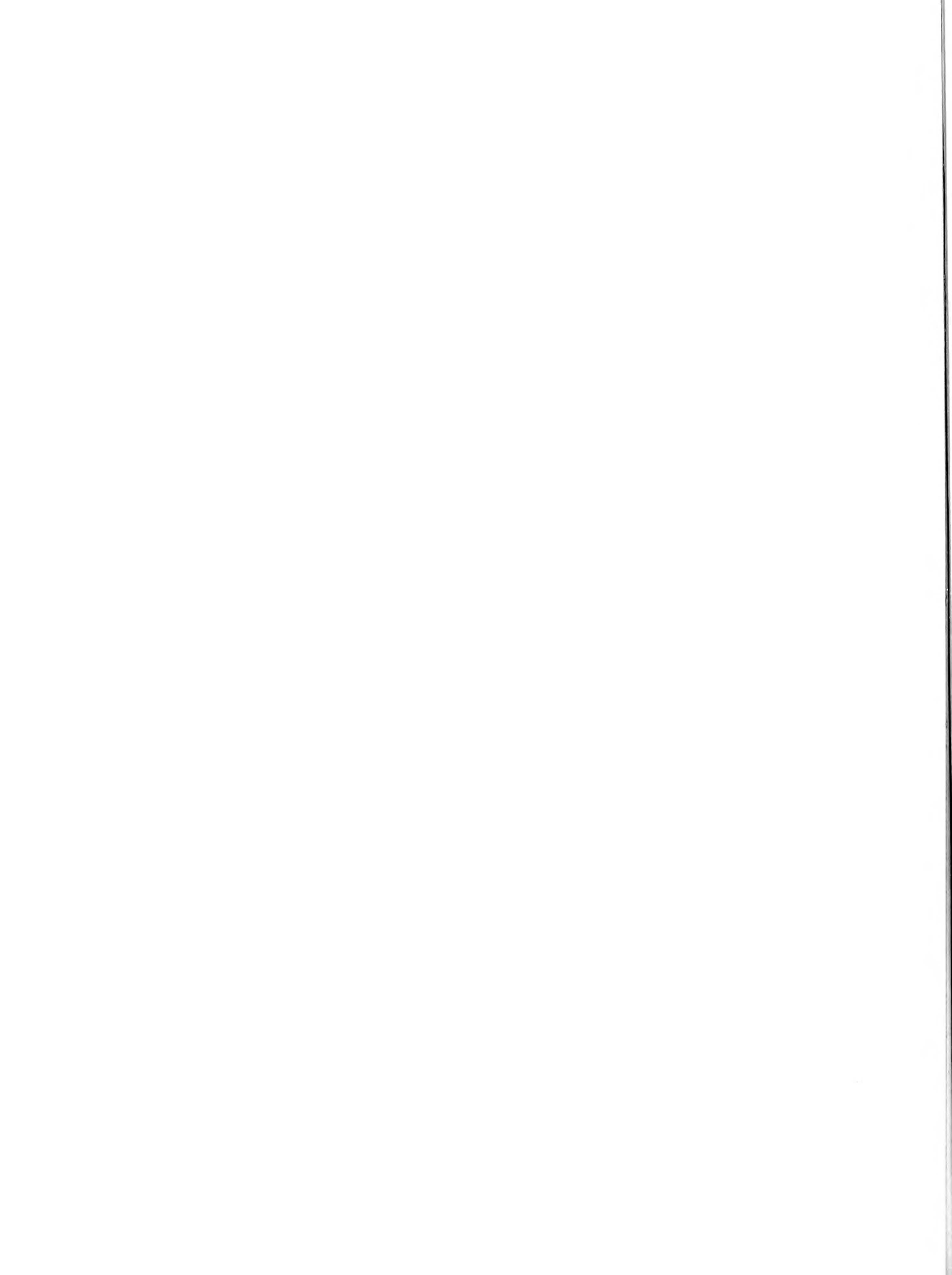

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SPAWNING CYCLE OF THE PEARL OYSTER, *PINCTADA MAZATLANICA* (HANLEY, 1856), (PTERIIDAE) AT ISLA ESPIRITU SANTO, BAJA CALIFORNIA SUR, MEXICO

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ABSTRACT The annual reproductive cycle of the pearl oyster, *Pinctada mazatlanica* (Hanley), from Isla Espíritu Santo, B.C.S., Mexico, was examined. Pearl oysters were collected at monthly intervals from June 1992 through August 1993. The gonadal development was analyzed by histological techniques and analysis of oocyte size. Spawning took place throughout the year, but at a lower rate in winter. A relationship between spawning and temperature was not observed The sex ratio was equal

KEY WORDS: Pearl oysters, spawning, *Pinctada mazatlanica*, reproduction

INTRODUCTION

The pearl oyster, *Pinctada mazatlanica* (Hanley, 1856), is a Panamic bivalve ranging from the outer coast of Baja California, through the Gulf of California, and south to Peru (Keen 1971). In the last century, near La Paz in the Gulf of California, this species was abundant and supported a thriving pearl-fishing industry. The quality of the pearls produced by this species was a factor of great economic importance in the foundation and colonization of South Baja California (Cariño and Cáceres-Martínez 1990, Monteforte and Cariño 1992). The Mexican government closed the fishery in 1938 because of overfishing (Sevilla 1969, Keen 1971, Monteforte 1990). *P. mazatlanica* is a potential mariculture species for the Gulf of California (Monteforte 1990).

For resource management or mariculture purposes, it is important to know the life cycle of the target species. Documentation of the reproductive cycle of *P. mazatlanica* is crucial for a better understanding of the population dynamics that regulate the remaining wild stocks. Studies of reproduction by using histological techniques and light microscopy have been carried out on pearl oysters like *Pinctada maxima* (Rose et al. 1990), *Pinctada fucata* (Tranter 1959), *Pinctada albina* (Tranter 1958a, Tranter 1958b, Tranter 1958c), and *Pinctada margaritifera* (Tranter 1958d). Previous to this, the only reproductive study in a natural population of *P. mazatlanica* was that of Sevilla (1969), who worked on a now extinct population located in La Paz harbor navigation channel. B.C.S. México. For cultured pearl oysters (*P. mazatlanica*), two studies of reproduction were made in Bahía de La Paz (Saucedo and Monteforte 1994, Saucedo 1995). The purpose of this study was to determine the annual reproductive cycle of a wild adult population of *P. mazatlanica* at Isla Espíritu Santo, B.C.S., Mexico.

MATERIALS AND METHODS

From June 1992 to August 1993 (except in November 1992), 17–20 adult specimens per month of pearl oysters, *P. mazatlanica*, were collected randomly from a wild population located near Isla Espíritu Santo, B.C.S., (Fig. 1) by SCUBA from a 3- to 4-m depth. A total of 308 organisms were captured ranging from 64 to 161 mm in shell length (mean, 126.5; SD, 18.6) and from 72 to 176 mm in shell height (mean, 136.3; SD, 20.6). At the time of the collection of biological samples, water temperature was recorded.

Before dissection, shell height and shell length was measured with a 0.01-mm resolution caliper. The visceral mass (gonad included) was fixed in a neutral solution of 10% formalin prepared with seawater, dehydrated in an alcohol series dilution, and embedded in paraffin (Luna 1968). Sections 7 to 9 μm thick were made and stained with Harris' hematoxylin & eosin (Luna 1968).

In addition, the diameter of at least 100 oocytes was measured, with an eyepiece graticule calibrated with a stage micrometer, in each of seven females per month selected randomly. The measurements were made along the longest axis in the oocytes sectioned through the nucleus containing clearly visible nucleoli. From these data, mean oocyte size and standard deviation were obtained. Individuals with few measurable oocytes and extensive phagocytosis ("spent" specimens) were not considered, following the criteria of Grant and Tyler (1983a, 1983b).

Sex was determined by histological analysis. The percentage of each sex during the study period was obtained. The sex ratio was determined and was examined for deviation from the expected ratio of 1:1 by χ^2 analyses (Snedecor 1950).

Categories of Gonadal Condition

Gametogenesis (either spermatogenesis or oogenesis) of *P. mazatlanica* was divided into five stages (indifferent, developing, ripe, partially spawned, and spent) on the basis of the classification cited for *P. mazatlanica* (Sevilla 1969, Saucedo and Monteforte 1994, Saucedo 1995), *P. albina* (Tranter 1958a, Tranter 1958b), *P. margaritifera* (Tranter 1958d), *P. fucata* (Tranter 1959), and *P. maxima* (Rose et al. 1990). The relative frequency of the gonad developmental phase was determined.

Developmental Stages

Indifferent Stage. All of the oysters examined were adults (Fig. 2). In both male and female individuals, there was no evidence of gonadal development. It was not possible to distinguish the sex. The connective tissues occupied all of the space between the empty and collapsed follicles.

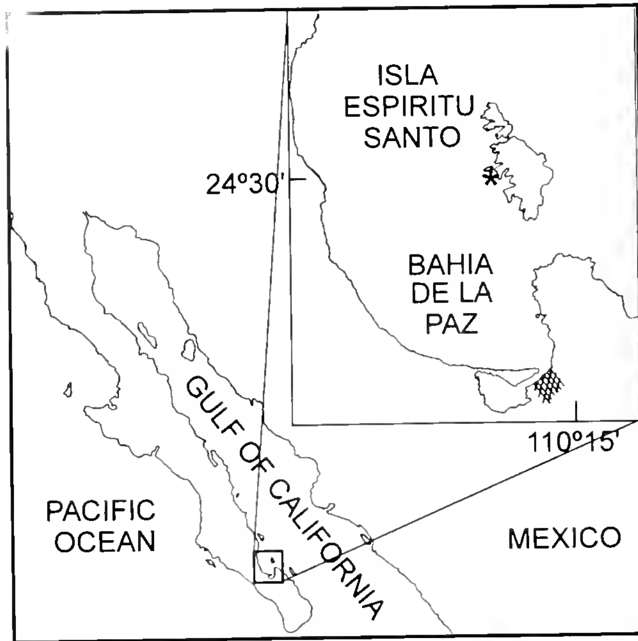


Figure 1. Study area. Isla Espiritu Santo, B.C.S., México. Asterisk marks the sampling area.

Developmental Stages of the Male

Developing Stage

The follicles, in different stages of development, occurred between the connective tissue (Fig. 3a). Inside the follicle, a variable quantity of germinal cells and ripe gametes were observed. Spermatozoa were stored as a dense mass in the lumen of the follicle, with the eosinophilic tails projecting into the central lumen. The area of connective tissue between the follicles decreases while follicles increase in area as the result of the accumulation of sperm.

Ripe Stage

The follicles were distended and filled with dense spermatozoa (Fig. 3b). Spermatocytes and spermatids were restricted to a thick layer on the follicular walls. Almost all connective tissue between follicles was replaced by follicles full of spermatozoa.

Partially Spawned Stage

During this reproductive stage (Fig. 3c), spermatozoa are expelled into the environment. The follicles were partially empty, and their walls became broken. There was a marked decrease in the number of spermatozoa filling the lumen.

Spent Stage

The follicles are collapsed, have decreased in area, and are invaded by amebocytes (Fig. 3d) which phagocytize the unspent spermatozoa. There was no evidence of active spermatogenesis taking place.

Developmental Stages of the Female

Developing Stage

Follicles were visible (Fig. 4a). Oocytes inside them increased in size and number. As the number of mature ova (free in the lumen) increased, the amount of connective tissue decreased. The developing oocytes, which began as hemispherical stalked cells attached to the wall of the follicle, became enlarged spherical cells, 52.5 μm (SD, 4.7) in diameter, as maturity approached.

Ripe Stage

The ripe ovary was characterized by the presence of distended follicles filled with ripe poligonal-shaped oocytes, some of which were attached to the follicular wall by slender stalks (Fig. 4b). Little or no connective tissue was present between the follicular walls.

Partially Spawned Stage

Some follicles contained oocytes, whereas others were empty (Fig. 4c). There was a large reduction in the number of free large oocytes present in the lumen. Little connective tissue was present. Some follicular walls were broken.

Spent Stage

The follicles were empty, with the exception of a few large, unspent oocytes free in the lumen being phagocytized by amebocytes (Fig. 4d). Follicular walls were reestablished.

RESULTS

Reproductive Cycle

The annual reproductive cycle of *P. mazatlanica* is summarized in Figure 5. The gametogenic development of this species indicated spawning activity throughout the year. Partially spawned individuals were observed all year except in January, March, and July 1993. The highest frequency of partially spawned individuals was observed in June, July, October 1992, and April 1993, when the spawning individuals were 28.6, 33.3, 29.2, and 21.2% of the population and the temperature was 28, 29, 30, and 23°C, respectively. Pearl oysters in the indifferent stage were observed in July 1992 through January 1993 and in August 1993. Developing pearl oysters were found every month, except August 1992. Ripe oysters were encountered throughout the study except in August

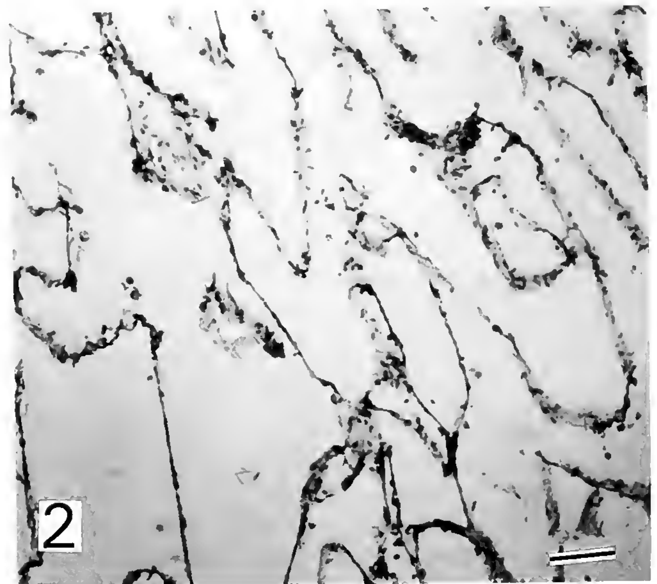


Figure 2. Indifferent stage. Scale bar = 50 μm.

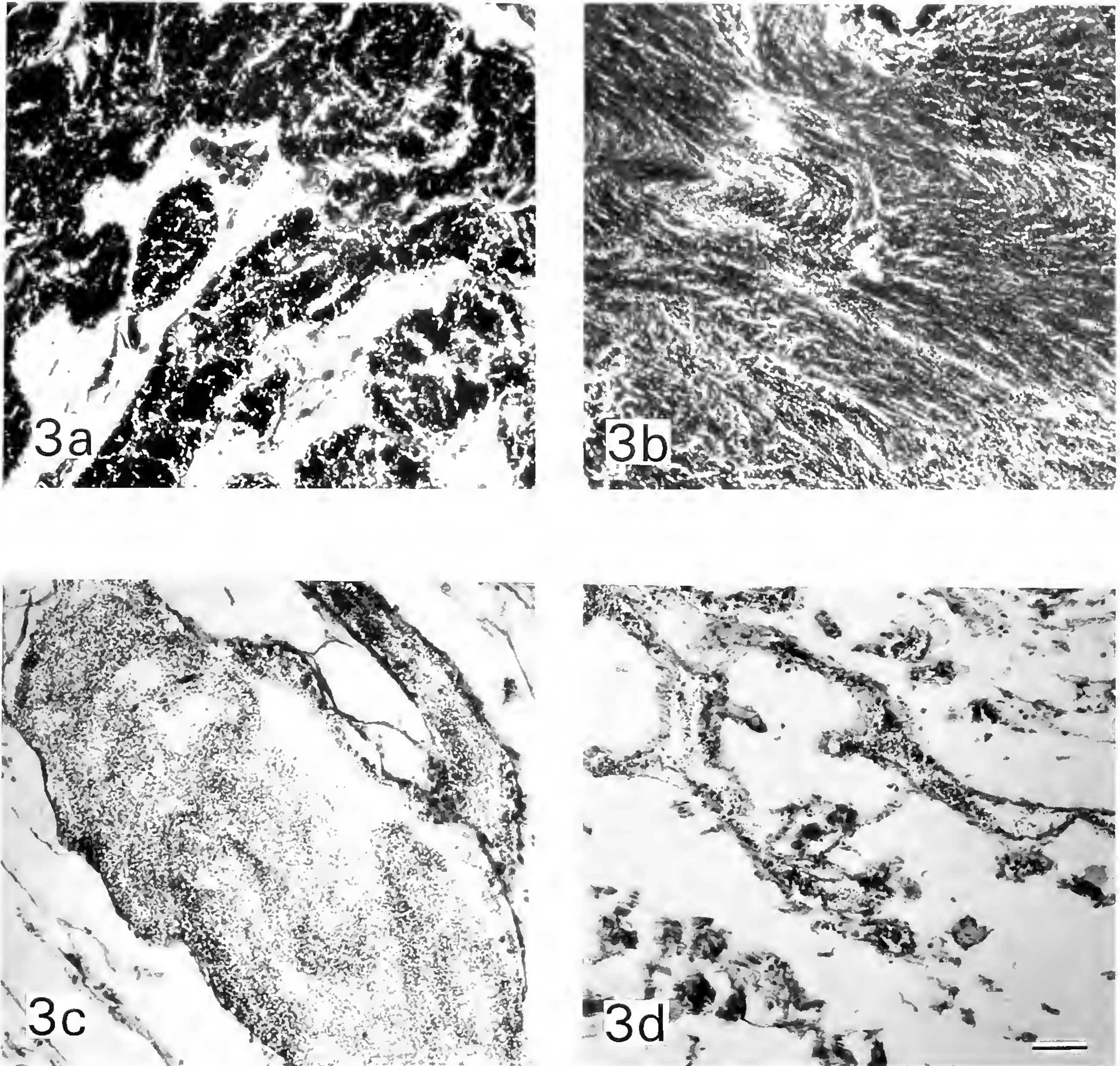


Figure 3. Photomicrographs of gonadal stages of the male pearl oyster *P. mazatlanica*. (a) Developing stage. (b) Ripe stage. (c) Partially spawned stage. (d) Spent stage. Scale bar = 50 μm.

through December 1992. The largest number of ripe pearl oysters occurred in March 1993 (52.9% of the individuals). The spent stage was recorded during June through December 1992 and May, July, and August 1993.

Two hermaphroditic specimens were encountered. The microscopic examination revealed the presence of oocytes in a gonad with primarily spermatogenic development (Fig. 6a). In the other gonad, spermatozoa were found in the center of follicles of obvious female development (Fig. 6b).

Analysis of Oocyte Size

The mean of oocyte diameter values for the study period can be observed in Figure 7. Maximum diameters were observed in June 1992, April 1993, and June 1993. All of these peaks were followed by a decrease in oocyte diameter, which indicates a spawning event.

Sex Ratio

Females outnumbered males. A total of 308 pearl oysters were sampled, of which 159 (51.62%) were females, 119 (38.63%) were males, two were hermaphroditic (0.64%), and 28 (9.1%) were undifferentiated. The sex ratio (1.33F:1M, n = 278) did not differ significantly ($P \geq 0.01$) from the expected ratio of 1:1.

Temperature

During the study period, the water temperature varied from 21 to 31°C. The highest values were in September 1992, and the lowest values were in February 1993 (Fig. 8).

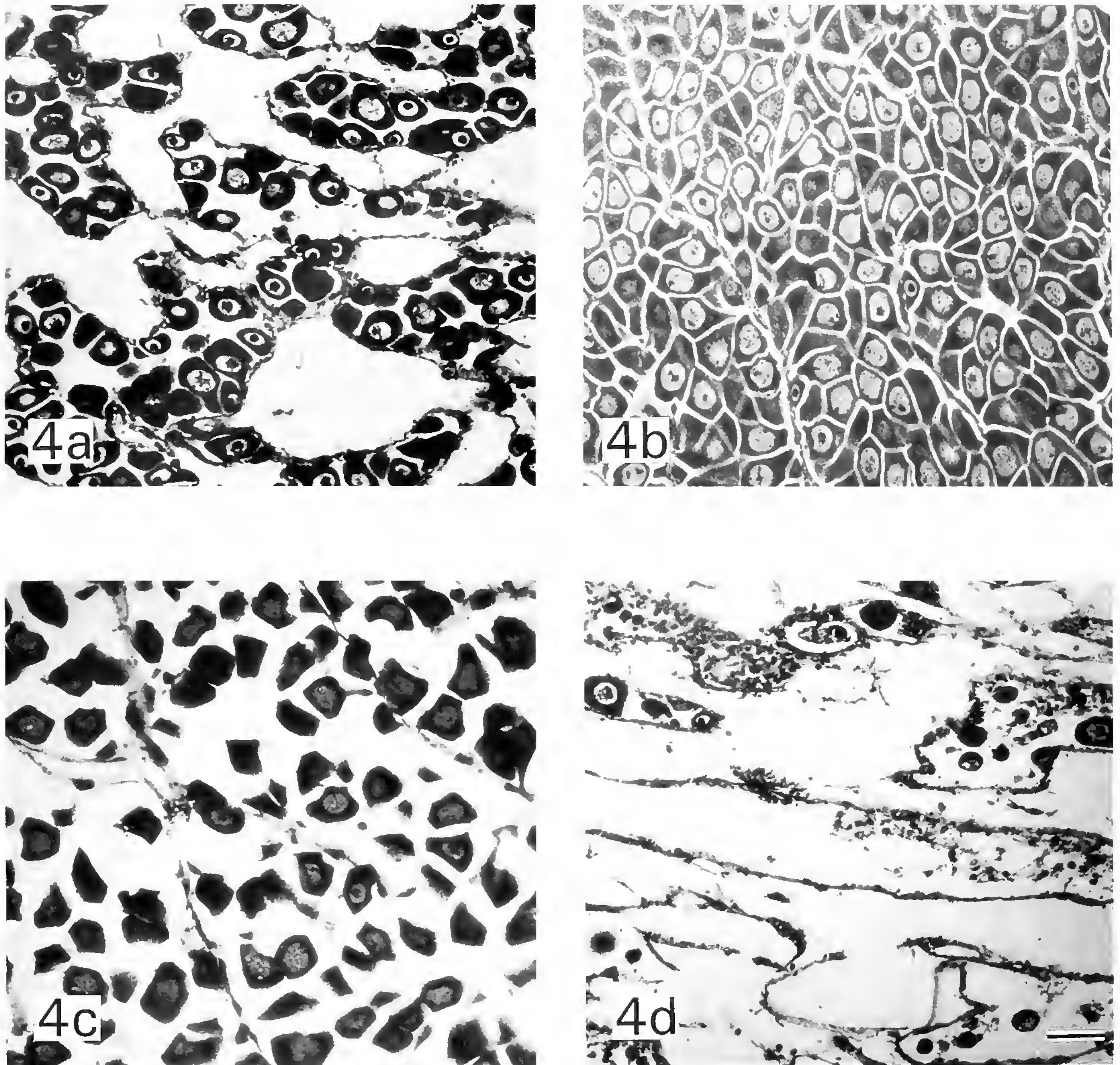


Figure 4. Photomicrographs of gonadal stages of the female pearl oyster *P. mazatlanica*. (a) Developing stage. (b) Ripe stage. (c) Partially spawned stage. (d) Spent stage. Scale bar = 50 μm.

DISCUSSION

The characteristics of gametogenesis in *P. mazatlanica* were similar to those described for *P. albina* (Tranter 1958b), *P. margaritifera* (Tranter 1958c), *P. fucata* (Tranter 1959), and *P. maxima* (Rose et al. 1990). During early gametogenic development, the follicles increased in length and volume as the number of mature ova or spermatozoa increased. As in *P. albina* (Tranter 1958a), in *P. mazatlanica*, sections across the gonad revealed that it consists of a system of branched tubules, the number and size of which depend on the stage of gonadal development. Older follicles contain fewer stem cells but many gametes.

The pearl oysters *P. maxima*, *P. albina*, and *P. margaritifera* are protandric hermaphrodites (Tranter 1958c, Tranter 1958d, Rose et al. 1990), whereas, in *P. fucata*, both protandric and protogynic sex changes were recorded (Tranter 1959). *P. mazatlanica* is also a protandric hermaphrodite (Sevilla 1969, Saucedo and Monteforte 1994). In a study with cultured young adults, oysters smaller than 100 mm matured as males, with females not occurring until animals attained a size larger than 100 mm. The sex ratio of cultured young adult pearl oysters was 0.12F:1M (Saucedo and Monteforte 1994). In this study, the sex ratio was 1.33F:1M. This work was carried out with larger individuals (mean height, 136.3 mm); however, females of 82-, 91-, 96-, and 98-mm shell height were found.

The results of histological gonad examinations and oocytes diameter analysis indicate that *P. mazatlanica* from Isla Espíritu Santo, B.C.S., collected from June 1992 to August 1993 showed no clearly defined seasonal reproductive cycle. Spawning occurred throughout the year, but on a minor scale in winter. Previous

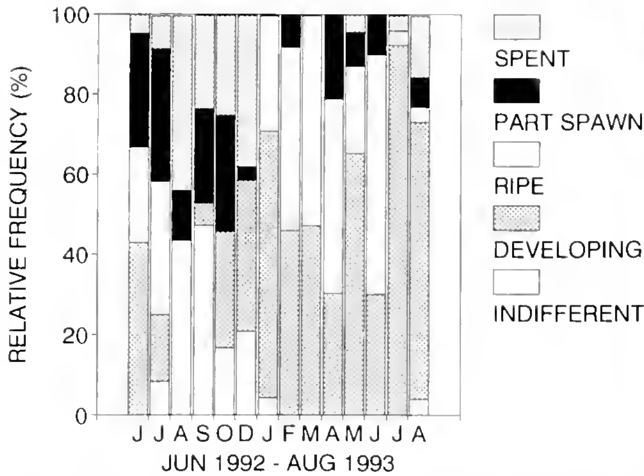


Figure 5. Relative frequency of gonadal stages of *P. mazatlanica* during June 1992 to August 1993. Observations of males and females were combined.

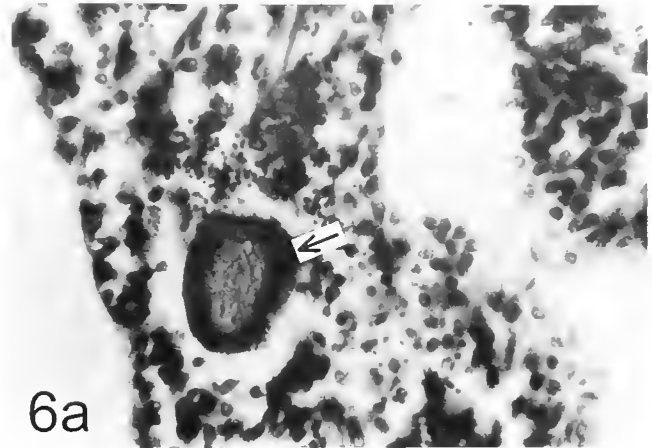
researchers using histological analysis have reported that *P. mazatlanica* spawn only in summer (Sevilla 1969, Saucedo and Monteforte 1994, Saucedo 1995). Those studies were performed on young individuals, predominantly males (77%), kept under conditions of experimental culture in bottom cages (Saucedo and Monteforte 1994, Saucedo 1995) or with only females collected in 1963 in the La Paz harbor navigation channel (Sevilla 1969). At this time, that population has disappeared (personal observation). Monteforte and Cariño (1992) also reported an absence of individuals in La Paz Channel as well an impoverished number in other local populations of La Paz Bay.

The histological observations of the spawning in *P. mazatlanica* are supported by studies of spat settlement. *P. mazatlanica* settlement was reported from July to November in spat collectors with 5 and 8 wk of immersion (Cáceres-Martínez et al. 1992, Monteforte and García-Gasca 1994), June to October in collectors with 8–10 wk of immersion (Monteforte and Wright 1994), and in January and August to December in collectors with 8–10 wk of immersion (Félix-Pico 1977). The age of the largest pearl oysters from a collector is approximately equal to its period of exposure (Tranter 1958a). The period of free-swimming larval existence in *P. mazatlanica* is about 3 wk (Masón-Suástegui 1987).

In *P. maxima*, mature individuals were observed outside the main breeding period, during the cooler months (Wada 1953, quoted in Rose et al. 1990). Similarly, in pearl oysters from Isla Espíritu Santo, ripe gonads were observed in winter, also outside the main breeding period. Histological data suggest that cultured *P. mazatlanica* (Saucedo 1995) were capable of spawning throughout the year.

Sevilla (1969) indicates that the spawning temperature of *P. mazatlanica* is 27–29°C, with a maximum spawning at 28–29°C. This author also reported that spawning ends when the temperature is less than 25°C. Saucedo and Monteforte (1994) reported that cultured *P. mazatlanica* spawned when water temperatures reached 28–30°C. We observed spawning at temperatures ranging from 21 to 31°C.

Temperature is one important environmental factor in the regulation of bivalve reproduction (Sastry 1979). The influence of temperature on the reproductive cycles of other bivalves from Baja California Sur, Mexico, has been well documented (García-Domínguez et al. 1993, García-Domínguez et al. 1994, Villalejo-



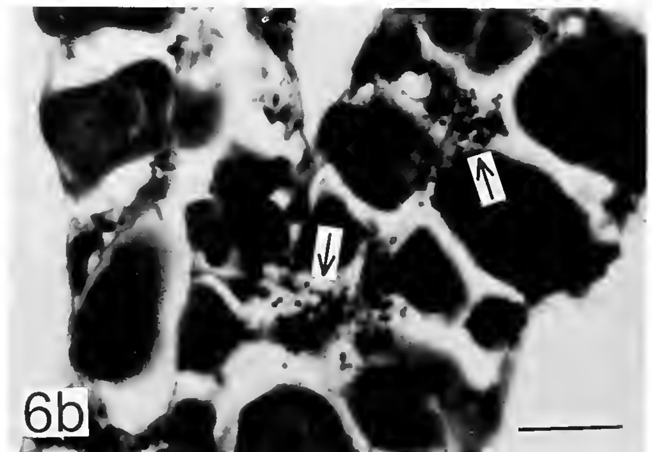


Figure 6. Hermaphroditic specimens. (a) Testis in gametogenesis with sperm surrounding an oocyte (arrow). (b) Ovary in gametogenesis with residual spermatozoa (arrows) occupying center of follicle. Scale bar = 25 μm.

Fuerte and Ochoa-Báez 1993, Villalejo-Fuerte et al. 1995). In this work, we did not observe a clear relationship between temperature and spawning. Similarly, García-Domínguez et al. (1994) did not observe a clear relation between temperature and spawning in *Megapitaria aurantiaca*, also from Isla Espírutu Santo.

The importance of food availability has been emphasized in the timing of bivalve reproduction (Bayne and Newell 1983, MacDonald and Thompson 1985, Jaramillo et al. 1993). Time of spawning may be related to food availability (Jaramillo et al. 1993). The spawning in bivalves might be synchronized to coincide with maximum food availability for larval development (Seed 1976, Jaramillo et al. 1993). In *Chlamys amandi*, the spawning time appeared to be related to high food levels rather than to water temperature (Jaramillo et al. 1993), whereas in *Hinnites giganteus*, the histological data suggest that there is no correlation between food availability and spawning (Malachowski 1988). In *P. mazatlanica*, the maximum spawning time (summer) did not coincide with maximum winter food availability. Signoret and Santoyo (1980) reported for La Paz Bay a maximum abundance of phytoplankton in winter (1,708,950 cells/l) and a minimum in spring (140,000 cells/l).

Tranter (1958d) reported the spawning period for *P. margari-*

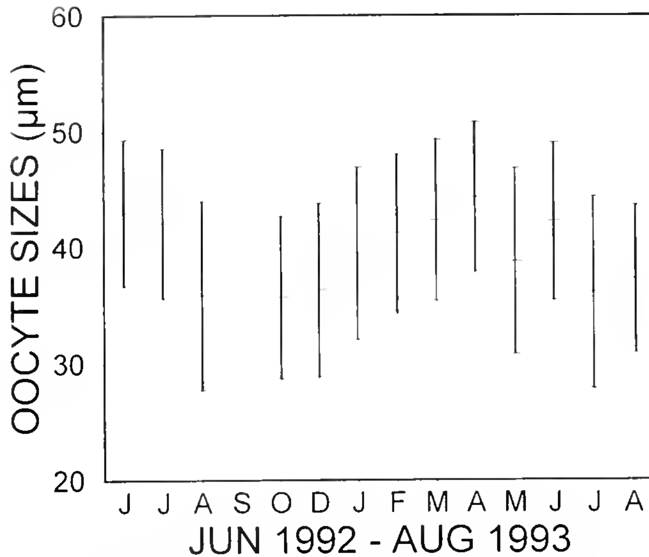


Figure 7. Mean oocyte sizes of *P. mazatlanica* between June 1992 and August 1993. Bar = standard deviation.

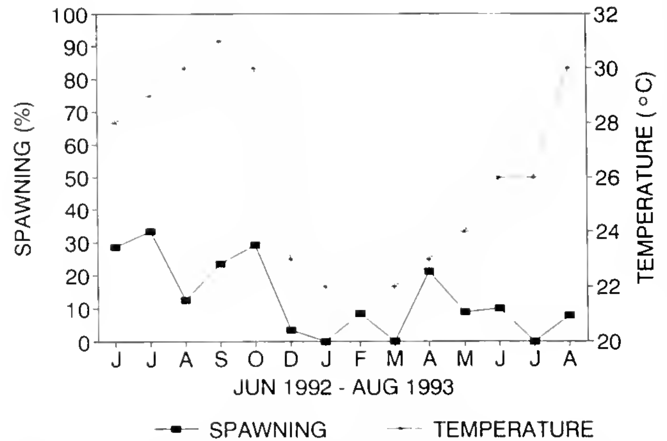


Figure 8. Relation of partially spawned stage with water temperature. Observations of males and females were combined.

tifera in summer and winter, and in intermediate periods at a reduced intensity. The similarity of their results with those obtained in this study for *P. mazatlanica* was most likely the result of the genetic closeness of the two species (Jabbour 1988). The breeding seasons of other species of *Pinctada* differ—*P. maxima* breeds annually, with maximal and minimal developmental periods consistent with correspondingly high and low temperatures (Rose et al. 1990); *P. albina* breeds continuously throughout the year, but most actively in April and May (autumn) when sea temperature begins to fall (Tranter 1958b); and *P. fucata* breeds in summer and autumn (Tranter 1959).

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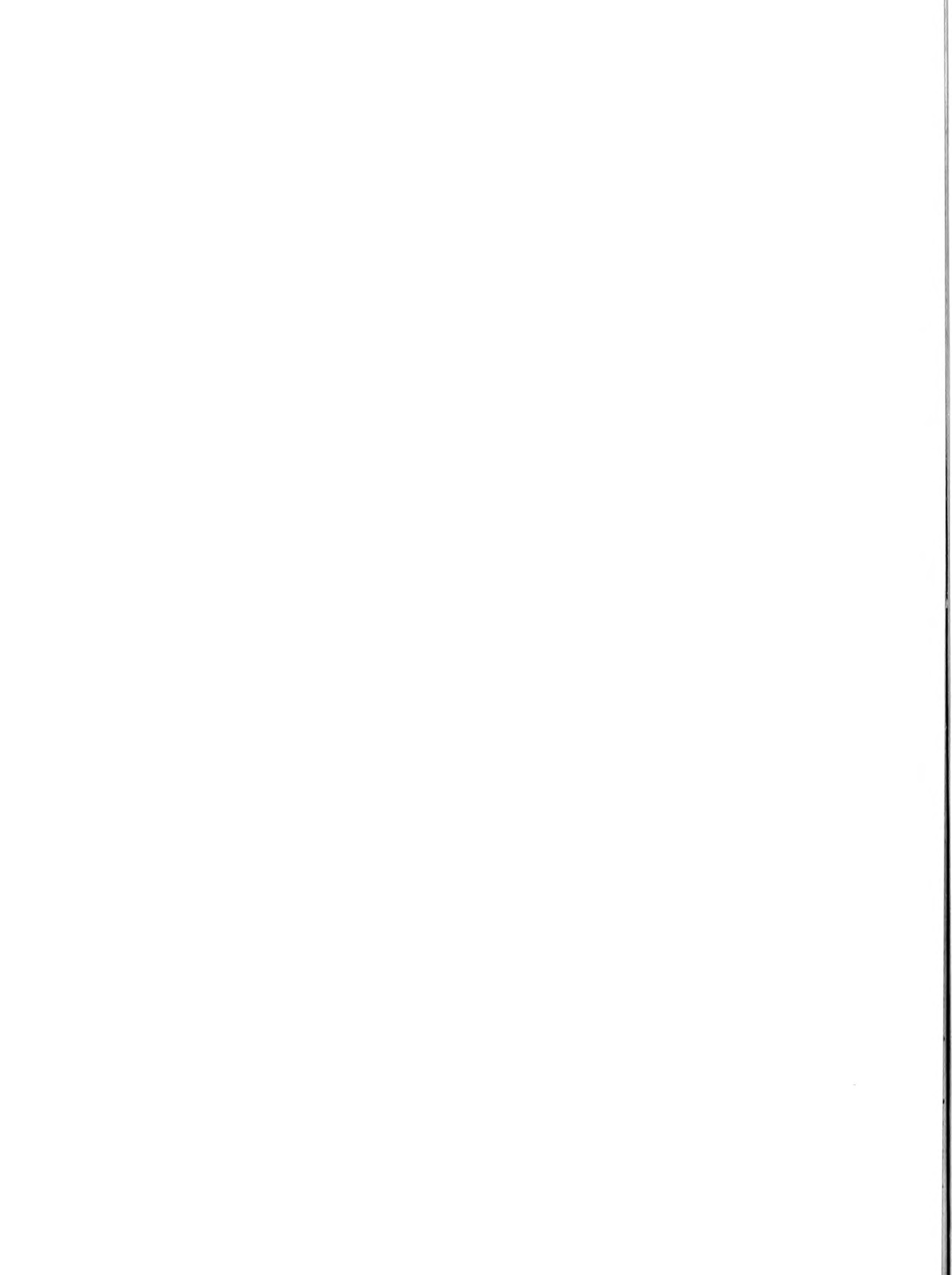

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OVERVIEW AND BIBLIOGRAPHY OF RESEARCH ON THE CHILEAN OYSTER *TIOSTREA CHILENSIS* (PHILIPPI, 1845) FROM NEW ZEALAND WATERS

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ABSTRACT An overview of the biology and research on the Chilean oyster (*Tiostrea chilensis* Philippi 1845) from New Zealand waters is provided along with a comprehensive bibliography. This complements the bibliography prepared by Toro (1995) for the same species in South American waters.

KEY WORDS: Chilean oyster, *Tiostrea chilensis*, bibliography, New Zealand flat oyster

INTRODUCTION

The Chilean oyster *Tiostrea chilensis* is one of four species of oysters commonly found in New Zealand waters (Jeffs 1995a). Extensive beds of this oyster formed the basis of one of the first substantial commercial fisheries in New Zealand over 130 years ago (M.A.F.) 1975b). In more recent times, there has been intense interest in the commercial development of this oyster for enhancement and aquaculture. This species has been the subject of numerous research projects, with the results mostly being communicated only in local publications. The purpose of this article is to draw together a bibliography of these publications and provide a synthesis of the current state of our knowledge for this species from New Zealand waters.

TAXONOMY

T. chilensis is known by a variety of common names in New Zealand, including tiopara, mud oyster, flat oyster, deep-sea oyster, dredge oyster, Foveaux Strait oyster, Bluff oyster, Stewart Island oyster, and southern rock oyster (Jeffs 1995b). The variety of common names in use is a reflection of the range of habitats and locations in which this species can be found.

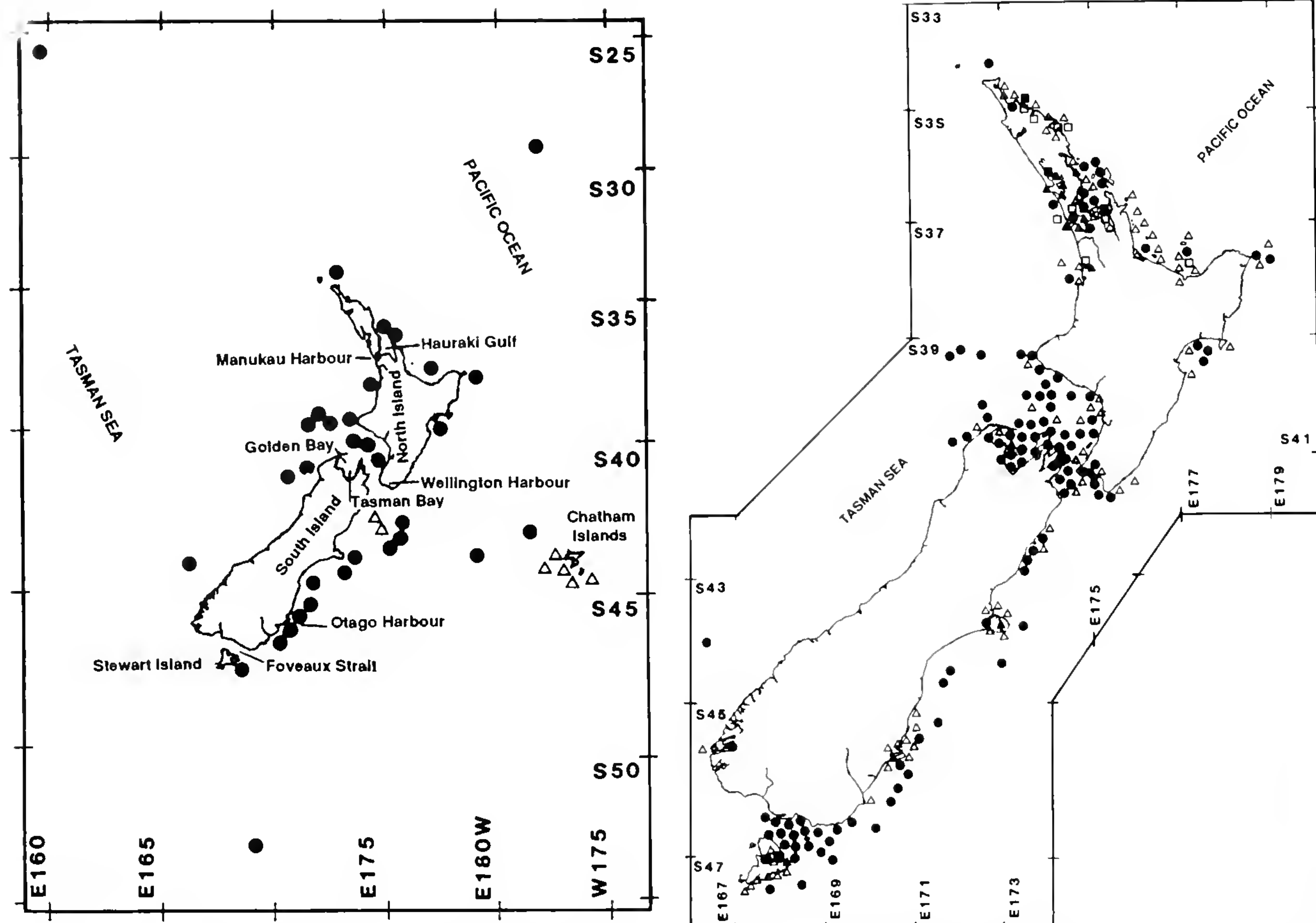
This species has also been known by a variety of taxonomic names since it was first described from New Zealand by Hutton (1873). A variety of geographic forms of the species were subsequently named as separate species: for example, *Ostrea heffordi* (Finlay 1928) for small and squat-shaped oysters found attached to rocks on the shores of Otago Harbour, and *Ostrea charlottae* (Finlay 1928) for individuals with broadly frilled shells often found in deeper water.

Chanley and Dinamani (1980) proposed a new genus, *Tiostrea*, containing two species that had previously been referred to as *Ostrea lutaria* from New Zealand and *Ostrea chilensis*, the Chilean oyster or "ostra," from the Pacific Coast of South America. The new genus was proposed on the basis of the highly distinctive larval shell structure shared by the two species. Subsequently, Buroker et al. (1983) synonymised the two species as *T. chilensis* on the basis of similarities observed in their ecology, life history, and biochemistry. These workers also confirmed that *O. charlottae* and *O. heffordi* were ecomorphs of this same widespread species. This nomenclature now has widespread acceptance in New Zealand (Beu and Maxwell 1990). Therefore, we have continued to use *T. chilensis* (Philippi 1845) when referring to the native species of New Zealand. However, debate on the taxonomic status of this species of oyster has continued with no clear consensus emerging (see Harry 1985, Toro 1995).

ECOLOGY

T. chilensis reaches its greatest natural abundances in the colder waters in the southern parts of New Zealand, where adult oysters can reach densities of over 150 m^{-2} in unexploited populations (Cranfield 1968a). Relatively extensive subtidal oyster beds have formed the basis of long-established commercial fisheries in Foveaux Strait and in Tasman and Golden Bays (Cranfield 1975a, Cranfield 1975b, M.A.F. 1975b, Warne 1989). The presence of these well-known southern fisheries has created a common misconception of a distinctly southern distribution for the species. However, *Tiostrea* has been found throughout much of New Zealand (Figs. 1 and 2) from intertidal sites to depths as great as 150 m (Beu and Maxwell 1990) and 549 m (Record A0910—Collection of the New Zealand Oceanographic Institute). The species has been observed to occupy a variety of habitats: attached to rocks and wharf piles in the intertidal (Morton and Millar 1973, Westerskov 1980); deeper water (60–100 m) and muddy substratum, such as off Otago Heads and Chatham Islands (Powell 1979); coarse sandy-pebble gravel bottom in waters of intermediate depths (20–50 m) (Cullen 1962).

Tiostrea appears to be able to tolerate a wide range of water temperatures and salinities. Salinities in Foveaux Strait are typically oceanic, ranging from 31 to 35 ppm, whereas oysters found in Stewart Island inlets can be subject to long periods of low-salinity water in the order of 3–5 ppm (Westerskov 1980, Buroker et al. 1983). Water temperatures in Foveaux Strait can range from 9 to 10°C in winter and 15 to 17°C in summer (Cranfield 1968b), whereas populations in northern areas, such as the Manukau Harbour, are subjected to winter lows of 11°C and summer water temperatures as high as 27°C (Jeffs, unpublished data). In addition to the natural populations in New Zealand, a small wild population exists in Wales, Great Britain, after being introduced there from collections of oysters from Foveaux Strait in 1963 and again in 1966 (Walne 1974). The oyster was introduced for scientific ex-



Figures 1 and 2. Maps of New Zealand showing the location of offshore and coastal *T. chilensis* records, compiled from a number of collections. Key locations mentioned in this article are also identified. Symbols: (●) Collection of the New Zealand Oceanographic Institute. (△) Collection of the National Museum of New Zealand. (□) Collection of the Auckland Institute and Museum. (■) Collections of members of the Conchology Section, A.I. & M. (▲) Personal observations (A.G.J.).

periments aimed at investigating the potential of alternative species for replacing exhausted beds of the native European flat oyster, *Ostrea edulis* (Linnaeus) (Taylor 1987, Utting 1987, Utting and Spencer 1992, Richardson et al. 1993). *T. chilensis* is also found as an almost ubiquitous fossil throughout New Zealand, usually deposited in Pliocene and Pleistocene rocks formed in past high-energy, shallow-water environments (Beu and Maxwell 1990).

LIFE HISTORY CHARACTERISTICS

The unusual reproductive behaviour of this oyster has attracted attention from some researchers (e.g., Roughley 1929, Millar and Hollis 1963). *T. chilensis* is a protandrous hermaphrodite that breeds in the spring and summer once water temperatures rise above the lowest winter temperatures: 9–10°C in Foveaux Strait and Otago Harbour (Stead 1971a, Cranfield 1968b, Westerskov 1980, Buroker et al. 1983), above 14°C in Tasman Bay (Tunbridge 1962), above 18°C in Wellington Harbour (Hollis 1963), and above 13°C in the Hauraki Gulf and Manukau Harbour (Jeffs, unpublished data).

In Foveaux Strait, only a small proportion of the adult population spawns as females each summer (as few as 10–12%), whereas as many as 70–90% will develop male gonads (Cranfield 1975a). Females produce between 7,000 and 120,000 eggs that, once mature, are 300–350 μm in diameter (Cranfield 1975a & d). The eggs are thought to be fertilised in the inhalant chamber of the adult oyster and are then retained in the gills, where they continue to develop through to late-stage pediveligers (Hollis 1962, Hollis 1963, Cranfield 1968a, Stead 1971a). Estimates of the larval incubation period vary from 15 to 38 days and are thought to be related to water temperature (Hollis 1963, Stead 1971a, Westerskov 1980).

After the release from the parent oyster, the late-stage veliger larvae are ready to settle and the prodissoconchs range in length from 416 to 514 μm, height from 318 to 400 μm, and from 195 to 250 μm in depth (Cranfield 1979d, Chanley and Dinamani 1980). The highly distinctive larval shells of *Tiostrea* lack the posterior dorsal sulcus, umbones, and all hinge structures, which are commonly used as diagnostic features within the family *Ostreidae* (Chanley and Dinamani 1980, Beu and Maxwell 1990).

The free-swimming larval life appears to be of only a few minutes duration (Cranfield 1968b, Stead 1971a), although some larvae may be released earlier and/or spend much longer in the plankton (Cranfield and Michael 1989). The growth rate of *T. chilensis* is extremely variable between individuals, between localities, and between years. In Foveaux Strait, oysters may grow from freshly settled spat to a height of 5–20 mm in their first summer, 15–40 mm in the second, 25–60 mm in the third, and

35–80 mm in the fourth (Stead 1971a). The oysters usually become sexually mature in their second or third year (Hollis 1963, Cranfield 1975a).

FISHERIES AND AQUACULTURE PRODUCTION

In prehistoric times, *T. chilensis* was harvested in small quantities by Maori in many parts of the country (Park 1969, Sullivan 1973, Ritchie 1980, Fox and Cassels 1983). Today, the customary Maori and recreational harvest of *T. chilensis* is minimal (M. F. Bull, pers. comm.).

Two commercial fisheries are based on beds of oysters found in Foveaux Strait and in Golden and Tasman Bays. Both fisheries rely on mechanical dredging to harvest oysters from the sea floor. Currently, most of these oysters are sold into the domestic market, which perceives the oysters as delicacy. Some small quantities of oysters, however, are currently exported from New Zealand to French Polynesia, Australia, and parts of Asia (N.Z. Fishing Industry Board export database).

The management of these two commercial fisheries has been the main impetus for research on *T. chilensis* in New Zealand. Starting in 1906, a large number of surveys, fishery assessments, and studies of fishery biology were undertaken by government agencies (e.g., Hunter 1906, Tunbridge 1962, Sorensen 1968, Stead 1971a & b, Street and Crowther 1973, M.A.F. 1974, Cranfield et al. 1993).

The Tasman and Golden Bays fishery was historically the smaller of the two fisheries and is not so well researched. Annual landings from this fishery currently remain stable at about 500 tonnes, although landings have increased to nearly 700 tonnes in the past season (M.A.F. unpublished data).

Annual landings from the Foveaux Strait fishery peaked during the late 1960s at 164,000 sacks; a sack may contain up to 70 dozen oysters and may weigh up to 79 kg (Cranfield 1979d, Michael and Cranfield 1983, Cranfield et al. 1991). In 1986, an endemic species of *Bonamia* (a haplosporidian parasite) was first identified as the cause of major mortality of oysters in Foveaux Strait (Doonan and Cranfield 1992, Doonan et al. 1994). From that time, the disease spread throughout Foveaux Strait, leading to closures in the fishery in 1992. Surveys during the late 1980s and early 1990s indicated that the oyster population had fallen below 10% of the estimated virgin stock and was in danger of collapse through recruitment failure (Cresswell 1993, Cranfield et al. 1993). The devastating effect of *Bonamia* on the Foveaux Strait oyster beds led to an intensive series of disease surveys and related studies (e.g., Hine 1986b, Hickman and Jones 1986, Dinamani et al. 1986, Dinamani et al. 1987, Hine 1991a, Hine 1992d, Cranfield et al. 1991, Cranfield et al. 1993).

Bonamia is also present in oysters from Tasman Bay, although it does not appear to have had any major effect on the commercial fishery (Hine 1992c). Other, less important parasites found in this oyster have also been the subject of further investigations (e.g., Howell 1965, Howell 1966, Howell 1967, Jones 1981). Various aspects of the food quality of *Tiostrea*, including food value, storage, handling, and the unusual capacity of these oysters to accumulate some heavy metals have also been investigated in several studies (Malcolm 1927, Malcolm 1929, Brooks and Rumsby 1965, Brooks and Rumsby 1967, Thomas 1969, Nielsen 1975, Fenaughty et al. 1988).

The collapse of the Foveaux Strait fishery and the increasing local price and demand for this oyster have led to greater efforts to culture this species (Smith et al. 1992). Difficulties in obtaining sufficient oyster spat and deaths of livestock due to *Bonamia* have hampered efforts to date. Consequently, the aquaculture and enhancement of this species have not yet become well established in New Zealand (Smith et al. 1992, Hayden 1988, N.Z. Trade Development Board 1989). More recent research efforts in New Zealand have focussed on developing *Tiostrea* for aquaculture and enhancement (e.g., Smith et al. 1992, Hickman 1992a, Hickman 1992b, Hickman et al. 1988, Jeffs 1995b, Street 1995). In Chile, however, the species has been farmed since 1975 and a number of government-operated experimental stations produce seed oysters for enhancing natural oyster beds (Osorio 1979, Winter et al. 1984, Chanley and Chanley 1991, Aiken 1993). Aquaculture production in Chile has also been hampered by difficulties in spat supply (Lépez 1983, Valencia Camp 1990).

DISCUSSION

There is considerable potential for the commercial development of *Tiostrea*, given that it is widely recognised as a excellent eating oyster that can command premium prices over and above other cultivated oysters, such as the Pacific oyster *Crassostrea gigas* (Thunberg 1793). Workshops of scientists and commercial interests held in New Zealand have all concluded that the full potential for the aquaculture and fisheries development of this species would only be realised through further research (Smith et al. 1992, New Zealand Marine Farmers' Association, pers. comm.). Of critical concern was research that led to improved spat supply, more efficient culture practices, and an improved understanding of the dynamics of the disease bonamiasis. Similar concerns have also been identified in Chile (Lépez 1983, Valencia Camp 1990, Toro et al. 1995a, Toro et al. 1995b).

Despite numerous studies, the reproductive biology in this species, particularly the factors influencing larval production, remains poorly understood. Also, low levels of post-settlement survival are reducing the potential effectiveness of fishery enhancement and hatchery operations in New Zealand (Drummond 1993a, New Zealand Oyster Company & M. F. Bull, pers. comm.). Further research in these areas would allow the development of hatchery techniques that would increase the spat production for aquaculture and fishery enhancement. In turn, this would create more opportunities for selective breeding programmes to improve important attributes of broodstock, such as faster growth and disease resistance. Improved production efficiency will also come from a greater understanding of how the performance of oysters is affected by culture practices and environmental factors.

The disease bonamiasis poses a continuing threat to *Tiostrea* aquaculture and fisheries, although active research into the disease has expanded in recent years. The results of these studies are beginning to clarify the nature of the relationship between the oyster and the *Bonamia* parasite, including the mechanisms that may be involved in triggering outbreaks of the disease (Hine 1996).

From a general ecological perspective, *Tiostrea* is unusual in that it maintains a widespread distribution that includes an enormous range of habitats. To date, most research has been concentrated on a few populations of commercial significance. Closer examination of other populations is likely to provide interesting and valuable results. For example, geographically isolated populations of oysters may exhibit characteristics well-suited to aqua-

ulture, such as faster growth, disease resistance, or higher fertility, as has been seen in other species of molluscs (Gjedrem 1983, Newkirk 1983, Castagna and Manzi 1989, McShane and Naylor 1995). Such populations are also likely to show marked genetic differences, given that gene flow between distant populations may be limited when the larval life is often extremely short (Buroker et al. 1993).

A number of scientists from around the globe are currently working on some of the research priorities outlined here. Recent cooperation between scientists from New Zealand and the United Kingdom has proved extremely fruitful (Utting and Hickman 1995). Further international cooperation can be expected to lead to greater gains in our knowledge of *Tiostrea* and should be actively encouraged.

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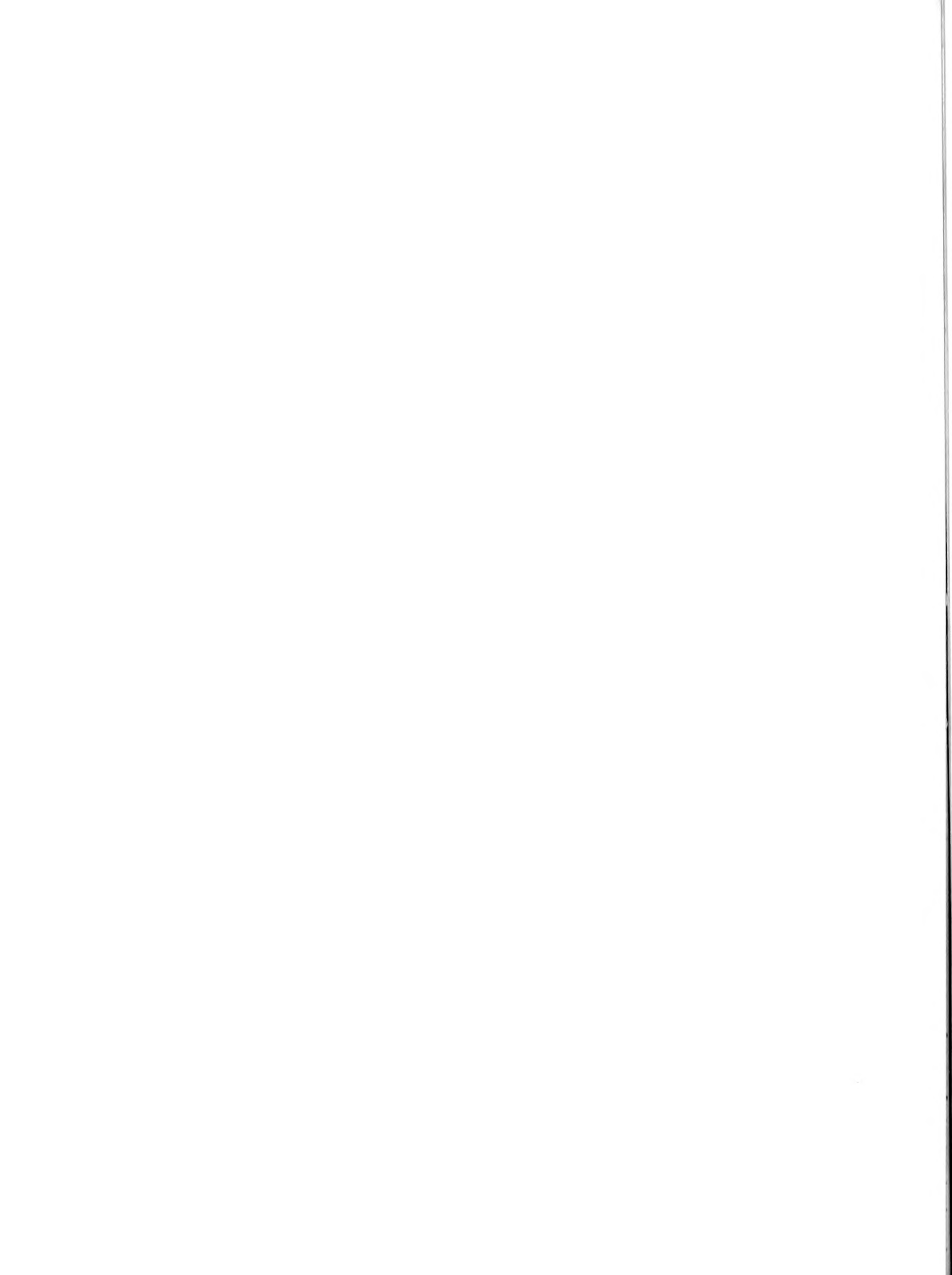

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ENHANCEMENT OF SUBTIDAL EASTERN OYSTER, *CRASSOSTREA VIRGINICA*, RECRUITMENT USING MESH BAG ENCLOSURES

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ABSTRACT Eastern oysters, *Crassostrea virginica*, in the southeastern United States are found predominantly in the intertidal zone. In this study, mesh bags (3 and 6 mm) were deployed over collecting frames, and the patterns of oyster settlement on these collectors were compared against unmeshed controls at three tidal heights (intertidal, low water, and subtidal) over three sampling regimes (biweekly, monthly, and seasonal) at two sites. Within the biweekly sampling regime, the meshed collectors and controls had similar patterns of settlement at the respective tidal heights. For monthly samplers, mesh treatments maintained higher settlement subtidally whereas controls had highest settlement on the collectors at mean low-water level. Controls had highest recruitment intertidally for seasonal collectors, whereas mesh treatments had higher recruitment lower in the intertidal zone. Conclusions from this experiment were that the use of mesh-covered collectors enhanced subtidal oyster recruitment. Causes of observed increases in subtidal settlement in mesh collectors over unmeshed controls over time could be the result of a combination of factors: predator exclusion, larval entrainment, or reduced desiccation, which seemed to overcome the detrimental effects of increased fouling, resulting in reduced flow and possible hypoxic conditions within the mesh bags. Given the degree of recruitment and the sizes of the recruits attained within the mesh bags, the use of these methods to attain juveniles for commercial purposes would appear to be both feasible and viable, particularly for long periods (up to 6 mo) of deployment.

KEY WORDS: *Crassostrea virginica*, mesh excluder, oysters, predation, recruitment

INTRODUCTION

The range of eastern oyster, *Crassostrea virginica* (Gmelin), extends from the Gulf of St. Lawrence south along the eastern seaboard of the United States and throughout the Gulf of Mexico (Galstoff 1964). Within this range, the oyster is found predominantly in the subtidal zone. However, in the southeastern United States, and specifically in South Carolina and Georgia, the majority of oysters are intertidal.

The primary reasons given for the lack of subtidal oysters have been disease, mismanagement of the resource, competition from other epibionts, and predation (Harris 1980, Ofiara and Stevens 1987, Michener and Kenny 1991). Macropredators are numerous and include mammals, fish, crustaceans, and molluscs (Linton 1968, Walker 1981, Walker 1993). However, relatively little is known concerning the effect of these predators on newly settled oysters. Predation on young oysters by blue crabs, *Callinectes sapidus*, was identified as a contributing factor in the 1946 failure of oyster recruitment in South Carolina (Lunz 1947). Galstoff (1964) observed that many young oysters were adversely affected by crabs feeding on larger oysters on which larvae had settled and attached.

Recruitment studies by O'Beirn et al. (1995 and 1996) suggested that events shortly after oyster settlement and metamorphosis may contribute to the confinement of oysters intertidally in coastal Georgia. It was observed that oyster numbers recorded on collectors were significantly higher in the subtidal than intertidal environment over short periods of sampler deployment (i.e., 2 wk and 1 mo), with the reverse observed for longer periods (up to 7 mo). Furthermore, it was suggested that with greater duration of deployment and consequently the submergence of subtidal collectors, the potential for predation or other mortality factors (such as competition or exposure to pathogenic organisms) on oysters was increased. We describe a field study to evaluate oyster settlement using protective mesh bags that in theory would allow oyster larvae to set on collectors while limiting the access of potential predators. Also, we anticipated that the use of protective meshes would increase the potential to harvest adequate numbers of spat from the subtidal zone in the southeastern United States.

STUDY SITES

The work was carried out from April to November 1993 at two sites and April to September 1994 at one site (for logistical reasons), in Wassaw Sound, GA (Fig. 1). The two sites were: House Creek, a sheltered tidal creek near the mouth of the sound; and Skidaway River, a less sheltered site located on the Intracoastal Waterway. A more detailed description of the hydrographic characteristics of the sites can be found in O'Beirn et al. (1995). The two sites have been used to monitor oyster recruitment since 1991 (O'Beirn 1995, O'Beirn et al. 1995, O'Beirn et al. 1996).

METHODS

The sampling apparatus and methods of deployment were similar to those already used in the established monitoring programs in coastal Georgia (O'Beirn 1995, O'Beirn et al. 1994, O'Beirn et al. 1995, O'Beirn et al. 1996). Briefly, longitudinally grooved poly-

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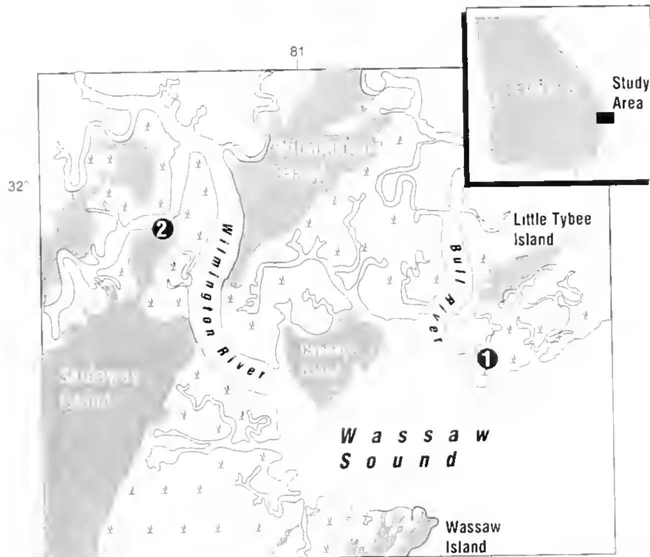


Figure 1. Map of sampling area in Wassaw Sound, GA, indicating the two sampling sites used throughout the study: (1) House Creek and (2) Skidaway River.

vinylchloride (PVC) tubing embedded with chips of calcium carbonate was used for collecting spat. A 12-cm section of tubing on each collector provided a sampling area of approximately 100 cm^2. Three collectors were arranged vertically on a sampling unit; each collector was exposed to one of three tidal heights (subtidal, mean low water, and intertidal). Four replicate sampling units were attached to a portable frame, which in turn was attached to a fixed frame (Fig. 2). After return to the laboratory, each collector was rinsed to remove extraneous material and examined with a binocular microscope at 10× to enumerate the number of oysters on each collector.

Collecting frames with four replicate sampling units were covered with 3- and 6-mm mesh bags (see Fig. 2). The open end of each bag was sealed, folded, and inserted into a PVC pipe that had been slit longitudinally. Hereafter, the 3- and 6-mm mesh bag–covered frames shall be referred to as the 3- and 6-mm treatments, respectively. An exposed control frame was also placed at each site. Collectors were retrieved and replaced at the two sampling sites on a biweekly and monthly basis. Thus, every 2 wk and once monthly, three frames (i.e., two mesh treatments and one control) were taken from each site and evaluated for spat. A separate set of

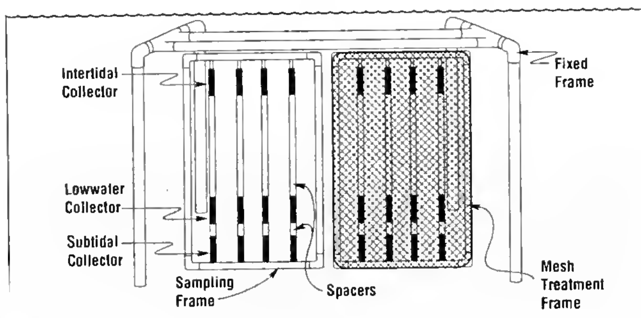


Figure 2. Schematic diagram of the sampling apparatus used in the study. For the 3- and the 6-mm treatments, mesh bags were slipped over the sample frame.

seasonal collectors was also deployed at the two sites. These were left on site for the duration of the study. These seasonal collectors gave an estimate of the overall recruitment of oysters for the entire spawning season. The experiment was repeated in 1994 at the Skidaway River site only.

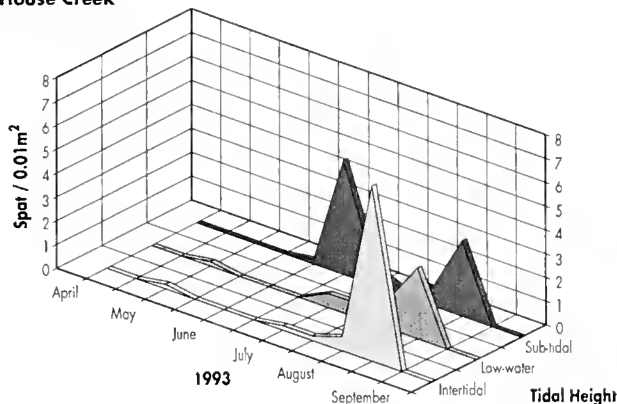
STATISTICAL ANALYSIS

The enclosure of each of the treatment frames within a mesh "bag" would result in some problems of analysis relating to pseudoreplication (Hurlbert 1984). Therefore, data at each tidal height, within each sampling period, were pooled and each period was then used as a replicate with which to carry out the analysis. The substantial variation in oyster numbers retrieved on the collectors throughout the study necessitated the log transformation [$\ln(x + 1)$] of these values within each of the data sets retrieved. To evaluate the patterns of settlement within each treatment, one-way analysis of variance (ANOVA) and the Tukey Studentized Range Test (when appropriate) were performed on these transformed data with tidal height as the main effect. Separate ANOVAs were performed on the data for each mesh treatment, within each sampling regime at each site, and in the case of the Skidaway River, each year. The primary goal was to evaluate the patterns of settlement within each treatment. It was hypothesized that the mesh treatments would retain proportionally greater numbers of spat, subtidally over time, than the unmeshed control. A standard significance level of 5% was chosen for all statistical tests ($\alpha = 0.05$).

RESULTS

Overall, oyster recruitment in Wassaw Sound in 1993 was considerably lower than that in 1991 and 1992 (O'Beirn 1995, O'Beirn et al. 1996). At House Creek, recruitment on the biweekly control collectors was first observed in late May 1993. However, peak settlement did not occur until late September ($\bar{x}$ = 7.4 spat/0.01 m^2; Fig. 3A). At the Skidaway River site in 1993, levels were extremely low until peak settlement in late September ($\bar{x}$ = 63.2 spat/0.01 m^2; Fig. 3B). Settlement was high on the monthly control collectors at House Creek early in the 1993 season, after which, settlement dropped off and showed no appreciable increase over the rest of the season (Fig. 4A). Peak monthly settlement on the intertidal collectors occurred in May at the House Creek site ($\bar{x}$ = 225.6 spat/0.01 m^2). Similar patterns were observed at the Skidaway River site; peak settlement occurred early in the 1993 season, followed by low settlement levels throughout the year. Peak settlement at the Skidaway site was on the low-water collectors in June ($\bar{x}$ = 154.4 spat/0.01 m^2; Fig. 4B). Oyster settlement on the biweekly subtidal collectors at the Skidaway River site in 1994 commenced in mid-May and increased to a peak in mid-June ($\bar{x}$ = 5.75 spat/0.01 m^2; Fig. 3B). Monthly settlement values had peak settlement on the low-water collectors in July ($\bar{x}$ = 26.3 spat/0.01 m^2; Fig. 4B).

Results of the ANOVAs on the transformed biweekly, monthly, and seasonal data from House Creek and Skidaway River are presented in Table 1. For the biweekly data at both sites in 1993 and at Skidaway in 1994 (Table 1), controls tended to retain greater number of oysters than both of the treatments. The lack of significance of many of the tests indicated no distinct patterns. The highest settlement was achieved on subtidal and low-water collectors for both treatments and controls at Skidaway in 1994 (Table 1).



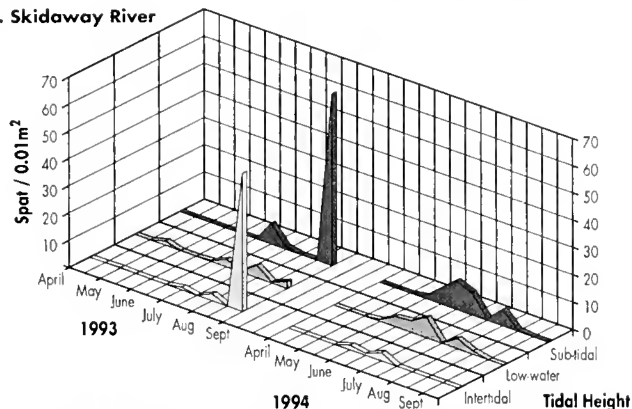


Figure 3. Mean number of spat per 0.01 m² from biweekly recruitment onto control collectors at (A) House Creek (1993) and (B) Skidaway River (1993/94).

The monthly data also tended to have substantially higher numbers of oysters on control collectors than treatment collectors (Table 1). Significantly higher oyster numbers were maintained on subtidal than on intertidal collectors on the Skidaway 3-mm treatments in 1993 and 1994 ($p = 0.0007$ and 0.0015, respectively). Neither differed from the low-water collectors. Control frames at Skidaway in 1994 did have significantly higher numbers of oysters on the low-water collectors ($p = 0.0003$; Table 1) than the other tidal heights.

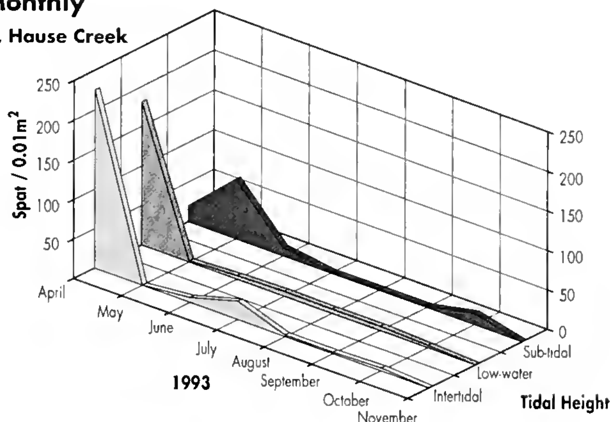
The seasonal data did reveal some divergence in patterns of recruitment between control and treatments, particularly at the Skidaway site (Table 1). Controls had predictably higher recruitment intertidally. Treatments at House Creek in 1993 closely mimicked the pattern observed in the controls, i.e., higher numbers of intertidal oysters (Table 1). Skidaway River, although having highest recruitment intertidally on control frames in both years, did have highest recruitment lower in the intertidal zone on treatment frames. In 1993, both treatments had higher numbers of oysters on the low-water collectors than the controls. In 1994, the 3-mm treatment had highest recruitment subtidally, whereas the 6-mm treatment had highest recruitment on the low-water collectors.

Analysis of the size data (Table 2) detected no substantial differences among treatments and controls at each tidal height and site for the biweekly regime. However, for the monthly sampling regime, treatment frames tended to have larger oysters than controls, particularly at the lower tidal heights. For seasonal returns, the 3-mm-treatment consistently had larger oysters at the two sites and at all tidal heights (Table 2).

DISCUSSION

The successful collection of wild molluscan spat for maricultural purposes is dependent on a consistent supply of larvae as well as a high rate of their settlement and subsequent survival. These latter two factors have been enhanced by the development of efficient means for collecting spat from natural spawning events in a number of bivalve species. Such means include monitoring adult gametogenesis, predicting times of larval availability, and deploying suitable collecting apparatus with which to collect spat.

In the study described here, oyster settlement on collectors covered with mesh bags was compared with that on uncovered control collectors. It was anticipated that subtidal recruitment would be enhanced on the mesh-covered collectors. For the biweekly sampling regime, no differences in patterns of settlement among the tidal heights were apparent at either site in 1993 and at Skidaway River in 1994 (Table 1). Some divergence was observed between the control and the 3-mm treatment for the monthly sampling regime at the Skidaway site in 1993 and 1994 (Table 1) whereby the treatment frames retained greater proportions of oysters subtidally.



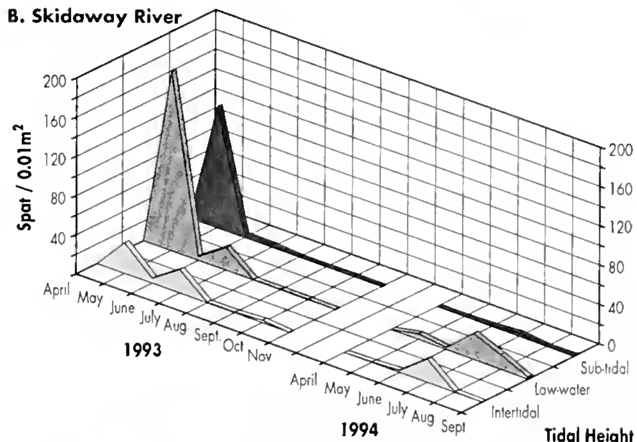


Figure 4. Mean number of spat per 0.01 m² from monthly recruitment onto control collectors at (A) House Creek (1993) and (B) Skidaway River (1993/94).

TABLE 1.

Biweekly, monthly, and seasonal mean oyster numbers from the two sites in 1993 and Skidaway River in 1994.

	Biweekly			Monthly			Seasonal		
Year/Group	SUB	LOW	INT	SUB	LOW	INT	SUB	LOW	INT
1993									
House Creek									
Control	1.1	0.6	1.0	35.7	29.9	18.5	1**b**	2.6**b**	63.8**a***
3 mm	0.2	0.1	0.1	1.6	10.3	1.6	0c	16.0**b**	57.0**a***
6 mm	0.1	0.2	0.1	0.8	5.0	0.5	1.8**b**	3.3**b**	20.3**a***
Skidaway River									
Control	8.0	1.5	6.0	9.2	26.0	18.5	0.6**b**	0.04**b**	17.8**a***
3 mm	0.2	0.1	0	1.6**a**	0.7**ab**	0.1**b***	14.3**b**	43.5**a**	37.0**a***
6 mm	0.8	0.6	1	0.4	0.4	0.2	14.5**b**	47.0**a**	12.8**b***
1994									
Skidaway River									
Control	4.7**a**	3.1**a**	0.8**b***	5.3**b**	10.2**a**	1.0**b***	0.3**b**	3.1**b**	11.8**a***
3 mm	1.8**a**	0.9**a**	0.1**b***	6.8**a**	3.1**ab**	1.0**b***	32.8	17.8	24.8
6 mm	1.9**a**	1.5**a**	0.3**b***	1.3	0.9	0.3	13.8	30.3	23.3

Given are the outputs from the Tukey test. Tidal heights with the same letter designation were not significantly different. * $p < 0.05$. SUB, subtidal; LOW, low water; INT, intertidal; 3 mm, 3-mm mesh bag treatment; 6 mm, 6-mm mesh bag treatment.

At the Skidaway River site, recruitment patterns observed on the seasonal collectors (Table 1) tended to support the overall theory that an external mortality factor on newly settled oysters results in their confinement to the intertidal zone. Although the control frame displayed higher recruitment intertidally, both of the treatment (3 and 6 mm) frames tended to have higher recruitment subtidally or at low water in both 1993 and 1994 (Table 1).

Both the 3- and the 6-mm seasonal frames from House Creek in 1993 developed tears in the mesh and were heavily inundated with silt below the low-water mark. Siltation was a result (in part) of the lower end of the frames resting on the bottom and, thereby, trapping silt in an area with high sediment loading (Virnstein 1978, Peterson 1979). A similar problem was encountered on the 3-mm seasonal frame at the Skidaway River site in 1993. Caging artifacts unfortunately were present, and consequently, the treatments were not maintained throughout the study. Despite this, the seasonal results from 1993 and 1994 at the Skidaway site indicated that the placement of mesh over collectors had an effect on the recruitment patterns of oysters. These data suggest that even with the problems encountered (siltation, torn mesh bags, and low recruitment), the "bagging" manipulation altered the distribution of oysters, such that survival tended to be higher below the low-water mark relative to controls.

The degree of variability in settlement patterns exhibited by biweekly and monthly data impedes the interpretation of observed trends. However, as already stated, more consistent trends were observed from the seasonal returns at the Skidaway River site. Factors affecting postsettlement oyster survival (e.g., predation) appear to take more than 1 mo to overcome the patterns set by "short-term" recruitment events. The bags may have other effects besides excluding potential predators and could be responsible for much of the inconsistencies observed over the shorter time scales.

TABLE 2.

Mean sizes of oysters (mm) from treatments and control collectors over the three sampling regimes at the three tidal heights.

	Biweekly			Monthly			Seasonal		
Sampling Area	3 MM	6 MM	CON	3 MM	6 MM	CON	3 MM	6 MM	CON
Intertidal									
House Creek	2.6**a**	1**b**	2.2**a***	2.1**ab**	2.6**a**	1.5**b***	45.1**a**	16.1**c**	37.9**b***
Skidaway River	—	—	—	—	—	—	44.7**a**	46.5**a**	14.5**b***
Skidaway River ('94)	2.7**a**	3.5**a**	0.9**b***	5.3**a**	3.3**ab**	1.2**b***	18.2**a**	17.0**a**	6.6**b***
Low water									
House Creek	2.6	2.0	3.2	1.4**b**	1.1**b**	2.5**a***	27.5	18.1	22.7
Skidaway River	5.3	5.6	2.6	2.2**ab**	3.7**a**	1.7**b***	56.3**a**	61.0**a**	16.0**b***
Skidaway River ('94)	2.6	4.2	3.4	5.2	5.8	4.2	23.9**a**	13.7**b**	8.4**b***
Subtidal									
House Creek	2.7	2.6	1.7	1.4**ab**	1.0**b**	2.4**a***	—	—	—
Skidaway River	2.5**ab**	0.7**b**	2.6**a***	1.7	2.2	2.5	39.3**a**	33.5**a**	18.0**b***
Skidaway River ('94)	2.3	3.2	2.6	5.6	5.8	4.3	27.0**a**	20.2**a**	8.3**b***

Also given are the outputs from the Tukey test. Treatments with the same letter designation were not significantly different, * $p < 0.05$. 3 MM, 3-mm mesh treatment; 6 MM, 6-mm mesh treatment; CON, control.

For example, the shading effects of the bags (a combination of the mesh itself with fouling that was observed) on intertidal collectors may reduce mortality and stress on newly settled oysters due to desiccation and temperature extremes (Roegner and Mann 1995). Consequently, this would obscure emerging differences resulting from higher survival on the subtidal collectors.

Negation of differences among the tidal heights in the treatments might also occur if water flow was restricted within the bags, resulting in hypoxic conditions, particularly at the lower tidal heights, where fouling was greatest. Hypoxia and anoxia have been shown to retard development and result in increased mortality of young oysters (Widdows et al. 1989, Baker and Mann 1994). It is assumed, however, that some oysters can survive these apparent stresses, and the cumulative effect of their settlement and survival results in the patterns observed on the seasonal collectors. Water flow restriction might also result in larval entrainment within the bags.

Neither the 3- nor the 6-mm meshes exclude predators such as flatworms, *Stylochus* sp., and juveniles of the oyster drill, *Urosalpinx cinerea*. Both had been observed on collectors inside the mesh bags, and their presence did coincide with low oyster numbers on the collectors. Also, holes characteristic of oyster drills were observed on many small oysters (ca. 5 mm). Both of these predators have been observed to prey voraciously on juvenile oysters elsewhere (Newell et al. 1991, Spencer et al. 1986). Other potential predators might be juveniles of known predators before an ontogenic shift to a larger food source, as is the case with blue crabs, *Callinectes sapidus* (Laughlin 1982), and the predatory drill, *Thais haemastoma* (Garton 1986). Blue crabs have been shown to prey heavily on juvenile (15 mm in shell height) oysters, *C. virginica* (Eggleston 1990). Other possible candidates include species of commercial penaeid shrimp as well as the grass shrimp, *Palaemonetes* sp. In studies with another bivalve, *Mercenaria mercenaria*, grass shrimp were shown to prey heavily on clams <0.6 mm in length (Uguccioni and Posey 1992). Also, Posey and Hines (1991) demonstrated that grass shrimp can prey on other thinner shelled bivalves such as *Mulinia lateralis* and *Macoma mitchelli* (up to 1 mm in shell length).

The enclosure of collecting materials (cultch) within plastic or wire meshes would serve two primary purposes: it retains the cultch in one cohesive unit, thus reducing the costs of subsequent handling, and it would protect newly settled oysters from some predatory organisms. Results from this study suggest that the recruitment of oysters lower in the intertidal zone (subtidally and/or at the low-water mark) can be enhanced by the use of mesh enclosures with the collecting apparatus. This is particularly apparent for collectors deployed for the duration of the sampling season. This would result in a high yield of oysters that are comparable in size to those found intertidally and larger than those found on collectors without mesh covers (Table 2). A combination of collecting strategies, whereby covered cultch is deployed subtidally and uncovered cultch is deployed intertidally, would yield high numbers of recruiting oysters with minimal effort and cost, thus, making oyster culture in the southeastern United States a more attractive venture.

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EVALUATION OF *VIBRIO* spp. AND MICROPLANKTON BLOOMS AS CAUSATIVE AGENTS OF JUVENILE OYSTER DISEASE IN *CRASSOSTREA VIRGINICA* (GMELIN)

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ABSTRACT Eastern oysters, *Crassostrea virginica*, cultured in the northeastern United States have experienced unexplained mass mortalities associated with a syndrome called juvenile oyster disease (JOD) for the past 7 y (1988–1995). Previous studies implicate bacteria, plankton blooms, or both as causes of this disease. The possibility that a bacterium in the genus *Vibrio*, common aquatic pathogens, is the causative agent was evaluated by weekly monitoring of *Vibrio* spp. concentrations in water, oysters, sediment, and debris associated with suspended oyster nursery trays at an oyster nursery on Long Island, NY, from May to September 1993. Juvenile oysters experienced mortalities totaling 20–60% from July through August. Total *Vibrio* spp. counts rose exponentially in juvenile oysters' tissues immediately after water temperatures exceeded 20°C, and preceding observed mortality by 1–2 weeks. The onset of oyster mortalities did not correlate significantly with blooms of the dinoflagellate, *Gymnodinium sanguineum*, or with salinity. *Vibrio* spp. concentrations in sediments rose significantly before oyster mortality was observed and decreased thereafter. However, trends in *Vibrio* spp. concentrations in water and debris samples did not correlate with the oyster mortalities. Healthy juvenile oysters were challenged with *Vibrio* spp. isolated from afflicted oysters before episodes of high mortality periods. Oysters injected with two of nine isolates experienced significantly higher mortalities than controls or than those injected with other isolates. *Vibrio* spp. that were phenotypically identical to the injected species were recovered from the experimentally infected oysters. Field and experimental observations strongly suggest a link between infection by a *Vibrio* strain and JOD.

KEY WORDS: *Crassostrea virginica*, juvenile oyster disease (JOD), hatchery mortalities, *Vibrio* spp., *Gymnodinium sanguineum*

INTRODUCTION

Since 1988, recurrent and widespread mortalities associated with a syndrome known as juvenile oyster disease (JOD) have occurred among nursery-reared eastern oysters, *Crassostrea virginica* (Gmelin, 1791), throughout the northeastern United States and have caused significant economic losses among aquaculturists. Total mortalities have ranged from 50 to nearly 100% of total stocks (Bricelj et al. 1992, Hillman and Elston 1992, Relyea 1992, 1994, Ford 1994, Davis and Barber 1994). The mortalities are age/size specific, preferentially affecting first-year oysters ranging from 6 to 30 mm in shell height, species specific, and site specific and are unrelated to broodstock origin. Mortalities generally occur during mid- to late summer after a period of relatively rapid growth when water temperatures exceed 20–22°C. Evidence for food or oxygen limitation, salinity, and direct temperature effects is not apparent for observed mortalities (Bricelj et al. 1992, Farley et al. 1992, Lewis 1993, Ford 1994). Histopathological examinations indicate that an irritant or toxin, probably produced by a pathogenic agent, affects the epithelial cells of the mantle edge (Bricelj et al. 1992). Mantle retraction is evident and may reflect the animals' attempt to "wall off" injured soft tissues from the irritant. Before dying, afflicted oysters also exhibit generalized symptoms of stress such as slow growth and "ring-like" conchiolin deposits, which include bacteria and cell debris in/on deposits on the inner shell surface (Bricelj et al. 1992, Ford 1994).

Blooms of the nonthecated dinoflagellate, *Gymnodinium sanguineum* (Hirasaka) (cell length, 43–50 μm; width, 31–43 μm) (=*Gymnodinium nelsoni* = *Gymnodinium splendens*), coincided with the time of initial oyster mortalities in Oyster Bay, NY, during the summer of 1991 study, suggesting some interaction between this potentially noxious phytoplankter and the disease (Bricelj et al. 1992). However, two other primary candidates for the cause of JOD have been suggested, a protozoan parasite (Farley et al. 1992, Farley and Lewis 1993) and a marine bacterium (Bricelj et al. 1992). Farley and Lewis (1993) and Lewis and Farley (1994) found that JOD is a transmissible disease and that mortalities are reduced by low salinity (10 ppt). Mortalities are also reduced by selected antibiotics including erythromycin, which stops the growth of many Gram-positive bacteria and strains of Gram-negative bacteria including some strains of *Vibrio* (Burdon and Williams 1968, Tubiash et al. 1965, Sindermann 1977, Brock et al. 1994). JOD symptoms such as mantle lesions, anomalous "ring-like" conchiolin deposits with adhering bacteria, and high mortality episodes are similar to the brown ring disease (BRD) that affects cultured Manila clams, *Ruditapes philippinarum* in Western Europe (Paillard and Maes 1990, 1994, Maes and Paillard 1992, Ford and Paillard 1994, Paillard et al. 1994). *Vibrio* P1 has been identified as the pathogenic agent of BRD.

The involvement of a pathogenic protozoan in JOD has not been conclusively demonstrated to date. In fact, a single recognizable protozoan has not been consistantly evident in afflicted oysters. To further confound the problem, the deposition of conchiolin and mantle retraction, which are used as symptomatic signs of JOD, may be caused by a variety of stressors (Palmer 1980) and cannot be readily linked to a specific agent. Bacteria, noxious dinoflagellates, protozoans, and environmental variables such as high water temperature may all have a synergistic role in JOD. However, similarities in the pathology of JOD and BRD and the absence of other demonstrable pathogenic organisms suggest that

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a species of bacterium or other noxious planktkon species may be involved in JOD. Thus, systematic studies of *Vibrio* and microplankton population dynamics within an affected nursery were warranted in order to examine their potential roles in JOD. Frank M. Flower and Sons Oyster Co. (FMF Co.) located in Oyster Bay, Long Island, NY, was chosen as the study site because it is the major producer (commercial operation) of oysters in the state of New York and has experienced severe oyster mortalities attributed to JOD since 1990.

MATERIALS AND METHODS

Experimental Design

Three oyster cohorts were produced (Table 1) and maintained according to protocols similar to those described by Bricelj et al. (1992). At this commercial operation, cultchless seed were used for experimental cohorts (i.e., oysters were set on 0.2- to 0.8-mm crushed hard clam shell chips). Oysters were kept in the hatchery in heated water for 4–6 wk while being fed cultured algae. When the oysters reached 3–4 mm, they were placed in ambient-water floating nursery trays for field growout in Oyster Bay, on the north shore of Long Island, NY. Oysters were placed in 0.8 × 1.2 × 0.08 m trays, open at the top and lined on the bottom with a 1-mm square mesh window screen. Initial densities were 36,000 oysters $tray^{-1}$, or 38,000 oysters m^{-2}. Trays (total, 2,692 trays at this commercial operation) were suspended in the water column (depth, 3.7 m at low water) in stacks of six trays, with the upper tray suspended about 12 cm below the surface. Oysters are typically removed from growout trays (usually 6–8 wk after deployment) and planted on the bay bottom at about 20–30 mm.

Water temperature and salinity were measured weekly. Mortality and shell heights of oysters (n = 100–400) were determined weekly or biweekly as described by Bricelj et al. (1992). Oyster samples were fixed in Davidson's fixative for the pathological examination of shell deposits. Results of a 1991 study (Bricelj et al. 1992) indicated that reducing the density of oysters significantly lowers the mortalities. This may have been because it permitted a greater degree of water circulation among oysters, reducing the buildup of rotting meats, feces, bacteria, and hydrogen sulfide that would exacerbate mortalities. To test this reasoning in a more practical way, part of cohort II was divided into two subgroups to compare the performance of oysters held in standard 1-mm standard mesh growout trays with that of those in 6-mm mesh trays.

TABLE 1.

Chronology of spawning, deployment of oyster, *C. virginica*, cohorts, detection of first mortality, and maximum cumulative oyster mortality at the study site.

Parameter	Cohort I	Cohort II	Cohort III
Spawning date	4/6/93	4/28/93	6/5/93
Deployment in field nursery	5/13/93	6/1/93	7/6/93
Mean initial shell height (mm)	3.2	3.2	10.6
(n, SE)	(100, 0.06)	(100, 0.05)	(100, 0.25)
Date of first recorded mortality	7/13/93	7/20/93	8/10/93
Elapsed time from deployment to maximum cumulative mortality (wk)	8.5	7	5
Cumulative oyster mortality (%)	21	35	60
Date of protocol change from whole oyster to meat & pallial fluid only (see Methods)	7/20/93	7/27/93	9/7/93

Sample Collection

All nursery samples (water, sediment, oysters, and tray debris) were collected weekly from May 19, 1993, to October 5, 1993. Reference samples (water, sediment, and oysters) were obtained monthly, approximately 0.5 miles away from the densely packed trays in the nursery. For microbiological analysis, a battery-powered peristaltic pump (Masterflex L/S™ Variable Flow Console) was used to obtain water from the same depth as the nursery float trays, approximately 0.5 m below the surface, and 0.5 m above bottom during early morning sampling. Water samples collected adjacent to the oyster floats were preserved in 2% glutaraldehyde and examined within 48 h to enumerate *G. sanguineum*, as well as other phytoplankton species, and the photosynthetic ciliate, *Mesodinium rubrum*, which bloomed during the study period. The vertical distribution of *G. sanguineum* was also determined by sampling at three depths in the water column, because this species is known to be highly motile and phototactic. Analysis of *G. sanguineum* data was performed by the use of two-way analysis of variance (ANOVA) without replication (Sokal and Rohlf 1981, Glantz 1992) to summarize distribution variations in the water column throughout the sampling period. Water samples from the hatchery well, which is used for larval, early postset, and algal cultures, and the microalgal food reservoir tank (~12,000 L) at the end of the exponential growth phase, as well as from the sewage treatment outfall (~3.2 km away from nursery) of the town of Oyster Bay, were also collected (Table 2) in order to identify potential sources of bacteria.

Triplicate sediment samples were taken below the growout trays with a gravity-type, messenger-activated core sampler (Cole-Parmer). Only the upper ~2 cm of the sediment was aseptically removed for analysis. Oysters were collected from growout trays before they were cleaned by hatchery personnel. All samples were stored on ice (<10°C) until processed, within 3–6 h. In general, four replicate oyster samples (i.e., two replicate samples from two replicate floats) were used for the determination of mortalities (n

TABLE 2.

***Vibrio* spp. concentrations in selected water samples.**

Samples	Date Sampled	Mean *Vibrio* Density ($CFU\ mL^{-1}$)	SE (n = 6)
Well water*	6/8/93	<1	<0.57
I. galbana culture medium	6/22/93	TMTC**	NA†
T. pseudonana culture medium	6/22/93	1	0.17
Sewage outfall	5/19/93	<1	<0.57
Water in larval setting tank	7/6/93	68	17.55

* Saline well water used in larval and early postset hatchery tanks (~27 ppt).

** TMTC, too many to count (>300 CFU/mL).

† NA, not applicable.

= 400) and shell growth (n = 100–400). Statistical analysis of mortality patterns among cohorts was performed by the use of one-way ANOVA and *a posteriori* comparisons to ascertain the effects of exposure time on percent-transformed data. Critical F values are indicated when the differences are significant. Differences in shell heights among cohorts at the end of the study were also analyzed by the use of one-way ANOVA (Sokal and Rohlf 1981, Glantz 1992).

Sample Processing

Oyster samples were cleaned and prepared according to methods modified from the Recommended Procedures for the Examination of Sea Water and Shellfish (APHA 1970). Oysters were homogenized as 10-g whole-oyster samples or as the meat and juice only of 12 oysters, depending on the size of the animals (Table 1). Homogenized oyster samples were then diluted decimally in sterile phosphate-buffered saline water (PBSW). Ten milliliters of each dilution of oyster homogenates was filtered through a sterile 0.45-μm-pore-size cellulosic membrane filter in triplicate by aseptic techniques. *Vibrio* trapped on the membranes were preenriched by placing the membranes on absorbent pads soaked with sterile alkaline peptone water for 6 h at 30°C. Membranes were then aseptically transferred onto thiosulfate-citrate-bile salts (TCBS) agar plates and incubated for 18–24 h at 30°C (Bryant et al. 1986, Venkateswaran et al. 1989). In the presumptive phase, the total number of colonies, most likely *Vibrio* spp. colonies, was enumerated on a colony counter to obtain the total number of colony-forming units (CFUs). In the confirming phase, selected colonies appearing in the highest (i.e., most abundant) dilutions were streaked onto new TCBS agar plates to isolate pure colonies from possible mixed colonies (Brock and Madigan 1991). Distinctive colony morphotypes were selected and transferred to marine agar (Difco™ 2216E) for storage and subsequent testing. These isolates were also streaked onto tryptic soy agar plates for further identification by use of the Biolog Inc. (Haywood, CA), and API 20E (BioMerioux, France) diagnostic systems, as well as other biochemical and physiological tests conducted according to Balows (1974), Cowen (1974), Difco (1991) and Holt et al (1994).

Bacteria in sediment samples were dissociated from the particles before analysis by the vigorous vortexing of 0.5-g subsamples in 10 mL of sterile sodium metaphosphate (25 mM; pH = 7.0) for 15 s and then the addition of 25 mL of sterile distilled water before being vortexed again for 15 s (modified from Saad and Cabelli, unpub. obs.). Suspensions were allowed to stand in a refrigerator, and then supernatants were diluted as required in PBSW. Sediment sample dilutions were then subjected to the same preenrichment, presumptive, enumeration, and confirming protocols as described for oyster samples. Debris (mostly biodeposits) collected from the external surface of oyster shells in nursery growout trays were treated like sediment samples. Water samples were also filtered and treated like oyster samples for bacteriological analysis. Three batches of 12 individuals from cohort III oysters collected on October 3, 1993, were assayed separately to examine the partitioning of *Vibrio* within the oysters. Batch 1 consisted of whole oysters crushed, batch 2 consisted of pallial fluid only, and batch 3 consisted of meat, palliall fluid, and shells. From batch 3, meat and pallial fluid were assayed together, whereas shells were crushed and assayed separately. All samples were subjected to the same procedures as regular oyster samples. Similarities in *Vibrio* spp. concentrations among cohorts were analyzed by the use of regression analysis.

Challenge Experiments

Juvenile oysters used in challenge experiments were obtained from the East Hampton Hatchery, Long Island, NY, for the first experiment (avg. height, 29.3 mm; ±0.22 SE) and from Haskin Shellfish Research Laboratory, Rutgers University, NJ, for the second (avg. height, 35.4 mm; ±0.25 SE) and third (avg. height, 17.3 mm; ±0.20 SE) experiments. JOD has never been reported from these sites. Animals were about 1 (first and second challenge experiments) or less than 1 y old (third challenge experiment). Two weeks before the experiments, oysters were acclimated to the experimental temperature in flow-through ambient seawater in a ~30-L (20–26 ppt salinity) aquarium supplemented with cultured agae, *Thalassiosira weissflogii* or *Isochrysis galbana*. During challenge experiments, batches of oysters were maintained in individual aquaria (~11-L capacity) in filtered seawater with constant aeration on a temperature-controlled seawater table at the State University of New York at Stony Brook, Flax Pond Facility.

A total of nine *Vibrio* spp. isolates were used in the challenge experiments. Three selection criteria were used to increase the probability of selecting pathogenic bacteria from 200 isolates. The nine species of *Vibrio* were isolated from oyster meats to minimize the selection of nonpathogenic *Vibrio* spp. from the general aquatic surroundings. They were also chosen during periods preceding the onset of high mortality by 1 to 3 wk in order to minimize the selection of strains that might be opportunistic secondary invaders of dead and dying oysters. A third criterion was the phenotypic resemblance to *Vibrio* P1 (because of the close similarities between JOD and BRD). At least the first two criteria were met by all nine isolates.

Small notches were cut into the ventral margin of the oyster shell edge with a Dremel tool. Random lots of ~30 animals were then segregated for each treatment, and each treatment was duplicated. From each experimental animal, pallial fluid was removed and replaced with a 50-μL inoculum of a *Vibrio* isolate in stationary growth phase (~10^6 cells per injection), as described in Paillard and Maes (1990). Tank water was changed every 7 d. In the first and second challenges, three negative controls were run simultaneously: (1) notched but no injection (blank), (2) filtered seawater injection (FSW), and (3) injection with a viable nonpathogenic bacteria, *Escherichia coli* (Difco Bactro Disks ATCC 25922) at the same cell density (*E. coli*). In the third experiment, negative controls were: (1) no notching and no injection (blank), (2) notched and no injection (notch), and (3) *E. coli* injection (E. coli). Each replicate treatment was placed in a separate aquarium containing 11 L of freshly filtered (0.2-μm-pore-size filter), aerated seawater. All aquaria were placed on a temperature-controlled seawater table.

Oysters in the first challenge experiment received a single inoculation of *Vibrio* spp., were maintained at 22°C under relatively uncrowded conditions (30–33 oysters per tank), and were batch fed twice daily (~66×10^6 cells $tank^{-1}$ or 2.0×10^6 cells $oyster^{-1}$ of *T. weissflogii*, or ~19×10^8 cells $tank^{-1}$ or ~6.0×10^7 cells $oyster^{-1}$ of *I. galbana* at each feeding) for 30 d. Biodeposits were removed daily with a pipette. Mortality was recorded daily, and dead oysters were removed. The second challenge experiment was run for 14 d under more stressful conditions. The animals were injected twice (days 0 and 7), fed only once a day, supplemented with 15 mL $tank^{-1}$ of *Vibrio* suspension (10^6 cells mL^{-1} final tank concentration) every other day, and crowded inside 1-mm mesh bags (9 × 10 cm enclosed area). Biodeposits

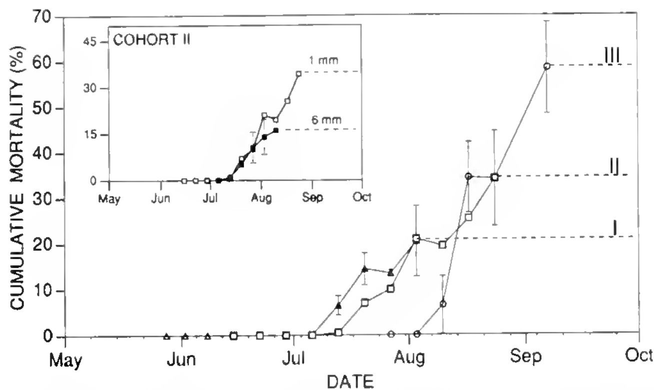

Figure 1. Mean (±SE) cumulative mortalities of three experimental oyster cohorts and cohort II grown in 1- and 6-mm mesh growout trays (inset) of duplicate samples from duplicate trays at the Frank M. Flower and Sons (FMF) Co. oyster nursery, Oyster Bay, NY, during 1993. The dotted lines represent estimated total mortality. (△) Cohort I. (□) Cohort II, 1-mm mesh trays. (■) Cohort II, 6-mm mesh trays. (○) Cohort III. Error bars smaller than the symbols are not indicated.

were not removed, and aquaria were maintained at a higher temperature of 24°C. At the end of the second experiment, oysters from the three treatments exhibiting highest mortality, as well as those from the three control batches, were processed and analyzed for bacterial abundances according to the protocols followed for field samples.

The third challenge experiment was run for 21 d under a different set of conditions. The animals received a single injection, were maintained at 25°C, fed twice a day, and were supplemented with *Vibrio* spp. suspension every other day. Oysters were crowded inside 1-mm mesh bags suspended in the water column, and the bottom of the aquaria contained sediment from Oyster Bay (175 g of dry weight sediment per tank, autoclaved 8–10 h at 121°C at 15 lb of pressure). Sediments were mixed with appropriate *Vibrio* spp. isolates before oysters and filtered seawater were added into each tank. Biodeposits were removed, and tank water was changed every 7 d. In this experiment, the two isolates that induced the highest mortality in previous experiments were used separately and in combination. Two other new isolates were also used separately and in combination. All treatments from the third experiment, including controls, were processed for total bacterial abundances and identification. To determine whether the bacteria that had been inoculated proliferated and thus satisfy Koch's postulates (Anderson and Sobieski 1980), the reisolated bacteria were identified and compared with the original inocula in the second and third experiments. One-way ANOVA and *a priori* multiple comparisons between controls and experimental batches, as well as *a posteriori* multiple comparisons among experimental batches and among all of the injected batches, were used to ascertain the effects of *Vibrio* injection. Critical F values are indicated when the differences were significant.

RESULTS

Oyster Mortality Patterns

Mortalities were negligible in all three cohorts until mid-July. They were first recorded in cohort I on July 13, in cohort II on July 20, and in cohort III on August 10 (Fig. 1). Mortalities peaked at 21, 35, and 60% for cohorts I, II, and III, respectively (represented by dotted lines in Fig. 1), 3–5 wk after mortality was first observed. JOD involvement in the mortalities was established by the presence of ridge-like conchiolin deposits on the inner shell (Fig. 6 in Bricelj et al. 1992). However, both conchiolin deposits and oyster mortalities were less severe than in previous years at this site. Only 0–40% of the population exhibited light to heavy shell deposits among live oysters during immediate premortality and mortality periods. Only on the shells of dead oysters did conchiolin deposits approach the 60–100% level recorded in live oysters during the same period in 1991. Cohort III, which had the highest mortality, also exhibited the lowest prevalence of conchiolin, only 11–17% of the population exhibited light to heavy deposition among live oysters. Histopathological study of tissues from dying cohort I individuals collected on July 20 indicated that 60% had some mantle lesions. Coccoid bodies (Bricelj et al. 1992) were evident in only 15%, and ciliates in another 15%.

Cumulative mortalities of oysters held in 6-mm mesh growout trays were half as much as those observed in oysters held in 1-mm mesh trays (16 vs. 35%, $P = 0.08$) (inset, Fig. 1). Mortalities for cohort II, held in larger mesh growout trays, ceased to increase 2 wk earlier than mortalities in smaller mesh trays, and final mortality was even lower than that of cohort I nursery oysters (24%). Anomalous conchiolin deposits were less prevalent in oysters from larger mesh size trays (0–25%) than in those from smaller mesh size trays (0–40%) before and during mortality.

Oyster Growth Patterns

All nursery cohorts experienced slower growth rates (slopes of shell height increase between sampling dates) during the period of mortalities (Fig. 2), although apparent growth did not cease entirely, probably because smaller oysters died. Maximum shell growth rates of 0.33–0.49 mm d^{-1} were observed among all cohorts before the onset of mortalities. Similar growth patterns were observed during a previous study at the same site (Bricelj et al. 1992). The average shell height of the nursery oysters in the larger mesh trays on September 28 was not significantly greater ($P = 0.125$) than that of oysters in the smaller mesh trays (41.6 vs. 38.8 mm; inset, Fig. 2), despite the differences in mortalities.

Phytoplankton

Two microplanktonic species occurred at high densities at the nursery site during 1993 (Fig. 3). A bloom of *M. rubrum* (1,150

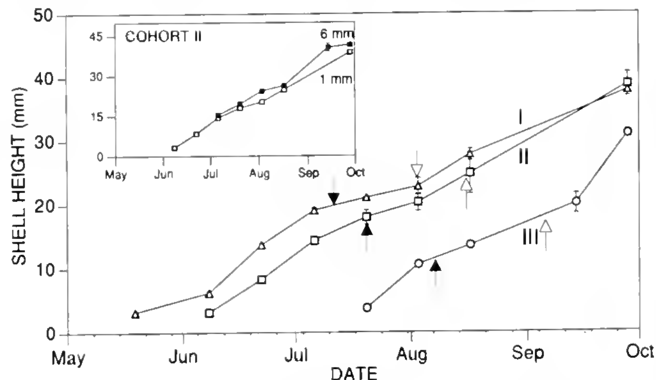

Figure 2. Mean (±SE) shell heights of three cohorts and oyster cohort II grown in 1- and 6-mm mesh (inset) at the FMF Co. oyster nursery during 1993. (△) Cohort I. (□) Cohort II, 1-mm mesh trays. (■) Cohort II, 6-mm mesh trays. (○) Cohort III. Error bars smaller than the symbol are not indicated. Arrows indicate the onset (closed arrows) and end (open arrows) of mortalities of each cohort.

cells mL^{-1}, on July 6), a red tide-producing ciliate (ranging in size from 15 to 70 μm), was observed before the onset of mortalities in cohorts I and II. *G. sanguineum* was not detected until July 20, 1 wk after oyster mortality began in cohort I, and peaked on August 3 (458 cells mL^{-1}), at the onset of mortalities in cohort III, and again on August 24 (212 cells mL^{-1}). The first bloom occurred concurrently with the maximum mortality in cohort I oysters. Comparable cell densities of *G. sanguineum* were observed at this study site in 1991 (Bricelj et al. 1992), suggesting that this species may be established in Oyster Bay. The cell concentrations of *G. sanguineum* were approximately the same at surface, intermediate, and bottom depths ($P = 0.56$). Diatom species, known to be of high food quality for bivalves, were abundant both before and after the midsummer *M. rubrum* and *G. sanguineum* blooms, when oyster growth was also good, but not during blooms. *Thalassiosira pseudonana, Skeletonema costatum, Chaetoceros* spp., and *Ceratulina bergonii* were dominant during early summer, whereas *S. costatum* comprised from 65 to 99% of total diatoms in late summer (inset, Fig. 3).

Environmental Parameters: Temperature and Salinity

Surface water temperature varied between 16.5 and 27.5°C during the study period and exceeded 20°C from mid-June through mid-September (Fig. 4). The outbreak of mortalities occurred during the period of elevated surface water temperatures (range 22–27°C) between mid-July and early September. Salinities ranged from 24 to 27 ppt. Salinity was within the optimal range for the growth of most *Vibrio* spp. (Baumann et al. 1984) and also for the growth of *C. virginica* (Mann et al. 1991). The variations recorded in surface water temperature and salinity were typical of previous years at this site.

Trends in Vibrio *spp. Densities*

Vibrio spp. concentrations in both surface and bottom waters at the nursery site were low (1—25 CFU mL^{-1}; inset, Fig. 5), with the exception of an unusually high peak observed in surface waters on August 3 (185 CFU mL^{-1}). Reference site measurements (1–5 CFU mL^{-1}) were always lower than measurements adjacent to the nursery floats. *Vibrio* spp. concentrations in sediments at the nursery site fluctuated markedly and were highest (500–1,300 CFU g^{-1}) between June 15 and July 27, before exponential increases of *Vibrio* spp. in oysters themselves and the onset of mortality were observed (Fig. 6). By late July and August, sediment *Vibrio* spp. concentrations had decreased (<200 CFU g^{-1}), whereas they escalated in oyster tissues during this mortality period. At the reference sites, *Vibrio* spp. concentrations in the sediment samples were typically two to three orders of magnitude lower (10–70 CFU g^{-1}) than those in the nursery sediment samples.

Vibrio spp. concentrations in nursery oyster tissues always increased at least one order of magnitude approximately 2 wk before mortalities were observed (Fig. 6). Concentrations in oysters from cohorts I and II were in the range of 10^3 CFU g^{-1} for the first few weeks after deployment in nursery trays. An exponential increase to 3×10^4 CFU g^{-1} occurred in cohort I oysters between June 15 and June 29, 2 wk before mortality was first observed (Fig. 6A). In cohort II, a similar exponential increase to 2×10^4 CFU g^{-1} was observed between June 15 and July 6, again 2 wk before the first mortality was observed (Fig. 6B). *Vibrio* spp. concentrations in cohort III were high (10^4 CFU g^{-1}) at initial deployment, decreased the following week, and then increased again 1 wk before mortalities began (Fig. 6C). *Vibrio* spp. concentrations declined after mortalitites began and then peaked twice in all cohorts generally a few days after the *G. sanguineum* blooms (arrows), suggesting a contributing stress effect of the dinoflagellate blooms on the oyster. Variation (i.e., timing of increases and decreases) in

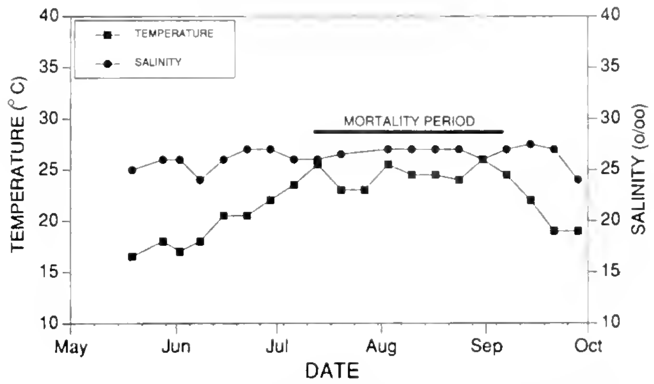

Figure 4. Surface water temperature and salinity at the oyster nursery, Oyster Bay, NY, during the 1993 study period. The thick horizontal line indicates the period of juvenile oyster mortalities.

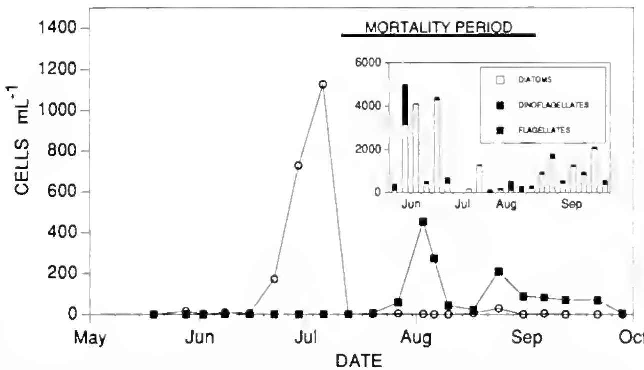

Figure 3. Cell densities of *G. sanguineum* (■) and *M. rubrum* (○) and relative concentrations (inset) of diatoms, dinoflagellates (including *G. sanguineum*), and other flagellates in surface water at the oyster nursery in Oyster Bay, NY, in 1993. The thick horizontal line indicates the period of juvenile oyster mortalities.

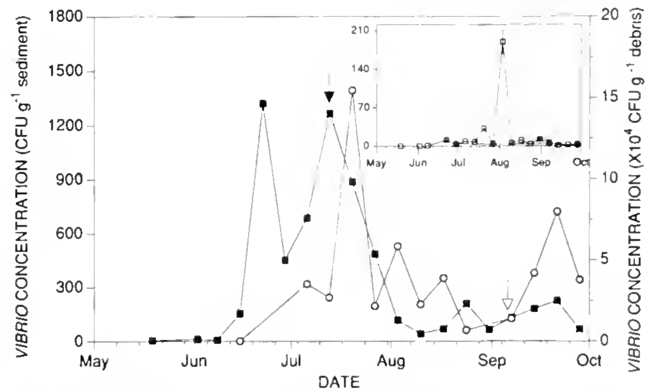

Figure 5. Mean total *Vibrio* spp. concentrations in nursery bottom sediment (■) and tray debris (biodeposit) from cohort I nursery oysters (○) in CFU g wet weight^{-1} and in nursery (□) and reference water samples (●) (inset) in CFU mL^{-1} during 1993. Arrows indicate the onset (closed arrow) and end (open arrows) of juvenile oyster mortality periods.

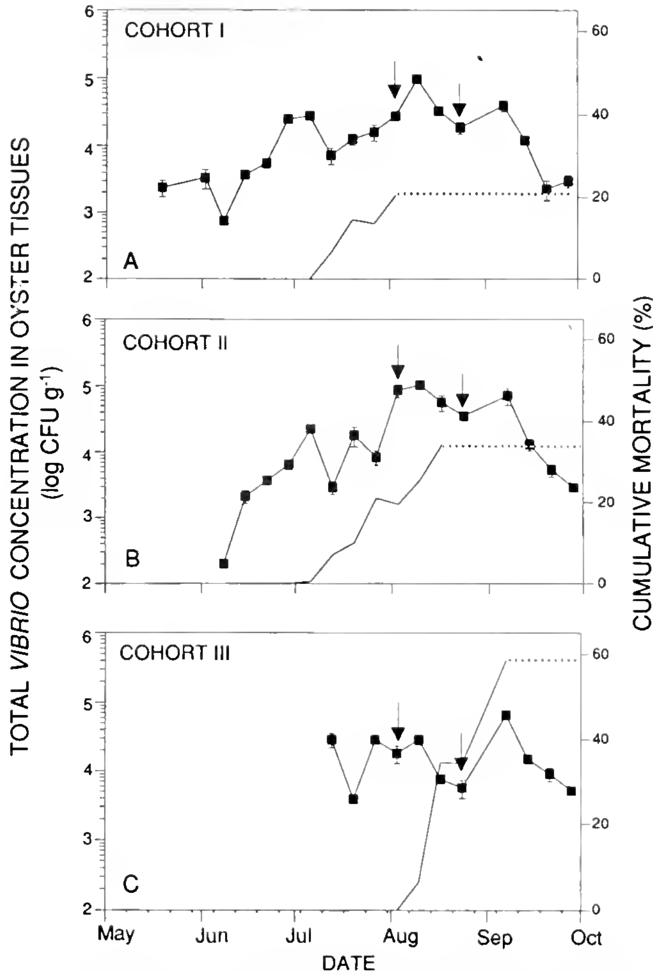

Figure 6. Mean cumulative oyster mortalities (–) and total *Vibrio* spp. concentrations in CFU g wet weight^{-1} (■) in three cohorts of juvenile oysters held in outdoor floating trays at the oyster nursery during the 1993 study period. The dotted lines represent estimated total mortality. (A) Cohort I, (B) cohort II, (C) cohort III. Arrows indicate dates when peak concentrations of *G. sanguineum* were observed.

Vibrio spp. concentrations among all three cohorts were overall similar (r^2 = 0.40–0.85), indicating that the effects of *Vibrio* spp. were similar among all three cohorts. However, the oyster mortality patterns with respect to onset, duration, and intensity were not completely consistent among cohorts, indicating the possible involvement of other variables in addition to *Vibrio* spp. *Vibrio* spp. concentrations in reference site oysters (30–2,500 CFU g^{-1}) were two- to three-fold lower than in the nursery oysters at the same sampling date. *Vibrio* spp. densities in tray debris from nursery oysters were typically higher than those of any other sample, varying from 210 to 140,000 CFU g^{-1} (e.g., cohort I in Fig. 5). Oysters from the reference site had no such debris. *Vibrio* spp. concentrations in the tray debris remained relatively high during the mortality period, even while declining in the sediments and remaining low in the water.

Examination of the partitioning of *Vibrio* within oysters collected on October 5, 1993, indicated that, per gram of wet weight, the *Vibrio* recovered appeared to be more or less evenly distributed between meats (20%), pallial fluid (32%), and shell (21%) (Table 3). In this analysis, oysters were sacrificed at the end of the sampling period, well after mortalities occurred. However, if the partitioning observed is representative of the whole sampling period, it would suggest that the pallial fluids may act as a transitional bacterial medium between the surrounding external environment and soft tissues; thus pallial fluid continually exposes oyster tissues to the potentially pathogenic bacteria.

TABLE 3.

Distribution of *Vibrio* spp. within cohort III oysters (sampled on October 5, 1993).

Sample	Mean (CFU/g wet wt)	SE (n = 3)	% of Whole
Whole	2926	527	100
Meat/fluid	1523	317	52
Fluid	944	102	32
Meat only*	579	215	20
Shell	628	80	21
Unaccounted			27**

* Meat only = meat and pallial fluid − pallial fluid.
** Unaccounted = whole − (meat + fluid + shell).

Potential Sources of Vibrio *spp.*

Vibrio spp. were undetectable in sewage outfall water, indicating that the local waste treatment plant was not a source of contamination at the time of analysis (Table 2). High *Vibrio* spp. counts (>700 CFU mL^{-1}) were found in the *Isochrysis* microalgal cultures usually fed to setting oysters (Table 2). A few of the numerically dominant *Vibrio* isolates from the flagellate and diatom cultures (although total *Vibrio* spp. concentration was low in the *T. weissflogii* tank) were similar to the isolates collected from the nursery float area (sediment, oyster and water samples) such as *Vibrio anguillarum* and *Vibrio splendidus*. Although this analysis was carried out at a single sampling time, it demonstrated that *Vibrio* spp. were also present within the hatchery. This finding was consistent with high *Vibrio* spp. counts from the hatchery larval settling tank (Table 2). Apparently, at the time of sampling, the larvae and postlarvae were being fed contaminated algal cultures, suggesting that contamination of oysters with some *Vibrio* spp. may have occurred even before they were exposed to bay water. Cohort III was present in the hatchery at the time of these samplings. This was consistent with the high *Vibrio* spp. counts observed in cohort III before they were even transferred to the nursery floats (Fig. 6C). However, the counts represent total *Vibrio* spp. populations and the actual pathogens may not have been present at all.

Oyster Mortalities and Reisolation of Vibrio *spp. in Challenge Experiments*

Average mortality in the first challenge experiment varied from 2 to 6% in controls and in five of the six treatments with bacterial isolates (Fig. 7A). One *Vibrio* isolate, ST-131, caused significantly higher mortality than controls (15%; F = 5.99; P < 0.001) and other treatment groups (F = 4.96; P = 0.002). The second experiment (Fig. 7B) produced much higher mortalities in all groups. Two batches of oysters injected with *Vibrio* isolates ST-78 and ST-131 showed higher mortalities (73 and 68% at F = 4.75;

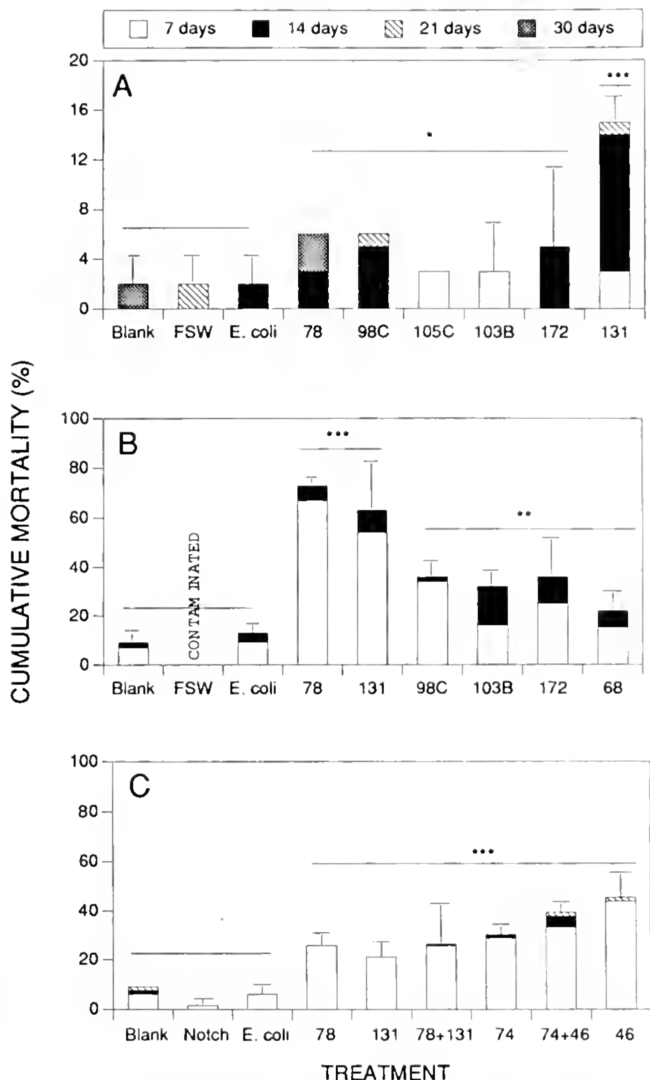

Figure 7. Mean (±SE) total mortalities of healthy juvenile oysters injected with *Vibrio* isolates in three experiments. (A) Juvenile oysters obtained from the East Hampton hatchery, NY, notched and injected with filtered seawater, suspensions of *E. coli*, or suspensions of *Vibrio* isolates or notched but not injected (blank), incubated for 30 days. (B) Juvenile oysters obtained from Haskin Shellfish Research Laboratory, NJ, subjected to similar experimental treatments except that they were crowded into mesh bags, supplemented with bacteria once every other day, and maintained for 14 d. Filtered seawater control in panel B is suspect because the filter was compromised. (C) Juvenile oysters were obtained from Haskin Shellfish Research Laboratory. They were crowded into suspended mesh bags in aquaria containing sediment and supplemented with bacteria once every other day; some were treated with a combination of bacterial isolates and maintained for 21 d. Mortalities in all challenge experiments are represented in 7-d increments. Probability ranges of significance in difference between controls and other groups are indicated by asterisks above the bars. Bars indicate that the sample mean is not significantly different.

$P < 0.001$ and $F = 4.75$; $P \leqslant 0.003$, respectively) than those injected with other isolates or control groups. All *Vibrio* spp. treatments also showed higher mortalities than control groups ($F = 7.71$; $P = 0.016$). Examination of shells revealed blister-like organic deposits in the second experiment, including controls injected with *E. coli*, but they were not the typical JOD deposits observed in field-deployed nursery oysters. Biochemically and physiologically identical *Vibrio* spp. were reisolated from ST-78 and ST-131 oyster batches at the end of the second experiment. The third experiment (Fig. 7C) showed that a combination of two isolates did not produce significantly higher mortality ($P = 0.67$) than single-isolate treatments, and there were no significant differences among treatments ($P = 0.2 \sim 0.9$), except ST-46, which showed higher mortality ($F = 4.96$; $P = 0.047$) than other treatments. However, all *Vibrio* spp. treatments showed higher mortalities than control batches ($F = 4.49$; $P < 0.001$).

In the third challenge experiment, most of the notched control oysters showed slight brown depositions around the notched area (Fig. 8A, arrow). Control oysters without notching showed no such depositions. Brown deposition is probably a defense response to the stress of being notched. Not all experimental oysters injected with *Vibrio* isolates displayed symptoms typical of JOD. However, those that did exhibited indications of mantle retraction (Fig. 3B, arrow) and conchiolin deposition (Fig. 8C, arrow).

DISCUSSION

The Role of Nonbacteriological Variables in JOD

Temperature and Salinity

Salinity and temperature were within the normal range for the study site and for the growth of eastern oysters. In this study, the onset of oyster mortalities followed a period of increasing temperatures and did not occur until they exceeded 22°C. This finding agrees with previous studies, in which high water temperatures (>20°C) were identified as the most important environmental parameter related to the timing of mortalities, but were nonetheless suggested to play only a secondary role in JOD (Bricelj et al. 1992, Ford 1994). The major consequence of high temperature is its effect on bacterial proliferation, because many *Vibrio* spp. actively grow and multiply at temperatures >20°C.

Phytoplankton Blooms

Nightingale (1936) reported an association between mortalities of oysters, *Ostrea lurida*, especially young stages, and red tides of *G. sanguineum* in Oakland Bay, WA. In this study, however, the timing of the first *G. sanguineum* bloom relative to observed oyster mortalities does not support the hypothesis that this dinoflagellate is the primary etiological agent for JOD. Lewis (1993) determined that JOD was a transmissible disease without the presence of *G. sanguineum*. Wikfors and Smolowitz (1994) found that an isolate of *G. sanguineum* from Oyster Bay did not produce JOD symptoms, i.e., abnormal conchiolin deposition in 10-mm juvenile oysters. However, copious pseudofeces production was observed during the first 2 wk of exposure and dinoflagellate cells were fed at a maximum concentration of only four cells per milliliter, two orders of magnitude lower than that attained in the field during our study. Thus, blooms of *G. sanguineum* may act as a contributing stress factor. It is also noteworthy that peak abundance of *G. sanguineum* and *M. rubrum* coincided with marked reductions in the concentration of other phytoplankton species, especially diatoms that commonly support good growth of bivalves. Furthermore, high *Vibrio* spp. densities in juvenile oysters and water samples from nursery floats coincided with *G. sanguineum* blooms. Hence, this species may indirectly affect the

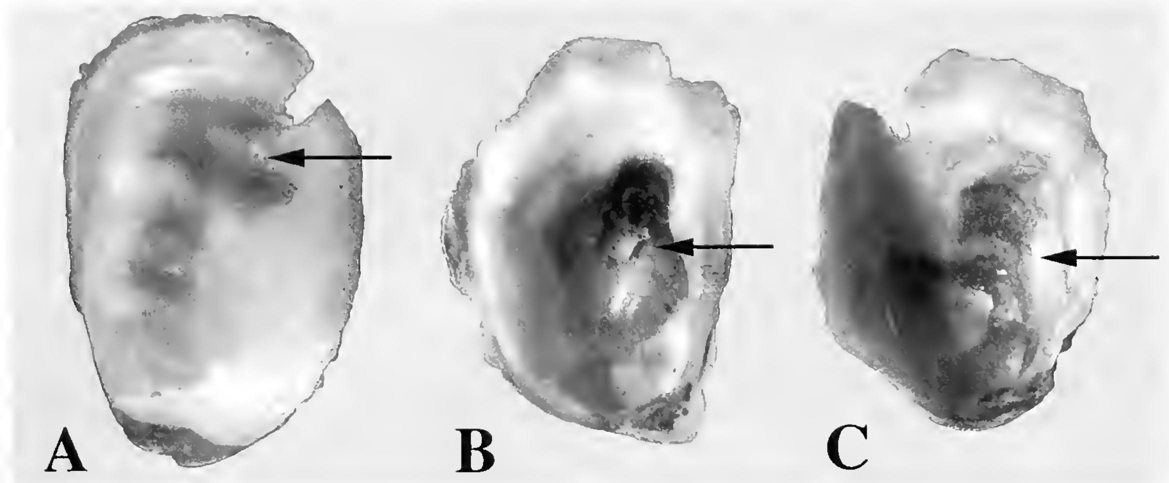

Figure 8. Juvenile eastern oysters from the third challenge experiment. (A) Notched control oyster. Note the slight conchiolin deposition around the notched area. (B) Oyster injected with *Vibrio* isolate (ST-78) exhibiting mantle retraction in the form of irregular depositions. (C) Oyster injected with a combination of two *Vibrio* isolates (ST-46 + 74) showing early stages of "symptomatic" conchiolin deposition. Arrows indicate deposition.

progression of JOD among nursery oysters by influencing bacterial densities in the water and in the juvenile oysters.

An association between phytoplankton or zooplankton blooms and elevated concentrations of *Vibrio* spp. and other bacteria have been reported in a number of previous studies (Kaneko and Colwell 1973, 1975, 1978, Huq et al. 1983, Hoppe 1984, Williams and LaRock 1985, Romalde et al. 1990a, 1990b, Montgomery and Kirchman 1993). During this study, a bloom of *M. rubrum* preceded *Vibrio* spp. increases in oyster and oyster mortalities. However, a stong association between *Mesodinium* cell densities and bacterial abundance in the water column, as observed by Crawford et al. (1993), was not apparent in this study. Furthermore, *M. rubrum* has not been reported to be toxic and is consumed by a wide range of organisms, from mysids to oysters (Lindholm 1985).

Oyster Age and Size

Mortality occurred among oysters within the susceptible size range of 10–16 mm. When mortality was first observed in the nursery floats, the mean size of oysters was ~20, ~16, and ~12 mm for cohorts I, II, and III, respectively. Mortalities of cohort I oysters did not occur until almost 9 wk after deployment, whereas deaths in the other cohorts took only 5–7 wk to appear (Table 1). Cohort I may have taken longer than the others to accumulate a critical level of pathogens because pathogen concentrations were relatively low in the plankton and/or because pathogen proliferation in oysters and oysters' potential filtration/pumping activity were reduced at lower temperatures. By the time additional determinants, e.g., higher temperatures and deleterious plankton blooms (stress), were present in the water column, cohort I oysters had presumably grown large enough to be less susceptible to these factors, as reflected in this cohort's lower overall mortality. The opposite may be true for the oysters deployed later. From an energetics standpoint, small individuals, which have a higher metabolic rate per unit body weight and relatively lower energy stores, are more likely to exhibit nutritional stress than larger individuals (Galtsoff 1964, Fisher 1988). Oysters spawned and deployed later in the growout season hence smaller individuals are thus more susceptible to JOD.

Vibrio *spp. Concentrations in Relation to Oyster Mortalities*

The initial exponential increases in *Vibrio* spp. concentrations were observed in all three cohorts before oysters began dying. Thus, bacterial proliferation was not the result of dead and dying oysters providing a suitable medium for *Vibrio* spp. growth. This observation provided the first evidence that *Vibrio* spp. may be involved in JOD. Before this exponential growth, relatively stable *Vibrio* spp. levels in all three cohorts were probably maintained by a dynamic equilibrium between filtration and egestion or destruction of *Vibrio* spp. Because of *Vibrio* spp. proliferation in the oysters' immediate environment, e.g., debris in tray and in/on the oysters themselves, stressed, crowded juveniles may eventually become vulnerable to disease. Quantitative bacterial analysis conducted in other studies has found high counts of *Vibrio* spp. in both "sick" oyster and clam intervalval fluids, ranging between 10^4 and 10^6 cells mL^{-1} (Lovelace et al. 1968, Maes and Paillard 1992). Similar concentrations of *Vibrio* spp. were found in this study in the pallial fluid between mantle and shell. Although not investigated, total *Vibrio* spp. increases in oysters during this study probably included the amplification of pathogenic *Vibrio* spp. The critical concentration of pathogenic *Vibrio* spp. can be different for each cohort because of the varying conditions of juvenile oysters and their environment. It appears that the intensity and timing of mortality are also influenced by oyster size and condition (i.e., prior history) at the time of infection. All other *Vibrio* spp. increases after the initial premortality rises were probably due to active bacterial proliferation inside already infected, dying, or compromised juvenile oysters or their immediate environment.

Mortality can be minimized by growout methods that may prevent a critical level of potentially pathogenic *Vibrio* spp. or other possible agents from accumulating around the oysters. Mortalities associated with JOD have been shown to be lower among oysters placed on a less densely packed raft (Bricelj et al. 1992). In this

study, oysters placed on rafts with a larger mesh size also showed lower mortalities. This phenomenon may be attributed to the dilution of bacteria caused by improved water exchange around the oysters or to the improved condition of oysters themselves reared under these practices. Another possible point of infection not related to the "contaminated environment" of the nursery site may have occurred during larval rearing and settlement inside the hatchery. This hypothesis is supported by high *Vibrio* spp. concentrations in microalgal cultures and hatchery larval and postset tank waters even before oysters (cohort III) were deployed to the raft system (Table 2).

It must be emphasized, however, that the correspondence between total concentrations of all *Vibrio* spp. and mortality in oysters within a given cohort does not establish causality. Many bacteria may simply represent background nonpathogenic populations, or opportunistic infections of compromised oysters, rather than pathogenic *Vibrio* spp. In this study, the most often identified species of *Vibrio* were *Vibrio parahaemolyticus*, *V. anguillarum*, *Vibrio vulnificus*, and *V. splendidus*—all known pathogens to varying degrees. However, concentrations of individual *Vibrio* species are unknown, and the frequency of the occurrence of specific bacteria was not determined.

The examination of temporal patterns in *Vibrio* spp. concentrations, especially before the start of mortalities, gives some insight into the *Vibrio*-JOD relationship. First, *Vibrio* spp. concentrations (1–185 CFU mL^{-1}) observed in the water column showed no relationship to oyster mortality patterns. Nevertheless, *Vibrio* spp. were present at all times, although at low concentrations. There was a significant correlation between *Vibrio* spp. concentrations in surface water and sediment before the onset of oyster mortalities ($r^2 = 0.869$), suggesting that the bay bottom may have acted as a source of *Vibrio* spp. to the oysters. An observed "pulse" of *Vibrio* spp. in the water samples is not necessary in order to postulate a transfer from sediment to oyster because of the large dilution effect of waters overlying the sediments and the high pumping rate of oysters. These observations do not, however, clearly demonstrate whether sediment was a source for *Vibrio* spp. found at the nursery site.

The FMF hatchery uses the same nursery location year after year. This presumably increases the inoculum of bacteria and organic nutrient loading to the nursery sediment compared with the reference site, through dead and dying oysters, debris, sedimenting planktonic blooms, and other aquatic animals attracted to the float area. All of these can act as a source for reinfection by providing a medium for bacterial proliferation and by sediment resuspension (Kaneko and Colwell 1973, 1978, Ruby and Morin 1979, Williams and LaRock 1985, Enger et al. 1989, Buck 1990). Under stressful conditions, e.g., low nutrients, extreme temperatures, crowding, *Vibrio* spp. can develop into viable but nonculturable resting stages, reinoculating the water column when environmental conditions improve (Parsons et al. 1984, Molitoris et al. 1985, Williams and LaRock 1985, Pathek et al. 1988). During this study, *Vibrio* spp. counts in the sediments declined late in the summer season, even though temperature and probably nutrient levels remained favorable for bacteria including *Vibrio* spp. One of the factors contributing to this decrease could be competition with other bacterial populations in the sediment (Hood and Ness 1982). Enough *Vibrio* spp. may have survived through the winter to grow and reinoculate the water and oyster during the next season. It appears that the sediment could act as a source of *Vibrio* spp. early in the growout season and become less important as the season progresses, especially after the oysters and tray debris are inoculated. Debris immediately surrounding the oyster most likely provides a habitat that promotes the growth of *Vibrio* spp. and other bacteria. Thus, growout tray debris is likely to be an important transient reservoir for potentially pathogenic *Vibrio* spp. that promotes additional hostile conditions for the oysters by creating anoxic microenvironments as oysters die and decay.

Vibrio spp. as Potential Pathogens

Challenge experiments showed that isolates phenotypically similar to *V. anguillarum* (ST-131) and *Vibrio alginolyticus* (ST-7-8) caused high juvenile oyster mortalities. Although there was only slight evidence of JOD-like anomalous conchiolin shell deposits in some of the experimental oysters examined, the high mortalities caused by injected *Vibrio* isolates strongly suggest that these bacteria are pathogenic under certain conditions. These conditions include high water temperatures, crowding, and high concentrations of specific bacteria. Thus, much higher mortalities were observed in the second and third challenge experiments, in which these conditions were amplified. The paucity of typical JOD symptoms among experimentally infected oysters is a liability in assigning a species of *Vibrio* as an agent of JOD and thus in fulfilling Koch's postulates. Such a discrepancy does not, however, invalidate the hypothesis. Environmental conditions imposed on oysters during the challenge experiments slightly misrepresented *in situ* nursery conditions. Oysters may have been stressed differently, so that symptomatic conchiolin deposits were not properly induced before mortality ("acute" JOD). The somewhat older and larger oysters available for use in the challenge experiments may not have responded in the same way as smaller juvenile oysters afflicted with JOD. The typical conchiolin deposit may be a defense mechanism of juvenile oysters that are in a "high-growth" mode. Larger oysters, as well as oysters in poor condition, may experience mortality without showing JOD signs (Lewis 1993). Alternatively, the trauma of injections and containment in a closed system may have caused oyster death to take place through "acute toxin-mediated infection" before oysters could use their defense systems to build conchiolin deposits (Elston et al. 1982, Elston 1984, Paillard and Maes 1994, Paillard et al. 1994). Another consideration is the fact that only nine isolates among 200 tentatively identified bacteria were used in these experiments. It is thus possible that the causative agent(s) of JOD may not have been among the selected nine isolates.

V. anguillarum and *V. alginolyticus*, both of which induced high mortalities in the challenge experiments, have been isolated from coastal environments (Tubiash et al. 1973, Sindermann 1977, DiSalvo et al. 1978, Sakazaki and Balows 1981, Kent 1982, Larsen et al. 1988, DiSalvo 1995). Their involvement as bivalve pathogens or in producing substances toxic to various bivalves, including oysters, is frequently reported (Tubiash et al. 1970, 1973, Sindermann 1977, 1990, Nottage and Birkbeck 1986, 1987, Birkbeck et al. 1987), but JOD etiology has not been previously described for these species. It is feasible that new pathogenic strains of these bacteria that produce toxins or other irritants (Kaper et al. 1979, Colwell 1984, Nottage and Birkbeck 1986, 1987) may have established themselves at this particular site.

Attempts to elucidate causes for mortalities in aquaculture often focus on a search for primary pathogens, when the real cause may be environmental, nutritional, or physiological and may in-

volve facultative, opportunistic microorganisms acting on compromised hosts. Factors such as water quality and other environmental variables and the physiological status of the animals must also be considered. In order to discover which species of *Vibrio*, if any, is the true etiological agent of JOD, additional challenge experiments should be performed with other *Vibrio* and non-*Vibrio* isolates to repeatedly and fully satisfy Koch's postulate. These experiments should entail varying environmental factors in an attempt to better simulate conditions found at the nursery site. Factors such as the continuous rather than batch delivery of food and bacterial sources and the inoculation of susceptible oysters with combinations of different bacterial isolates in the presence and absence of high plankton densities should be incorporated into future experimental designs. Oyster strains resistant to pathogenic *Vibrio* spp. (Farley et al. 1995) and conditions favoring JOD also need to be identified to provide aquaculturists with additional means for reducing losses due to JOD. Although not yet conclusively demonstrated to be the definitive pathogen, species of *Vibrio* appear to have a strong involvement in JOD development, as suggested by field observations and challenge experiments, and still must be considered to be a potential candidate for the etiological agent of JOD.

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BIOMASS AND DISTRIBUTION OF FIVE SPECIES OF SURF CLAM OFF AN EXPOSED WEST COAST NORTH ISLAND BEACH, NEW ZEALAND

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ABSTRACT In 1992, the total biomass of five species of surf clam, between depths of 1 and 9 metres below chart datum, along 27.5 km of west coast North Island, New Zealand, open sandy coast was conservatively estimated as being 1,032 t. Approximately 50% was *Dosinia anus*, 19% was *Mactra discors*, 16% was *Paphies donacina*, 14% was *Mactra murchisoni*, and 3% was *Spisula aequilatera*. This biomass equates to approximately 37.5 t km^{-1} of coastline, and the potential harvest from this standing crop is correspondingly limited. The only other common macrofaunal species taken was a sand dollar, *Fellaster zelandiae*. The numerical density of each surf clam species varied with water depth, each having an optimal range. A trend of increasing density along the surveyed coast (increasing with latitude) was found with *F. zelandiae*, which suggests that recruitment processes are not always uniform or random along the coast at the scale of this study. A less marked trend was also found with *P. donacina*. No trends of density with latitude were found with *D. anus*, *M. discors*, *M. murchisoni*, or *S. aequilatera*. Good recruitment was evident from length frequency distributions in all species except *P. donacina*. It is suggested that the smaller size classes of this species live in shallower water than that sampled. Small *S. aequilatera* were more common in shallow, inshore waters than offshore, whereas the reverse pattern of size with depth was exhibited by the two *Mactra* species and by *D. anus*. *P. donacina* showed no trend of mean length with depth. All species exhibited highly positively skewed histograms of frequency of abundance within dredge tows, indicating that they all had highly aggregated distributions. The relative "patchiness" or degree of clumping for each species appeared to be positively related to its the relative abundance. Target fishing for single species is unlikely to be possible on a commercial basis owing to the high degree of overlap in their distributions and the high levels of patchiness for all species. The facts that the five species have different productivity levels and a wide range of growth rates, combined with the inability to target for particular species, have implications for the management of the resource. The sand dollar, *F. zelandiae*, can be so abundant that in some areas it could clog fishing gear during prolonged tows and become a severe nuisance to commercial fishing. Any fishery based on this coast would best be managed as a mixed species fishery, and the variability in available biomass suggests that a constant fishing mortality harvesting strategy (such as current annual yield) would be more appropriate than a constant catch strategy (such as maximum constant yield).

KEY WORDS: Surf clams, biomass survey, *Mactra*, *Spisula*, *Paphies*, *Dosinia*, *Fellaster*

INTRODUCTION

The biological communities present in the surf zone off exposed sandy shores in New Zealand are poorly known (Morton and Miller 1973). Until recently, in New Zealand, the difficulties of working in such an environment have precluded anything other than cursory investigations. Occasional storm strandings (Powell 1979) and analysis of Maori middens (Carkeek 1966, Butts 1982a, Butts 1982b) indicated that considerable populations of relatively large bivalve molluscs lived subtidally along these shores. However, in most areas, little more was known beyond the mere presence of these "surf clams."

In New Zealand, the term "surf clam" refers to a number of bivalve mollusc species. The five main surf clams on the Wellington West Coast are the yellow tuatua *Paphies donacina* (Mesodesmatidae), the venus clam *Dosinia anus* (Veneridae), two similar trough shells *Mactra discors* and *Mactra murchisoni*, and the triangle clam *Spisula aequilatera* (Mactridae). Two other species, *Bassina yatei* and *Dosinia subrosea* also occur and can be locally abundant at other localities around the country (Cranfield et al. 1994). Other species occurring on open coasts include the now uncommon toheroa, *Paphies ventricosa*, which usually occurs intertidally, and the grey tuatua, *Paphies subtriangulata*. The latter is the principal species now taken by recreational harvesters and is common close inshore in water shallower than 1.0 m below chart datum. A small commercial fishery for *P. subtriangulata*, using hand picking, is already established in northern New Zealand. In the mid- to late 1980s, interest developed concerning whether any surf clam species could form the basis of a commercial dredge fishery (Michael et al. 1990), as with similar species in Italy, North America, and other countries (Caddy 1989). Investigations were therefore begun to determine the distribution and abundance of each species and methods of harvesting them efficiently under New Zealand conditions.

A number of studies had already been carried out by the New Zealand Ministry of Agriculture and Fisheries along with the New Zealand Fishing Industry Board staff. A preliminary investigation testing two different hydraulic dredges, concentrating primarily on

a dense bed of tuatua, *P. donacina*, was carried out at Peka-peka (Fig. 1) on the Wellington West Coast (Michael et al. 1990). The authors did not produce a biomass estimate from these preliminary trials, but the high catch rates reported (an average of approximately 58 animals m^{-2}) may have fueled hopes for the existence of a large virgin resource along New Zealand oceanic beaches. Surf clam resources in Cloudy Bay (Cranfield and Michael 1987) have also been investigated (Fig. 1); however, the survey design used was neither stratified nor random and the biomass estimate produced was acknowledged to be "at best very approximate." A detailed tag/recapture study of growth rates has also been carried out successfully in central New Zealand, providing information on the relative productivity of the main species (Cranfield et al. 1993). Exploratory fishing to establish the presence of harvestable concentrations of surf clams, over a broader geographic scale, has been carried out around New Zealand (Cranfield et al. 1994). In that study, highly detailed, small-scale, stratified random surveys were made of the surf clams found along 450-m-wide strips of coast between 1 and 9 m below chart datum at 16 sites around the New Zealand coast.

The results of three such detailed site surveys on the Wellington west coast suggested that the resource could possibly sustain a viable commercial fishery (Cranfield et al. 1994). However, the biomass estimates were originally made with an assumed dredge efficiency of 65%, determined in a previous survey (Michael et al. 1990). If these estimates are extrapolated to tonnes (t) per kilometer of coast (to 9-m depth) and are adjusted for 100% dredge efficiency, they become 25, 95.8, and 53.2 t, respectively, or an average of 57.98 t km^{-1}. However, because extrapolation beyond the extent of the three narrow surveyed areas would be invalid, the true size of the resource remained unknown.

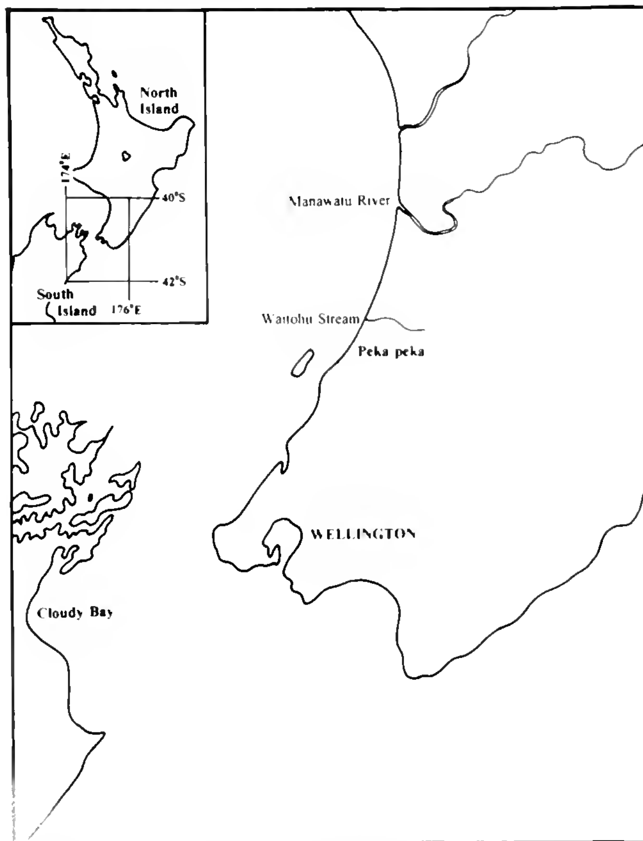

Figure 1. Location map showing the general position of the study area in relation to the rest of New Zealand and the detail of the coast between the Manawatu River and the Waitohu Stream.

This study was based on a stratified random survey of biomass and population sizes conducted along a 27.5-km stretch of coast and was designed to facilitate the setting of total allowable catches for the five main surf clam species in that area. Potential recruitment was investigated by determining the population size structure of each species, as indicated by length frequencies. The areal distribution of the five surf clam species was also investigated to determine the degree of patchiness in their distributions, as was the effects of depth on distribution, and to determine whether their distributions would allow each species to be individually targeted. Thus, for each species, the survey samples were also used to investigate how both clam density (biomass) and shell length related to depth. The patchiness of each species was examined first by consideration of how density varied relative to geographical position, and second, by graphical examination of the relative frequency of different levels of abundance.

METHODS

General Methods

This study extended over a distance of approximately 27.5 km of coast between the Manawatu River and Waitohu Stream (Fig. 1) along the Wellington west coast. Sampling began 1.0 km south of the Manawatu River because of dangerous fishing conditions near the river mouth. The analyses were restricted to the five species in the area sufficiently abundant to have commercial potential: *D. anus*, *P. donacina*, *M. murchisoni*, *M. discors*, and *S. aequilatera*. The echinoderm *Fellaster zelandiae*, a sand dollar, was also considered because it proved to be the most numerically abundant macroorganism occurring in the samples, with the highest biomass in some areas.

Survey Design

A stratified random sampling design was adopted for this study. Because surf clams are not evenly distributed relative to depth (Anderlini and Wear 1991, Cranfield and Michael 1992), the survey area was stratified by depth. Before any dredge sampling, a bathymetric survey was made of the coastline between the Manawatu River and the Waitohu Stream from 1.0 and 9.0 m chart datum (Fig. 1). This was done from a 6.0-m boat with a depth sounder (accurate to 0.2 m) and a Magellan PRO 1000 GPS receiver to determine geographical position. The depth strata produced by this method were only approximate indicators of depth.

All depths are described as metres below chart datum. The four depth ranges selected were 1–3 m, 3–5 m, 5–7 m, and 7–9 m. The depth range of each stratum was 2.0 m, which, on that coast, meant that each stratum would have a minimum width of between 100 and 200 m. This was necessary in order that dredge tows, which, because of the influence of wind and/or current, were rarely parallel with the coast, would fit comfortably within a single stratum. The strata limits were interpolated from the map of depth soundings. Although they all tended to be parallel to the shore, there were some local deviations both inshore and offshore, particularly near river and stream outlets.

The survey area was divided into geographical sectors along the coast in order that each stratum was no more than approximately 3 km^2. The sector boundaries were based on the location of river and stream mouths because such boundaries are accepted by

the Maori people as legitimate boundaries in matters relating to land claims and fishing rights. This division produced five geographical sectors along the coast, each with four depth strata, providing a total of 20 strata. The stratum areas varied between approximately 0.9 and 3.1 km^2 with an average area of approximately 2.12 km^2, whereas the total survey area, between 1.0 and 9.0 m, was approximately 38.5 km^2.

A total of 127 samples were taken, each of 50-m tow length. Initially, 100 random stations were allocated to the 20 strata, apportioned according to their relative area. A further 27 random stations were later allocated in strata where catch rates were highly variable. This was done as the opportunity arose and not as a distinct second phase to the survey (Francis 1989). The position of each sampling station within each stratum was selected randomly by the use of a custom computer program. All positioning in this survey was carried out with a Magellan PRO 1000 Global Position System receiver (GPS).

Dredge Sampling

The 6-m vessel *Susie*, used for the population survey, was powered by twin three-stage Hamilton water-jet units. During dredging operations, one jet unit was used to propel the vessel while the other was used to provide water to the hydraulic dredge. A Japanese design hydraulic "Rabbit Dredge" was used in the sampling. The dredge was 1.3 m wide and constructed according to the design specifications in Michael et al. (1990, their Fig. 7). The hydraulic dredge operates by injecting seawater into the sand at a rate sufficient to liquefy the sand in front of the dredge and permit its easy towed progression through the substrate at a depth of approximately 200 mm.

Clams >10 mm in length were retained by fitting a 10-mm steel mesh screen across the top frame, side grills, and filtration grill area posterior to the digging bit, and the catch bag was fitted with a 10-mm knot-to-knot liner so that small clams would be retained once caught. To ensure comparability of samples collected on different occasions, all dredging was restricted to calm seas with swells of less than 0.75 m.

The latitude and longitude of the start of each standard 50-m tow were recorded with the GPS receiver. Depth, to the nearest 0.2 m, was determined by echo sounder. Depths at the start of each tow were converted to chart datum by the use of the appropriate published tide table corrections. Where a randomly selected station was due to start within 50 m of a stratum boundary, and extend in a direction where the tow might cross the boundary should the start position be in error, the tow direction was constrained to be into the stratum. This only occurred in 4 of 127 tows, so a systematic error is unlikely. By detailed position plotting, it was found that none of the 127 samples used in the analysis crossed a stratum boundary.

To measure distance towed, a lead line attached to a 3-kg weight, with weights approximately every 200 mm, was thrown overboard to mark the start of the tow once operational water pressure was reached. During the tow, the lead line was continuously paid out by hand and the tow was stopped after precisely 50 m of the weighted line had been paid out in a straight line over the bottom (allowance was made for the water depth). The area swept was thus constant at 65 m^2 (1.3 × 50 m).

Propulsion was adjusted and controlled to achieve slow but approximately constant speed over a period of not less than 5.0 min for the 50-m tow. Tow duration was recorded on each occasion. Care was taken to ensure that all tows were made in a straight line. Tow orientation varied primarily according to the direction of along-shore current flow and sea breeze. For safety reasons, tow orientation generally headed into any swell and so tended to be offshore. Every effort was made to standardize the manner of dredge operation with the aim of obtaining consistent dredge performance. No tows were made where the dredge was retrieved completely full, although in the sand dollar–rich areas longer tows would have been full.

For each tow, the surf clams were separated from other macrofauna and placed in labelled bags; all samples were stored in large insulated bins containing frozen coolant pads until the end of the sampling day. Sand dollars were counted aboard before being returned to the sea.

Sample Processing

After landing, all samples were frozen until laboratory processing, which occurred usually within 1 wk of the date of sampling. The surf clams from each tow were separated into the five species, counted while defrosting, and weighed by species to the nearest gram while still partly frozen so as to avoid water loss. The maximum anterior-posterior length of each surf clam was measured to the nearest 1.0 mm with vernier callipers.

Data Analysis

Standard methods were used when estimating biomass and population sizes from the stratified survey design (Snedecor and Cochran 1967). In these analyses, the data from each stratum were weighted according to relative stratum area. Length-frequency data from each stratum were also weighted according to relative stratum area and were used to describe the size structure of the five species of surf clam. This latter information suggests the extent of potential recruitment and the size ranges potentially available to the fishery.

The surf clam species are thought to differ in their depth of burial in the sand and in their distribution with respect to river outlets and different sediment types. Thus, each surf clam species may differ in their vulnerability to being taken by the hydraulic dredge. This may also vary with water depth and sediment type. In practice, vulnerability to capture would be measured by estimating the dredge efficiency. Because dredge efficiency was difficult to estimate with sufficient precision to be useful at all depths and in all sediment types, a vulnerability (dredge efficiency) of 100% was assumed in the biomass and population estimates. This leads to conservative estimates.

Density and Size Relationships With Water Depth

The mean frequency per tow of each species was estimated for all stations in each of eight 1-m depth intervals: 1–2 m, 2–3 m, ..., 8–9 m. Mean counts were plotted against depth interval to provide a visual indication of whether a species exhibited a modal depth band and, if so, where it lay.

Similarly, the mean shell length of each species for each tow was plotted against depth of tow to indicate any relationship with depth. Linear regression lines were fitted to these data to indicate any trend present. These regression lines are only intended to suggest the depth trends because they are weakened by the presence of larger animals in all depths where the species are found. For comparison, moving averages of average shell length against depth were also plotted.

Distribution Patterns of Each Species

The biomass of each of the five species in each of the tows was plotted against the geographical position of the tow to compare the relative distribution of each species. This was also done for the counts of *F. zelandiae*. To assess the degree of aggregation visually, frequency versus abundance histograms were plotted and inspected. These were compared with the total weighted variance to mean ratios of abundance. Finally, to produce a broad overview of the degree of mixing of species, the cooccurrence of species per tow was determined by collating the number of species per station for all stations.

RESULTS

Biomass Estimate

D. anus was the most abundant species by weight, and *S. aequilatera* was the least abundant (Table 1). The total biomass of surf clams between the Manawatu River and the Waitohu Stream was estimated at 1032.698 t. This implies an average biomass of approximately 37.55 t km^{-1} of coastline (assuming 100% dredge efficiency). *D. anus* contributed 47.6% of the biomass, followed by *M. discors* (18.9%), which had only about a third of the *D. anus* abundance. *P. donacina* (16.5%) and *M. murchisoni* (14.1%) contributed very similar proportions of the overall catch, followed by the least common species, *S. aequilatera* (2.9%; Table 1).

The relative proportions of individuals of each species differed from those of the biomass estimates. *D. anus* dominated even more strongly, with a population of approximately 28 million, whereas the remaining four species were within 2% of each other, with approximately 4 million individuals each (Table 2). *M. discors* was slightly more common than the rest, but *S. aequilatera* greatly increased its relative importance, indicating that its population was made up of mostly smaller, lighter individuals (Fig. 2). The coefficients of variation for the estimates of both biomass and population numbers were less than 20% for all five surf clam species.

Length Frequency Distributions

As indicated by their length frequency distributions, juveniles of all species were abundant except for *P. donacina* (Fig. 2). The only clear mode for *D. anus* was between the lengths of 43 and 60 mm, with a peak at 50 mm (Fig. 2). There are suggestions of numerous other modes at smaller sizes but none were clear. Three modal classes were found in *M. discors* (Fig. 2), only one of which was clearly marked. The first ranged between 7 and 24 mm, centred approximately at 15 mm, the second ranged between 25 and 38 mm, centred at approximately 33 mm, and the third and largest ranged between 39 and 72 mm, centred at approximately 51 mm. However, these major classes appeared to be made up of a number of components.

In *M. murchisoni*, there appeared to be three or four distinct size classes (Fig. 2), all of which had approximately the same abundance as the smaller modes of *M. discors*. The first was between 10 and 28 mm, centered on approximately 18 mm, the second was very broad and ranged from 28 to 58 mm, centered on

TABLE 1.

Biomass Estimates for Each of the Five Species of Surf Clam.

Species	Average g m^{-2}	Standard Error	CV %	Biomass Tonnes
D. anus	12.76	1.7234	13.51	491.381
P. donacina	4.44	0.7982	17.96	171.105
murchisoni	3.77	0.4042	10.72	145.238
scors	5.07	0.7853	15.48	195.319
quilatera	0.77	0.1508	19.58	29.655
Total	26.82			1032.698

Data are from 127 stations, in 20 strata. CV, coefficient of variation.

TABLE 2.

Population Numbers for Each of the Five Species of Surf Clam.

Species	Mean Clams m^{-2}	Standard Error	CV %	Population Size	Percentage of Total
D. anus	0.7280	0.0799	10.97	28,034,578	63.46
P. donacina	0.0932	0.0163	17.47	3,587,200	8.12
M. murchisoni	0.1039	0.0086	8.30	4,002,290	9.06
M. discors	0.1220	0.0189	15.53	4,696,094	10.63
S. aequilatera	0.1002	0.0132	13.20	3,856,551	8.73
Total	1.1473			44,176,713	

Data are from 127 stations, in 20 strata. CV, coefficient of variation.

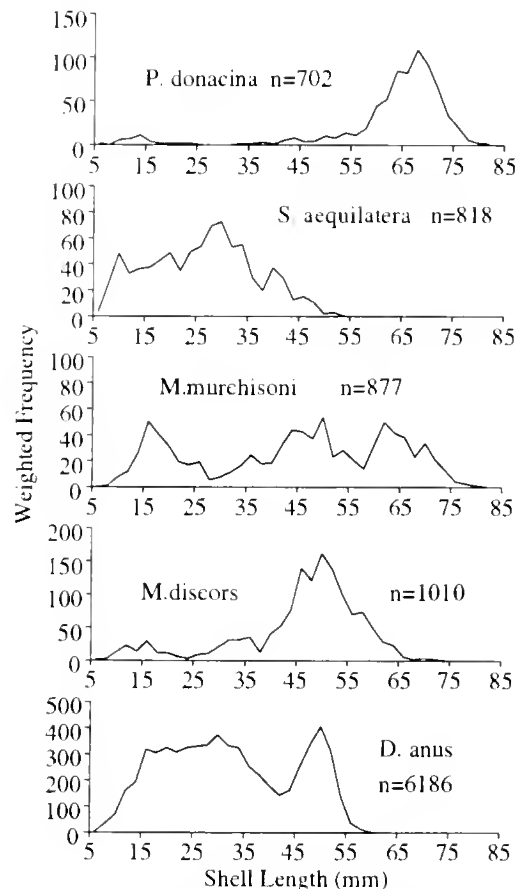

Figure 2. Weighted shell length frequencies of each species of surf clam. The distributions are shown as a connected whole to emphasize which size ranges were most common. Note that the frequency scales differ between species.

approximately 46 mm (although this may be made up of two main component classes each with different modes), and the last class ranged from 59 mm up to 83 mm in length, centered on approximately 67 mm.

For *S. aequilatera*, the length frequency distribution was very noisy despite measuring 818 individuals (Fig. 2). Three possible major classes may be identified, with modal groups approximately in the centre of the range of each: the first between 7 and 13 mm, the second between 14 and 23 mm, the third between 24 and 38 mm. Beyond that, shellfish were found with lengths up to 54 mm but only in low numbers.

With *P. donacina*, a small peak of new recruits centred on 15 mm was found between 7 and 25 mm (Fig. 2). Beyond that, there was only the final collective modal group, ranging from 55 to 80 mm, with a modal value of approximately 70 mm.

Depth-to-Size Relationships

When average shell lengths for each tow are plotted against tow depth, the five species exhibit different distributions (Fig. 3). *P. donacina* is concentrated in waters <4 m in depth, but the mean size (length) of animals had no relation with depth so that the regression line was effectively horizontal. *S. aequilatera* had more smaller individuals in shallow waters than in greater depths, so the regression line had a slightly positive gradient (Fig. 3). *M. discors* exhibited a distribution that was the inverse of *S. aequilatera* in that there were more smaller animals in deeper water so the regression line had a negative gradient. Finally, both *M. murchisoni* and *D. anus* had a pattern similar to that of *M. discors*, except that the negative regression lines were steeper because there were fewer larger animals in deeper water as well as fewer smaller animals in shallow water (Fig. 3). Thus, *P. donacina* and *S. aequilatera* are both relatively shallow water species and the other three surf clam species are all relatively deep water species. The same trends were found when all individual lengths from each tow (instead of mean lengths) were plotted against depth; the diagrams, however, were less clear because of the profusion of points. Again, similar trends were found in the plots of moving average of average length versus depth, with the changes seen remaining gradual.

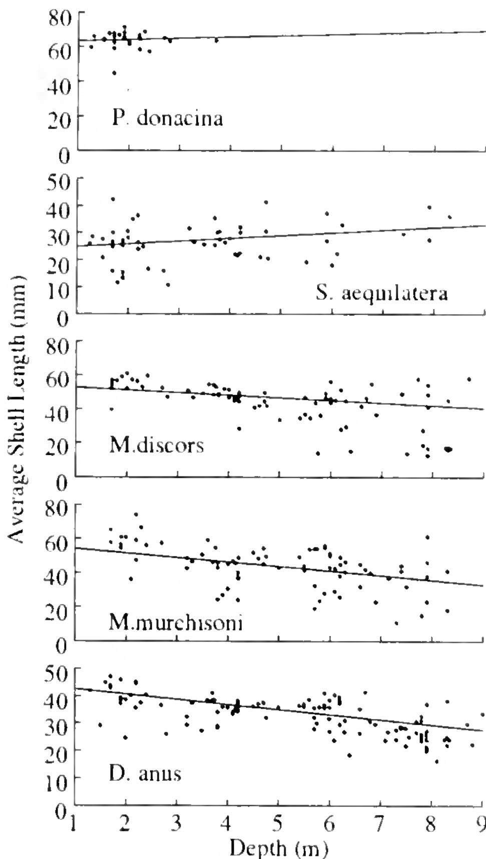

Figure 3. Average shell length of each tow plotted against chart datum depth for each tow, for each species. The lines are the least squares regression lines.

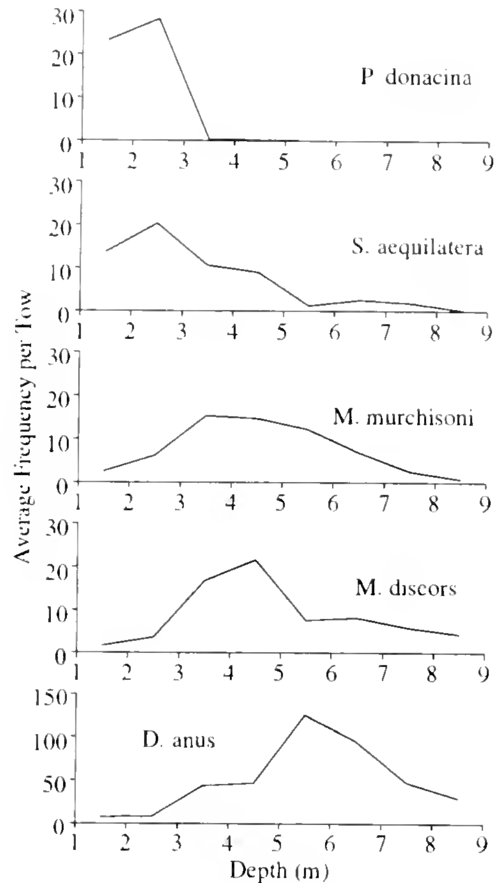

Figure 4. Average catch in numbers of shellfish per tow within 1-m depth categories showing the depth ranges within which the highest densities were found for each species.

Depth and Density Relationships

Each species exhibited a different optimal range of depth, as defined by modes of numerical abundance (Fig. 4). These presumably optimal depth ranges permit an ordering of species with increasing depth starting with *P. donacina*, having peak densities between 2 and 3 m and no animals deeper than 4 m. *S. aequilatera* was also found at its highest densities between 2 and 3 m but was also found out to 8 and 9 m, although in lower numbers. *M. murchisoni* had a broad mode with high abundance between 3 and 6 m in depth and slightly higher numbers at 3–4 m. *M. discors*, on the other hand, exhibited a clear mode between 3 and 5 m in depth, although it was also found at relatively constant medium densities to depths of 9 m. Finally, *D. anus* had a clear mode lying between

5 and 7 m below chart datum (Fig. 4), although the species was also found in all depths. Changes in the average relative abundance of the different species were most obvious between 3 and 4 m. In shallower water, *P. donacina* and *S. aequilatera* dominated catches, whereas below 3 m, the two *Mactra* species and *D. anus* were the most abundant, particularly the latter (Fig. 4).

Geographical Distribution

The distribution of biomass of the different species in relation to depth and geographical distribution was illustrated by plotting catch weight categories against position of tow (Figs. 5–7). Stations with relatively high catch levels of *D. anus* were found along the whole of the surveyed coast, especially in the 5- to 7-m strata, and were absent from only three stations. Low- and high-density tows were often close together, indicating a high degree of aggregation or patchiness (Fig. 5).

The two *Mactra* species were both generally distributed, with relatively low catch levels throughout the survey area, although there were two areas, at opposite ends of the survey area, where high catches of *M. discors* were made (Fig. 6). The average low catch levels of *S. aequilatera* combined with its wide distribution over the range of depths and latitudes included in this survey contrast with the concentration of relatively high catch levels of *P. donacina* in the inshore areas (Fig. 7). Although *P. donacina* was found along the full length of the surveyed coast, catches were highest in the south (Fig. 7a). The high catch levels of *P. donacina* along with none being caught in tows from water deeper than 4 m imply that this species also had a relatively high degree of aggregation. None of the areas of high or low catch levels were obviously related to river or stream outlets, and a crude latitudinal gradient in biomass was apparent only in *P. donacina*. In terms of absolute densities over all stations of all surf clams combined, the average density was 1.232 animals m^{-2}, and the minimum density found at a station was 0.015 clams m^{-2}, whereas the maximum was 9.969 surf clams m^{-2}.

A more obvious gradient of density of individual was observed with *F. zelandiae* (Fig. 8). These were distinctly more abundant in the southern parts of the survey area.

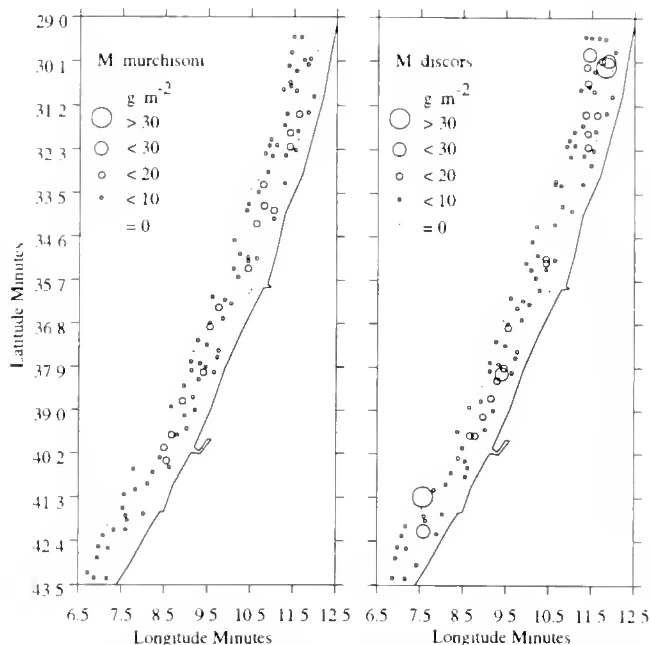

Figure 6. Schematic diagram of the surveyed coast. Axis values relate to latitude 40°S and longitude 175°E, respectively. The distributions of different densities by weight of both *M. discors* and *M. murchisoni* are indicated. Units are grams per square meter.

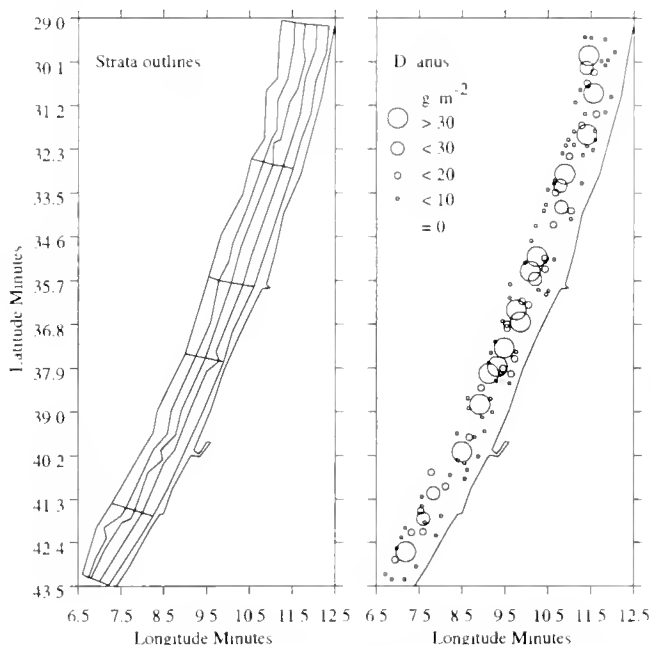

Figure 5. Schematic diagram of the surveyed coast. Axis values relate to latitude 40°S and longitude 175°E, respectively. The strata outlines indicate the 20 strata used in the survey. The inshore strata are between 1 and 3 m, the next are 3 and 5 m, then 5 and 7 m, and finally 7 and 9 m. *D. anus* illustrates the distribution of different densities by weight. Units are grams per square meter.

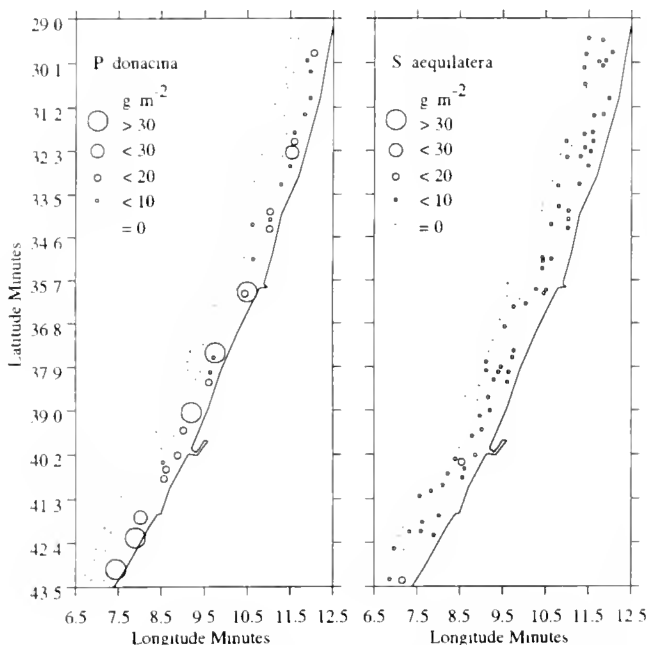

Figure 7. Schematic diagram of the surveyed coast. Axis values relate to latitude 40°S and longitude 175°E, respectively. The distributions of different densities by weight of both *P. donacina* and *S. aequilatera* are indicated. Units are grams per square meter.

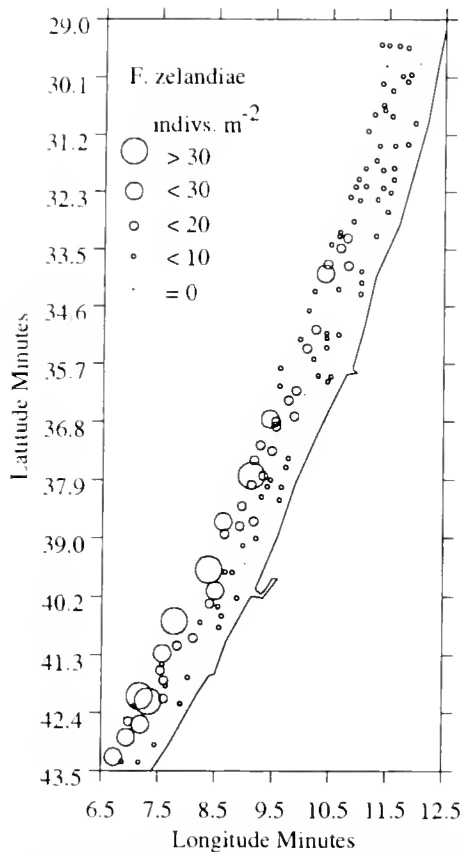

Figure 8. Schematic diagram of the surveyed coast. The axis values all relate to latitude 40°S and longitude 175°E, respectively. The distributions of different densities of counts of *F. zelandiae* are indicated. Units are individuals per square meter.

Other Indications of Spatial Distribution

All five species of surf clam exhibited highly positively skewed frequency versus abundance histograms, suggesting they were all highly aggregated. A comparison of the ratio of the overall weighted variance to mean densities (Table 3) indicated that the most highly aggregated species was *D. anus*, with *P. donacina* next, followed by *M. discors*, then *M. murchisoni*, and finally *S. aequilatera*. This is consistent with the visual assessment of the distribution maps (Figs. 5–7).

Cooccurrence of Species

Eight of 127 stations contained only a single species of surf clam. One of these yielded only *P. donacina* (a single individual), and the other seven contained only D. anus. None of these eight stations had high densities of surf clams. Eighteen tows contained two species, which was similar to the 17 that contained five species, whereas the most common situation was where a tow contained either three or four species (Fig. 9).

TABLE 3.

The Total Weighted Variance, Mean, and Variance/Mean Ratio for Biomass ($g \cdot m^{-2}$) for Each Species.

Species	Mean	Variance	Variance/Mean
D. anus	12.76	12,549.3	983.5
P. donacina	4.44	2,691.8	606.3
M. discors	5.07	2,605.6	513.9
M. murchisoni	3.77	690.2	183.1
S. aequilatera	0.77	96.1	124.8

The larger the ratio the greater the degree of aggregation found in the samples.

DISCUSSION

Biomass Estimation

This survey was the first large-scale (27.5 km of coast), stratified random survey of surf clams on the Wellington West Coast. A total biomass of 1,032 t implies that the average biomass per linear kilometre of coast (between 1 and 9 m chart datum) is approximately 37.5 t km^{-1}. This value is lower than the average of the three estimates made by Cranfield et al. (1994), but it is greater than their lowest estimate. Our estimate is likely to be conservative because of the assumption of 100% dredge efficiency. Michael et al. (1990) calculated a dredge efficiency of 65% with respect to a predominantly *P. donacina* bed containing approximately 58 surf clams m^{-2}. This was up to 10 times the maximum densities found elsewhere (Cranfield and Michael 1987) and over 5 times more dense than the maximum and 47 times more dense than the average density of surf clams found in this study. The relationship between the rabbit dredge's catching efficiency and surf clam density is unknown. Other hydraulic dredge designs have had catch efficiencies estimated between 80 and 100% (Meyer et al. 1981, Smolowitz and Nulk 1982). Notwithstanding adjustments with respect to dredge efficiency, the total biomass along the surveyed stretch of coast is lower than the earlier sampling suggested might be present. The possible harvest from this resource will be correspondingly limited, depending on the relative productivity of the species concerned.

Potential Recruitment

A number of size classes, which with some relatively fast growing species, especially *S. aequilatera* and *M. discors*, may relate to age classes (Cranfield et al. 1993), were found in all species except *P. donacina*. Recruitment of juvenile size classes is clearly occurring in the deeper water species. Very few juveniles of *P. donacina* were found, indicating that these occur elsewhere. Adults of the related *P. subtriangulata* occur mostly between the low-tide line and 1.0-m chart datum. Juveniles of that species

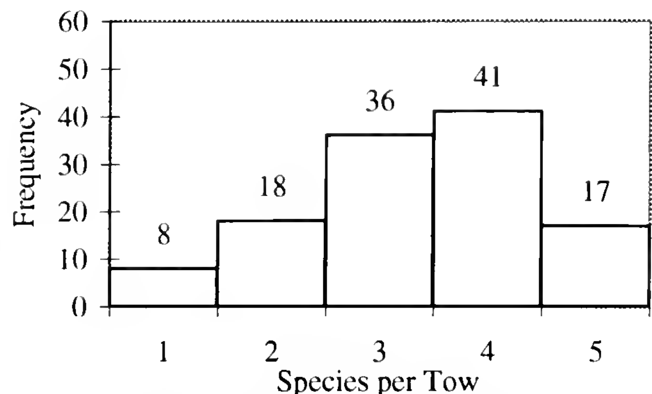

Figure 9. Relative frequencies of tows containing different numbers of species of surf clam. The numbers contained above each bar are the number of tows in each category.

mainly occur towards the high-tide mark and migrate to lower levels only when they have attained a size of approximately 25 mm (unpublished data). Juvenile *P. donacina* have also been observed both intertidally and in shallow subtidal water. It appears that prerecruits of *P. donacina* mainly settle and grow in water shallower than 1.0-m chart datum. Their absence from the observations of length frequency are therefore to be expected. However, why no *P. donacina* were observed between approximately 20 and 40 mm is unknown.

Distribution of Shellfish Lengths Relative to Depth

The manner in which the mean length of shellfish varied with depth reflected whether the species belonged to either the inshore group (*P. donacina* or *S. aequilatera*) or the offshore group (the two *Mactra* species and *D. anus*). The lack of any relation between the depth and mean length of *P. donacina* reflects both its restricted depth distribution and the fact that its juveniles develop inshore of the 1.0-m survey boundary. It implies that as they grow in length, juveniles of this species must, at some stage, migrate offshore to the adult beds. *S. aequilatera* has a far wider depth distribution, but smaller individuals were found predominantly in shallower water. This species is also found in water less than 1.0-m chart datum. Clearly, some of the smaller animals must be moved by wave action or migrate offshore as they develop and grow. The reverse is true for the two *Mactra* species and for *D. anus*. Because smaller individuals of these three species are more common offshore, then to maintain the distribution of larger animals across all depths, some of the smaller individuals must move inshore as they increase in size. In both *M. murchisoni* and *D. anus*, there is some indication that larger animals are also less common in the deepest stations, which implies such movement. With *S. aequilatera* and *M. discors*, on the other hand, the movement of individuals would only be facultative.

An alternative hypothesis, that the observed distributions were brought about by differential settlement or the survival of larvae over a number of years, is less likely. If this were the case, all species might be expected to exhibit the same distribution of size versus depth.

Depth and Density Relationships

There is a succession of modes of maximal relative abundance from shallow to deep for the five different species. *D. anus* dominated the absolute numbers in all depths except the shallow range of 1–3 m, where *P. donacina* predominated. Despite the clear succession in maximum relative abundance, there was a great deal of overlap between all species (except *P. donacina*, which is restricted to shallow water) across all depths, especially out to 7 m. In waters deeper than 7 m, the numbers of surf clams were relatively low, but at least medium densities were found at all depths between 4 and 7 m. The most marked division of surf clams is the relatively shallow water (1- to 4-m chart datum) group of two species (*S. aequilatera* and *P. donacina*) and a deeper water (4- to 8-m chart datum) group of three (*M. murchisoni*, *M. discors*, and *D. anus*). In depths where potentially commercially viable (fishable) densities occur, it appears that it would be almost impossible to fish without catching a mixed bag of surf clams. However, without further information concerning how the different species are distributed along a more extensive section of the coast, no general conclusions can be made as to whether it is possible to target a particular species at all sites along the coast.

Geographical Distribution by Density of Surf Clams

The domination of the biomass by *D. anus* and the concentration of this species in the 3- to 7-m depth strata is clear from the distribution maps (Fig. 5). The close proximity of stations with high levels of biomass to those with very little biomass indicates the high degree of patchiness in this species. The two *Mactra* species are more evenly distributed and are well mixed with the stations containing *D. anus*. Visually, these three species appear to form a well-mixed group that predominates in the 3- to 7-m strata.

The inshore group differs from the offshore group in that its members are very different in their abundance and relative distributions. *P. donacina* is concentrated in the shallow strata and has a gradient of increasing density from north to south. *S. aequilatera* differs in being at consistently low densities throughout the survey area, with increasing numbers of zero count stations in the deeper strata.

The distribution bubble maps indicate the mixed nature of the communities present at any single location along the coast. The geographical information is consistent with the information concerning depth distribution in that both imply that target fishing would be difficult. It might be possible to locate a patch of surf clams rich in a particular species, especially with the inshore group, but it would be very difficult to fish extensively without obtaining high proportions of at least two other species. Thus, the closest approach to target fishing would be to concentrate effort within patches known to have high densities of the species of interest. Generally, however, the distribution maps and high levels of aggregation suggest that one would only be able to dredge a short distance before leaving a concentrated patch of a particular species.

The Effect of Fellaster zelandiae *on Surf Clam Fishing*

In the northern parts of the survey area, the presence of the New Zealand sand dollar was only a minor nuisance because it was present in relatively low densities. In the southern area, however, their high densities (sometimes more than 2,000 individuals in a 65-m^2 tow) meant that they became a severe nuisance, hampering fishing operations by clogging the catch net with this nontarget species. Commercial fishing operations would either have to find an efficient means of separating the surf clam catch from usable surf clams or avoid areas of high densities of sand dollars. The extent and distribution of this species should therefore also be examined when developing a New Zealand surf clam fishery.

Management Options

Tagging experiments have demonstrated that growth rates vary considerably between surf clam species (Cranfield et al. 1993). Estimates of size-at-age have been made that indicate that, for the Wellington West Coast, *S. aequilatera* (4 y) and *M. murchisoni* (5 y) grow the most quickly to reach a "marketable size," whereas *D. anus* (14 y) grows the most slowly (Cranfield et al. 1993). The growth rate for the first two species suggests relatively high levels of natural mortality, which, in turn, implies that these species could sustain a relatively high fishing mortality with minimal risk of harming the stock. Unfortunately, it would be necessary to be able to target for particular species when fishing before being able to manage the surf clam species separately.

With the low catch rates possible along this coast, it is likely that continuous towing with some mechanism bringing the catch to the surface for continuous sorting would be necessary for the fish-

ery to be economical. This is a further reason why it would be both difficult and impractical to target fish for particular species.

To date, only the East Coast United States surf clam fishery uses a total allowable catch (as quota) to control the fishery. The U.S. fishery is for *Spisula solidissima*, which is relatively long lived and slow growing and has variable recruitment. Accordingly, the annual quota is set at a low proportion (0.045) of the recruited biomass (Murawski and Serchuk 1989, Cranfield et al. 1994).

Setting a total allowable catch appears to work with the United States Atlantic Coast surf clam fishery. However, the patchy nature of the distribution of surf clam species along the Wellington West Coast, combined with the reported differences in productivity and growth, as well as the occasional beach strandings of large numbers of surf clams, implies that the New Zealand surf clam community may be more dynamic in yield and composition than the U.S. East Coast situation. If the relative proportions of the different surf clam species can alter markedly from year to year, then managing the fishery by the use of a constant catch strategy, such as a maximum constant yield, to set total allowable catch levels for each species may not be the best strategy. It is suggested that because New Zealand surf clam populations appear to be dynamic in terms of both population size and relative proportion of species, a management strategy closer to a constant fishing mortality be adopted. To implement this, the fishable biomass of each species within a fishing area would need to be determined, before each annual fishing season, and each year's survey would need to be used to determine the potential catch.

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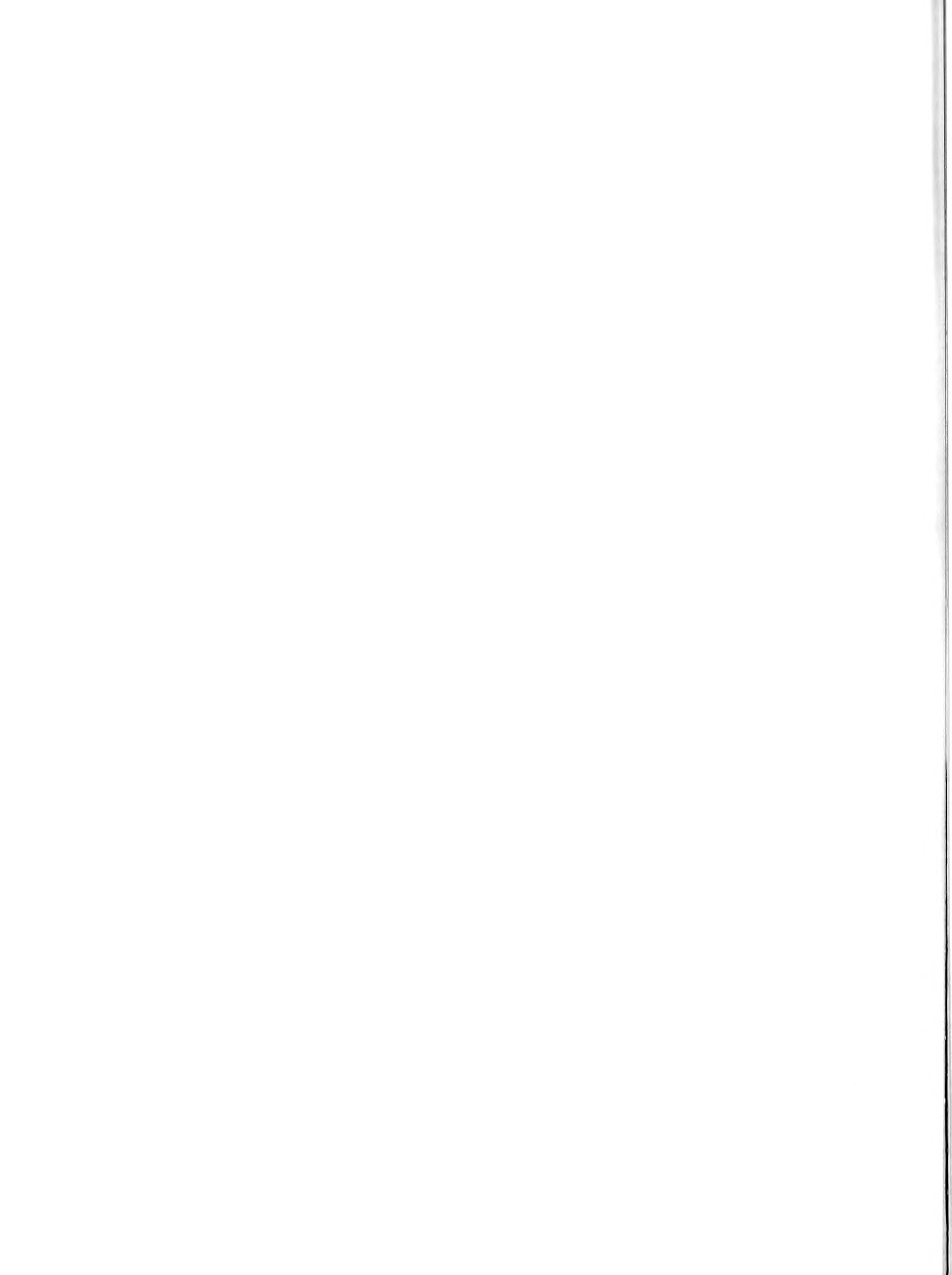

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EFFECT OF GROWOUT DENSITY ON HERITABILITY OF GROWTH RATE IN THE NORTHERN QUAHOG, *MERCENARIA MERCENARIA* (LINNAEUS, 1758)

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ABSTRACT Realized heritability for the increase in the rate of growth in the northern quahog, *Mercenaria mercenaria*, was determined under conditions of moderate growout density (<90 per sq. ft.) independently for two lines by Crenshaw et al. (1993). For one line, termed Group A, a mean estimate of heritability of 0.402 was obtained. This estimate was based on a single generation of selection in which a standardized selection differential (i) of about 1.5 standard deviations was used, representing a selection intensity of about 16%. When Select and Control progeny of Group A were maintained in growout at high densities (>350 per sq. ft.), Control progeny grew at significantly greater rates than Selects, thus resulting in negative estimates of realized heritability. Clam stocks were collected in House Creek, Little Tybee Island, Wassaw Sound, in coastal Georgia. Same-age cohorts of F_1 progeny were established in April and May 1986. Progeny were reared in the laboratory until December 1986, when they were transferred to growout cages in an intertidal creek. Selection was carried out for F_1 cohorts in March 1988. Select and Control parental groups were identical in number, the latter randomly chosen from the entire population before ascertaining the Select-line parents. Spawnings of the Control and Select Group A F_1 parents occurred on July 6 and 7, 1989, respectively. Clam progeny were transferred from nursery to growout cages on September 12, 1990, and density was reduced for Group A moderate-density cohorts on April 1, 1991. Other Group A cohorts of Select and Control lines were maintained at high density in growout cages similar to those holding moderate-density cohorts. For clams reared at moderate density, heritability estimates were calculated on September 12, 1991, for clams of Group A. Group A clams maintained in high-density growout developed more slowly than those reared at moderate density, and it was not appropriate to take measurements for estimating heritability until March 16, 1992.

KEY WORDS: Genetic selection, heritability, growth rate, crowding, quahog

INTRODUCTION

Quantitative genetic selection may be used in a living organism to improve any trait for which there exists additive genetic variance. The process is particularly useful in that selection may be carried out over many generations, with progress resulting in each, until genetic variance for the trait is exhausted. Newkirk (1980) reviewed genetic research involving commercially important bivalves, emphasizing the potential of selective breeding. Humphrey and Crenshaw (1989) reviewed subsequent genetic research involving bivalve shellfish, emphasizing the genetics of the northern quahog, *Mercenaria mercenaria,* and outlined simplified procedures that could be used to develop estimates of realized heritability with shellfish.

The realized heritability estimate resulting from genetic selection is particularly useful as "an empirical description of the effectiveness of selection" (Falconer 1981). This estimate can be used to estimate the number of generations of selection of a given intensity that would be required to achieve a particular goal. In a mariculture operation, successful selection for rate of growth in shellfish would result in reduction of time in growout, reduced rearing expenses, and improved profitability. Efforts to estimate a realized heritability for growth rate in the northern quahog or hard clam were initiated in our laboratory a number of years ago. Subsequently, estimates of heritability of growth rate in the hard clam have been published by ourselves and others (Hadley 1988, Hadley et al. 1991, Crenshaw et al. 1993).

Many factors may affect the outcome of a given selection program. We have reported on negative growth and survival effects in the larval and embryonic progeny of northern quahogs and of bay scallops selected for rapid growth rate (Heffernan et al. 1991, Heffernan et al. 1992). However, reduced larval rate of growth in both cases was more than offset by subsequent high growth rates in juveniles and young adults.

The effect of environment on selection response is unpredictable. It is well known that selection carried out in a given environment may produce different results in different environments (Falconer 1981). The heritability of a trait estimated in a given environment predicts response to selection most accurately in that same environment. In this study, we demonstrate the importance of crowding or population density on response to selection.

MATERIALS AND METHODS

Two lines of *M. mercenaria*, here termed Groups A and B, were established form mass spawnings of wild stock clams from House Creek, Little Tybee Island, Wassaw Sound, in coastal Georgia, on April 4 (Group A), and May 8 (Group B), 1986. This report deals only with cohorts of Group A. Postsettlement (juvenile) stages were reared in downwelling nursery systems following standard nursery culture procedures for bivalve molluscs (Castagna and Kraueter 1981, Heffernan et al. 1988). In order to retain all genetic variance possible for rate of growth, cohorts were never subjected to size culling, as is standard practice in commercial hatcheries.

Larval and juvenile progeny were reared in the laboratory in ambient filtered seawater with food provided by Wells-Glancy

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cultured phytoplankton, supplemented by single-species algal cultures until December 1986, when they were transferred to growout cages at densities of approximately 1,000 clams/m^2 in a sheltered, intertidal creek (House Creek) in Wassaw Sound, GA. Selection was carried out at approximately 2 y of age and mean shell length of about 32 mm for F_1 cohorts of Group A on March 16, 1988. At that time, densities had been reduced by mortality and escape to about 52 and 24 clams per sq.ft. for the two cages involved. Spawning of 177 Select parents and an equal number of Controls was induced by thermal stimulation. The Control parents had been randomly chosen from the entire population before the designation of the Select-line parental group (Crenshaw et al. 1988, Heffernan et al. 1991). Select-line parents were identified as those clams within the upper 16.6% of the shell length distribution, thus lying at or above a truncation cutoff point of 0.97 standard deviations above the mean. The mean shell length of Select-line parents was 1.50 standard deviations above the overall mean of the population from which they were taken.

Reared under the same conditions as the previous generation, F_2 Group A progeny were transferred from nursery to growout cages in the field on September 12, 1990. On April 1, 1991, density was reduced by dividing clams in each single cage approximately equally between two cages of the same size, resulting in a decrease in density from less than 90 clams per sq. ft. to less than 30 clams per sq. ft., termed here moderate density. On September 5, 1991, F_2 clams of Group A Control line reared at moderate density were found to be approximately the size of the F_1 parental array at the time selection was carried out, and F_2 Select- and Control-line measurements were made. It is on the basis of these Select- and Control-line groups, reared at moderate density, that our first estimates of realized heritability for growth rate in the northern quahog were computed (Crenshaw et al. 1993).

On March 11, 1991, shortly before densities were reduced for Group A to bring about moderate-density conditions for the determination of heritability of growth rate, three growout cages were established for Group A Select and one for Group A Control populations to determine the effects on growth of very crowded conditions. The growout cages were wooden boxes, 4′ × 4′, covered with plastic-covered wire mesh. Established with 5,700 animals in each cage, density was initiated at slightly less than 360 per sq. ft. (approximately 3,200 clams/m^2) for clams reared under these high-density conditions. On March 6, 1992, after 18 mo in growout, sample measurements of Controls grown at high density indicated that they had reached approximately the same size as clams grown at moderate density at the time they were measured for the purpose of determining heritability. Thus, clams grown at high density required about 6 mo longer to reach this size than was required for clams grown at moderate density. At that time, densities had been reduced by mortality and escape to between about 80 and 130 clams per sq. ft. On March 16, 1992, random samples of 200 from each cage were removed and measured to determine the effect of high density on heritability.

RESULTS

Mean shell length measurements and selection statistics of the F_1 generation of Group A are provided in Table 1. As indicated above, a cutoff point of approximately 0.97 standard deviations above the mean was used, meaning that, after random selection of a Control group of F_1 parents, all remaining clams with shell lengths equal to or exceeding the mean plus 0.97 standard deviations were chosen to comprise the Select F_1 parental group. For the overall F_1 population, at 2 y of age, the weighted mean shell length of two replicates was 31.27 mm. The mean of the Select-line parents was 42.42 mm or 1.50 standard deviations above the mean of the population from which Select- and Control-line parents were taken. This figure of 1.50 represents the standardized intensity of selection (i) or "reach" for Group A. Mean shell length measurements of the F_2 generation of Group A, Select and Control lines, moderate- and high-density groups, are provided in Table 2.

Response to selection or "gain" is estimated by the difference between the means of the F_2 progeny from F_1 Select and Control parents. For Group A, F_2 progeny of Controls reared at moderate density were found to have a mean shell length of 31.97 mm; those of Select parents also reared at moderate density had a mean shell length of 35.57 mm. The difference, 3.60 mm, is highly significant by analysis of variance (ANOVA) ($P < 0.0001$) and is equal to 0.61 standard deviations of the Control progeny distribution (Table 2). This figure represents an estimate of response to selection or "gain" for four growout replicates of Group A.

Realized heritability (h^2) may be computed as the ratio of response (="gain") to selection (="reach"). For Group A clams reared at moderate density, it is estimated that $h^2 = 0.613/1.499 = 0.409$.

For clams reared under crowded conditions in growout, the situation is quite different. F_2 progeny of Controls were found to have a mean shell length of 35.63 mm, whereas progeny of Select parents also reared at high-density conditions had a mean shell length of the only 33.78 mm. The difference, 1.85 mm, is highly significant by ANOVA ($P < 0.0001$), but clearly is in the wrong direction to represent a positive response to selection. The shell length of two of the three replicates of progeny of Select parents was significantly different from one another (Turkey's HSD Multiple Comparison), but all were lower than the mean shell length of Controls, and we have lumped them for simplicity in our analysis. A calculation of heritability on the basis of the "gain" to "reach" ratio would produce a negative figure, which would be meaningless.

On March 6, 1992, 10 d before measurements were taken from

TABLE 1.

Size attained by F_1 generation same-age populations of the northern quahog at time of selection in Group A

Group	Number Measured	Mean shell Length in mm ± SE	Standard Deviation (SD)	CutOff Point Above Mean	Mean of Selected Parents
A	1,232	31.27 ± 0.20	7.14	0.97 S.D.	42.42 mm (mean + 1.499 SD)
Replicate 1	842	31.10 ± 0.24			
Replicate 2	390	33.05 ± 0.37			

TABLE 2.

Size attained in the northern quahog by F_2 generation progeny of F_1 generation Select and Control parents of Group A, reared under conditions of high and moderate density, at approximately the same size as their parents at the time selection for increase in growth rate was carried out.

Density	Group	Date of Measurement	Number Measured	Mean Shell Length in mm ± SE	Standard Deviation (SD)	Difference in mm/ Significance	Gain in SD of Control
Moderate	A Control	9/09/91	200	31.97 ± 0.42	5.87	3.60/	0.613
	A Select	9/09/91	800	35.57 ± 0.23	6.60	$P < 0.0001$	
	Replicate 1		200	37.08 ± 0.44			
	Replicate 2		200	36.82 ± 0.45			
	Replicate 3		200	34 55 ± 0.46			
	Replicate 4		200	33.81 ± 0.47			
High	A Control	3/16/92	200	35.63 ± 0.42	6.0	−1.85/	−0.308
	A Select	3/16/92	600	33.78 ± 0.27	6.55	$P < 0.0001$	
	Replicate 1		200	32.76 ± 0.47			
	Replicate 2		200	34 36 ± 0.44			
	Replicate 3		200	34.21 ± 0.47			

the clams reared at high density, counts indicated that the mean survival of three Select-line replicates was 58.04%, as compared with 67.49% survival for the Control group. However, one of the Select-line replicates had a survival rate of 76.68%.

DISCUSSION

As reported earlier, the estimate of heritability of growth rate for Group A reared at moderate density, about 0.40, may be interpreted to indicate that about 40% of the total phenotypic variance in growth rate is attributable to the average effects of genes under the environmental conditions of the experiment (Crenshaw et al. 1993). The selection event for the Group A F_1 generation, which was spawned to produce both moderate- and high-density F_2 groups, was carried out under conditions of moderate density. The density factor was introduced for the F_2 generation after about half a year in growout, and clams reared at moderate density remained in growout another 6 mo before measurements were made to calculate the heritability of growth rate. Clams of the high-density growout group, by contrast, remained in growout another year before reaching the same approximate size. The effect of crowding is clearly indicated by the relative performance of Controls in the two situations. It took nearly twice as long for clams reared under high-density conditions to reach the same size as clams reared at moderate density.

More interesting is the growth performance of Group A clams selected for rapid growth under two density conditions relative to their appropriate controls. The group reared under conditions of moderate density, approximating the conditions under which their parents were grown before selection, showed the expected response to selection by exhibiting more rapid growth than controls, but not quite as rapid as their parents, which had been selected as the most rapidly growing members of their cohort.

The progeny of clams selected for rapid growth, when reared under conditions of high density in growout, by contrast, showed slower growth than Controls reared at high density indicating that something about the array of genes that determines rapid growth under conditions of moderate density actually has a negative effect on growth rate under conditions of high density. We suggest that selection for allelic combinations that foster rapid growth in moderate-density growout, which could be fairly termed nearly optimal conditions, will, at the same time, select against genotypes that perform best under conditions of stress. The control groups in the situation described here are very close genotypically to wild stocks, which have been fine tuned over thousands of generations of natural selection to withstand a variety of stressful conditions that occur in the natural clam habitat. It is reasonable, then, that a Control line, when subjected to stressful crowding, would do better at surviving and growing than a line selected for rapid growth, where there is an abundance of food and a low concentration of waste products. Although there was found fairly high survival in one Select-line replicate, the mean survival of Select-line replicates was less than that of the Control group.

Falconer (1981, p. 322) prefers to regard the same trait as measured in two different environments not as one but as two different characters. His argument is that the "physiological mechanisms [involved] are to some extent different, and consequently the genes required for high performance are to some extent also different."

In our original report of a determination of heritability of growth rate for the hard clam (Crenshaw et al. 1993), we discussed the related situation of a low estimate of heritability obtained from a second line, Group B, in which it could be shown that the low estimate was associated with the last 5 mo of growout, a period during which our data showed an appreciable reduction in the rate of growth of Select-line progeny relative to the growth rate of Control-line progeny. We attributed this difference in growth rates as due most likely to one or a combination of stressful factors associated with this period: 1) a change of tidal creek environment at the beginning of the period, or the new environment itself; 2) several stays in the laboratory with marginal food and low salinity at the beginning of the period; or 3) excessive population manipulation associated with the laboratory stays or the change of residence.

The critical lesson for the mariculturist desiring to use stocks selected for desirable traits is that the selection process itself should be carried out under conditions comparable to those existing in the hatchery, nursery, and growout facilities in which they will be used. It is well known that response to selection is maximized in groups reared under conditions most similar to those under which their parents were reared and that stress of any sort tends to affect selected lines more profoundly than unselected lines (Falconer 1981).

It has been pointed out that in this research we are not actually selecting for growth rate broadly in the quahog but, to be precise, for growth rate to a given size, as reflected by shell length. The result is, however, the same. Indirectly, we are selecting for growth rate, admittedly up to a specific size. In selection programs involving warm-blooded domestic animals, it is often fairly simple to select for a character at a specific stage of development, e.g., "at birth" or "at one year of age." With invertebrate poikilotherms, growth and development may vary enormously depending not only on temperature, but also on food supply as well as other factors. We would have preferred to use "attainment of sexual maturity or spawnability" as an appropriate milestone, and in fact, we have picked target shell lengths because animals of this size are usually sexually mature and spawnable. This correlation between size and sexual maturity is generally accepted. Determination of sexual maturity for each individual clam would require cytological examination and would be both excessively time consuming and somewhat traumatic. In essence, then, we use target shell length as a reflection of a given stage of development, and we have found that it is both practical and effective.

An important problem emerges from the considerable variance in the time at which different members of a same-age cohort will reach a given shell length. In this work, we have found that when the mean shell length of our line selected for increased growth rate reaches the target size, most of the cohort are sexually mature. However, at the same time, the mean shell length of the control cohort will be smaller, and significantly more members will not be spawnable. The problem that this poses for the determination of heritability and our solution has been discussed elsewhere (Crenshaw et al. 1993).

ACKNOWLEDGMENTS

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CYTOLOGIC SEXING OF MARINE MUSSELS (*MYTILUS EDULIS*)

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ABSTRACT Four hundred eighty mature marine mussels (*Mytilus edulis*) were collected in late April from a mussel lease in an estuary in Prince Edward Island, Canada. Squash preparations made of reproductive gland (mantle) tissue of each mussel were stained with Wright-Giemsa stain and were examined by one investigator using light microscopy for sex determination. A strip of mantle tissue was also removed from each mussel, fixed in 10% buffered formalin solution, and processed for histologic evaluation. The sex of each mussel was determined independently by evaluation of the histologic preparations by a second investigator. The evaluation of cytologic preparations of mantle tissue was highly accurate in determining mussel sex, in that all 480 mussels (261 females and 219 males) were correctly identified when compared with the traditional standard of histologic sexing. No hermaphrodites were observed. The advantages of the cytologic procedure over traditional histologic processing include ease of sample preparation and evaluation, short preparation time, low cost, and sparing of tissue for other studies.

KEY WORDS: Cytologic, histologic, marine mussel, *Mytilus edulis*

INTRODUCTION

The classification of marine mussels (*Mytilus edulis*) as male or female cannot be determined by morphology, size, or shell color. The sex of individual mussels or groups of mussels may have effects on physiologic parameters and the biochemical content of tissues. At present, the standard method available for determining the sex of mussels is to fix reproductive gland (mantle) tissues in fixative solution and then use histologic processing and light microscopic examination for the presence of oocytes or spermatozoa. Histologic processing is expensive and permanently alters the tissue sample. A biochemical procedure for sex determination evaluating color change after the exposure of mantle tissue to boiling thiobarbituric acid has also been reported (Jabbar and Davies 1987). This procedure destroys tissue and involves the use of a caustic chemical. The cytologic evaluation of tissues is an established diagnostic procedure used in veterinary and human medicine. In this procedure, cells obtained from squash preparations of small amounts of solid tissue or from needle aspiration of tissues are spread on glass slides, air dried, stained and examined by light microscopy. Compared with histologic processing and biochemical procedures, this procedure is rapid and inexpensive and requires minimal tissue. The goal of this study was to determine the accuracy of classifying the sex of marine mussels (*M. edulis*) using squash preparations of mantle tissue compared with sexing using traditional histologic processing and evaluation.

MATERIALS AND METHODS

Mussel Selection and Processing

Four hundred eighty mature (5- to 7-cm shell length) marine mussels (*M. edulis*) were collected in late April from a mussel lease in an estuary in Prince Edward Island, Canada. The mussels were removed from their shells, and each mussel had a small amount (approximately 1 mm^2) of tissue removed from the mantle with a scalpel. The tissue was gently squashed, smeared on a glass slide, and allowed to dry at room temperature. Dried smears were stained using a Wright-Giemsa stain pack (Fisher Diagnostics, Montreal, Quebec, Canada), a Romanowsky type of stain routinely used in cytologic processing (Tyler et al. 1992). Each mussel also had a strip (approximately 0.5×2 cm) of mantle tissue removed and placed into 10% neutral buffered formalin solution. After fixation in the formalin solution (1 day or more), the mantle tissues were removed. They were dehydrated through an ascending series of graded ethanols, cleared in xylene, embedded in paraffin wax (Paraplast Plus; Oxford Laboratories, Sherwood Medical, St. Louis, MO), sectioned at 5-μm thickness with a Reichert Histostat rotary microtome (Reichert Scientific Instruments, Warner-Lambert Technologies, Inc., Buffalo, NY), and placed on glass slides. Sections were stained with hematoxylin and eosin, as is routinely done in histologic processing (Luna 1968).

Determination of Sex

A classification of sex as male or female was made by evaluation of the Wright-Giemsa-stained squash preparations by a cytopathologist (S.A.B.). A classification of sex as male or female was made independently by the evaluation of paraffin-embedded tissue sections by a histopathologist (G.R.J.).

Statistical Analysis

The number of mussels to be evaluated (480) was chosen by the use of a sampling formula (Martin et al. 1987), in which we assumed a possible 5% error rate and in which we wished to be 93–97% confident of results.

RESULTS

Cytologic evaluation of mantle tissue squash preparations correctly classified all 480 mussels (261 females and 219 males) when

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compared with independent histologic evaluation. The distinction between sexes was easily made by the evaluation of stained smears, because preparations from male mussels had numerous small, angular, dark purple spermatozoa seen (Figs. 1 and 2), and the preparations from female mussels had large, thin-walled, pale pink oocytes observed (Fig. 3 and 4). Interestingly, the squash preparation slides could even be roughly classified as male or female by visual inspection without microscopy, because stained smears from the male mussels had a blue tinctorial quality compared with stained smears made from the female mussels, which were purple. This was an incidental observation, and determination of the accuracy of stain color to determine the sex of the 480 mussels was not performed.

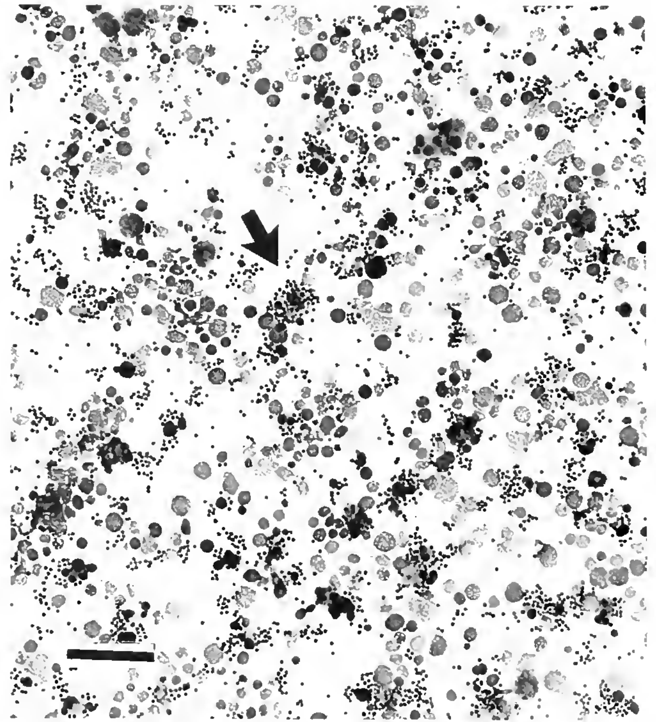

Figure 2. Photomicrograph of squash smear (cytologic) preparation of reproductive gland tissue from a male mussel. Note spermatozoa (arrow). Wright-Giemsa stain. Magnification, 234×. Bar = 50 µm.

DISCUSSION

Cytologic sexing of marine mussels (*M. edulis*) was 100% accurate in identifying the sex of individual mussels as compared with the traditional standard of histologic evaluation in this study. The cytologic evaluation was very simple, requiring only a few moments of scanning the smears to make the differentiation of male or female. No obvious mussels that could be classed as hermaphrodites were observed in our study. Hermaphrodites may occur at very low prevalence in mussel populations and may cause inconsistencies in cytologic versus histologic evaluation if present.

The mussels in this study were evaluated in late April. At that time in eastern Canada, water temperatures are still cold and mussels are sexually mature but have not yet spawned. A similar study comparing cytologic and histologic sexing of marine mussels may be less successful if performed at other times of the year, particularly in the post-spawning period. The accuracy of the cytologic sexing of blue mussels throughout the year would require further study.

It should be noted that the evaluation of unstained smears of mantle tissue (Jabbar and Davies 1987) or direct microscopic evaluation of mantle tissue from mussels (Nichols 1991) has been used previously by other investigators. However, the use of Romanowsky-type staining and the rigorous correlation of the cyto-

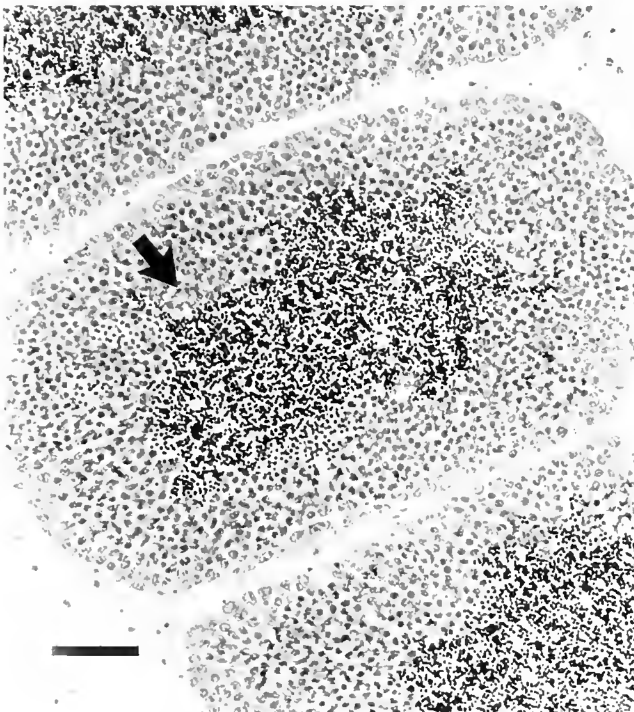

Figure 1. Photomicrograph of fixed tissue (histologic) section of reproductive gland tissue from a male mussel. Note spermatozoa (arrow). Hematoxylin & eosin stain. Magnification, 234×. Bar = 50 µm.

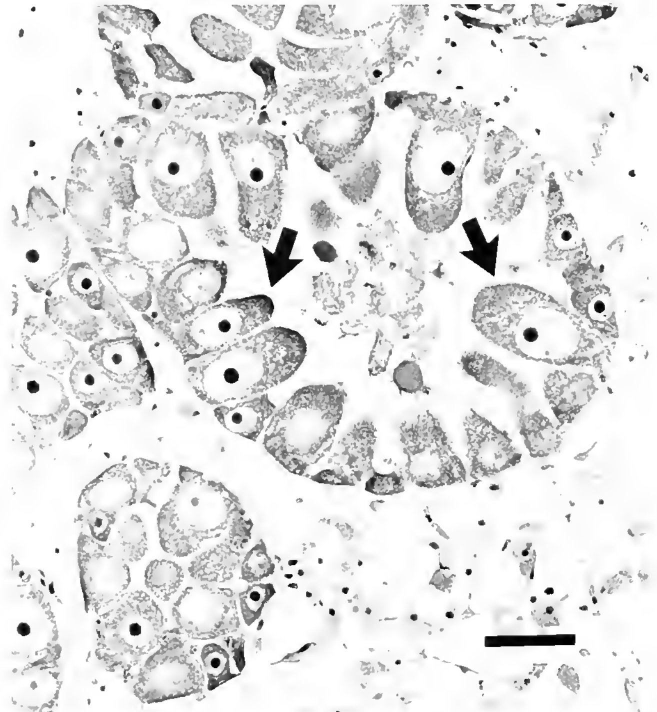

Figure 3. Photomicrograph of fixed tissue (histologic) section of reproductive gland tissue from a female mussel. Note oocytes (arrows). Hematoxylin & eosin stain. Magnification, 234×. Bar = 50 µm.

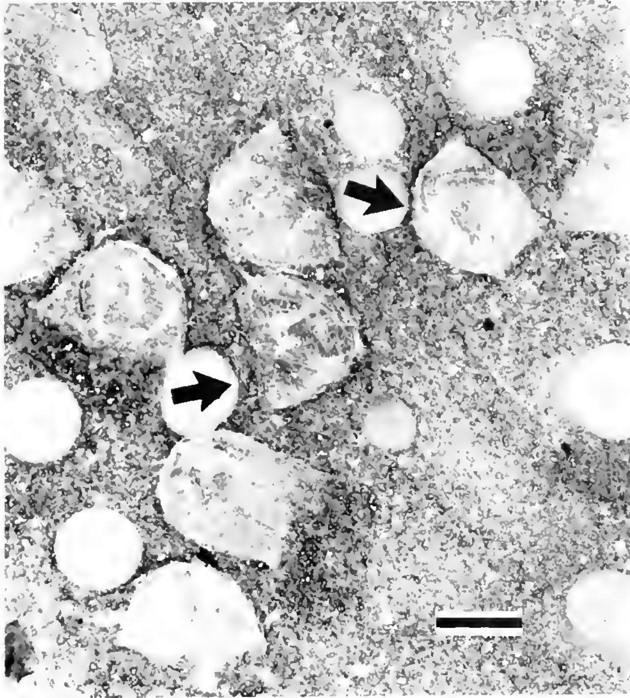

Figure 4. Photomicrograph of squash smear (cytologic) preparation of reproductive gland tissue from a female mussel. Note oocytes that have ruptured in smear preparation (arrows). Wright-Giemsa stain. Magnification, 234×. Bar = 50 µm.

logic method to the traditional standard of histologic sexing are features that distinguish this study from earlier ones.

The advantages of the cytologic sexing of marine mussels are the ease of sample preparation and evaluation, the low cost of slides and stain, short time (approximately 10 min for drying and staining), and minimal tissue required. Although this study was initiated to answer producer concerns related to marketing, this procedure may be of use to researchers interested in determining if sex influences the biochemical content of tissues, growth, or physiologic parameters. Although the sexing in this study was a terminal procedure (to attain sufficient samples for histologic processing), cytologic sexing requires very minimal tissue and could possibly be attained by needle aspiration, as is routinely performed in veterinary and human medicine. Fine-needle aspirates of mantle tissue (using a 21-gauge needle and 6-ml syringe) may result in diagnostic cytologic preparations with minimal effect on the mussel, allowing the cytologic sexing of live mussels in which further evaluations requiring living mussels were to be performed. Adequate preparations were achieved by us when doing fine-needle aspirates of mantle tissues (data not reported), but this technique was not consistently evaluated in this study.

In summary, the cytologic evaluation of squash preparations of mussel tissue was highly accurate in determining the sex of marine mussels (*M. edulis*) when evaluated in late April in Prince Edward Island, Canada. The advantages of the cytologic procedure over traditional histologic processing and biochemical determination include ease of sample preparation and evaluation, short preparation time, low cost, and the sparing of tissue for other studies.

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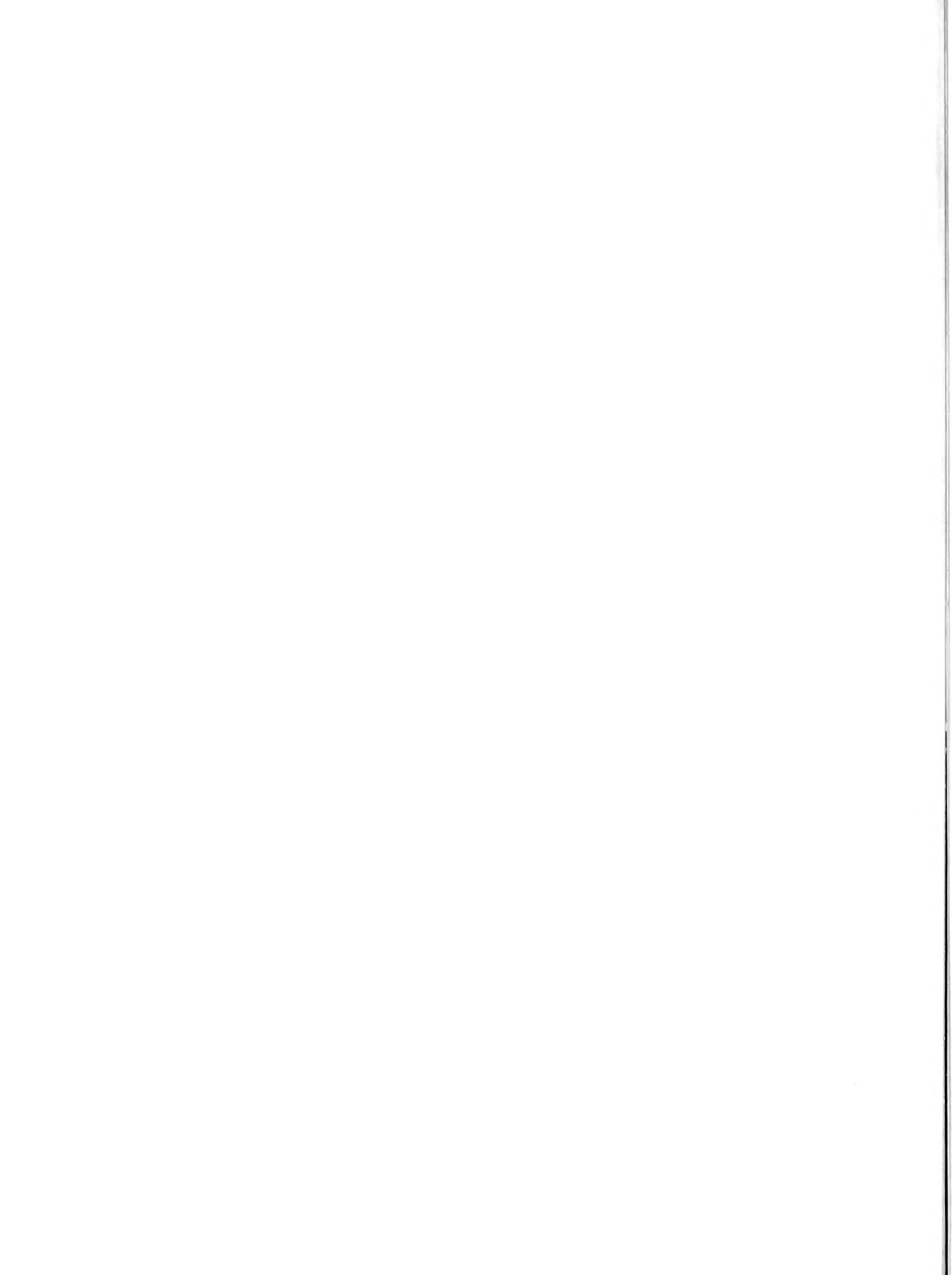

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COMPARATIVE ALLOMETRIES IN GROWTH AND CHEMICAL COMPOSITION OF MUSSEL (*MYTILUS GALLOPROVINCIALIS* Lmk) CULTURED IN TWO ZONES IN THE RIA SADA (GALICIA, NW SPAIN)

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ABSTRACT A study was carried out in two zones in the ría de Sada (Galicia, NW Spain) with mussels (*Mytilus galloprovincialis* Lmk) coming from the same stock and cultured in rafts, from seed sized to the first split (individuals with sizes between 15 and 65 mm), in order to evaluate a) the environmental differences in each location and b) their influence on growth (length and dry weight) and on chemical composition (protein, carbohydrates, glycogen, lipid classes, and fatty acids of total lipids). Allometric relations regarding those parameters for mussels on both locations were also determined. The mussels cultured in the inner zone (Arnela), with a higher concentration in chlorophyll *a* during the period studied and a smaller number of cultured mussels, present the highest performance in dry weight and a nearly doubled amount of glycogen than those in the outer one (Lorbé). There are also significant differences in saturated, monounsaturated, nonmethylene-interrupted dienoic, and total fatty acids and sterols, the lowest content being shown by those cultured at Arnela. The mussel is an important dietetic source of polyunsaturated ω-3 fatty acids, as eicosapentaenoic acid and docosahexaenoic acid; both of them are beneficial for health (i.e., the treatment and prevention of cardiac ischemia). The values found range from 1 to 2% of dry weight in both locations. An indicator of the greatest importance if the product is to be used for human food is the ratio of ω-6/ω-3 fatty acids. Habitual diets with a ratio of ω-6 to ω-3 fatty acids of 12–50 are associated with a greater risk. The value observed in this study was about 0.2 in both locations.

KEY WORDS: Mussel, growth, chemical composition, dietetic value

INTRODUCTION

The growth rate of bivalve molluscs is mainly determined by the environmental conditions in the zone of culture. Different environmental factors (temperature, salinity, primary production, and food availability among others) have a clear influence on their growth (Pérez Camacho et al. 1991). Zones with different latitudes, with few climatic changes (northern and southern Galician bays), present, northwards, a delayed gradient in the sexual maturation period (Villalba 1995).

Taking this as an aim, a study has been carried out on two zones of the ría de Sada (Galicia, NW Spain) with mussels (*Mytilus galloprovincialis* Lmk.) coming from the same stock and cultured in rafts, from seed size to the first split, comprising the period from winter to spring (individuals with sizes between 15 and 65 mm), in order to evaluate a) the environmental differences in each location and their influence on growth (length and dry weight) and b) the biochemical composition (protein, carbohydrates, glycogen, lipid classes, and fatty acids of total lipids).

Another one of the authors' objectives is the reassessment of mussel in order to improve its consumption. Hearn et al. (1989), Ackman (1990), and Cronin et al. (1991), among others, have clearly demonstrated that polyunsaturated ω-3 fatty acids (ω-3PUFA) help diminish both the risk of cardiovascular illnesses and the level of tryglicerides and can increase the level of high-density lipoproteins. Concerning ω-6 fatty acids (ω-6PUFA), these authors say that they have strong effects on humans; however, some of these effects are antagonists to the ones proposed for the ω-3PUFA. This explains the importance of studying the biochemical characteristics of organisms that will be used as human food.

MATERIALS AND METHODS

Culture Conditions

Ropes with mussel seeds from collectors (seeds fixed in the ría) and with an initial average size (length) of 27.61 ± 7.28 mm were placed in two rafts in the ría de Sada (Galicia, NW Spain), one at the inner zone (Arnela) with an area of 490,899 m^2 on 30 rafts and another at the outer one (Lorbé) with 1,339,200 m^2 on 90 rafts. The density estimated for mussel culture was 89.5 kg/m^3 at Lorbé and 43.1 kg/m^3 at Arnela.

The initial sample was carried out in November 1992 (size frequency and dry weight by size), together with a subsampling of the different size groups, to perform the analyses concerning biochemical composition. The final sampling at each of the rafts was performed in April 1993.

Temperature and chlorophyll *a* in the water column were made by means of CTD (Sea Bird 25) cast with a fluorometer (Sea Teach). The fluorescence values were calibrated against acetone extracts of chlorophyll *a* (Yentsch and Menzel 1963). The chlorophyll *a* was expressed in milligrams per cubic meter. Condition index was calculated according to Davenport and Chen (1987).

Biochemical Composition

A mean of 50 mussels for the lower class of size (15–35 mm) and 25 mussels for the upper class of size (40–65 mm) were freeze dried and stored at −70°C under inert nitrogen atmosphere for later analysis. The samples were sprayed in at pulverisette 6 (Fritsch) and homogenized with water in an ultrasonic vibrator Sonifier 250. Proteins were studied following the method described by Lowry et al. (1951), after hydrolysis with NaOH 0.5 N for 24 h at 30°C. Carbohydrates were quantified as glucose by the phenol-sulphuric acid method (Stickland and Parsons 1968). Glycogen is also quantified as glucose after precipitation with 100% ethanol.

Lipids were extracted following a modification of Bligh and Dyer (1959), described by Fernández Reiriz et al. (1989). Fatty acids from total lipids were transesterified to methyl esters with HCl in methanol and later analyzed by gas chromatography as described by Fernández Reiriz et al. (1993). Nonadecanoic acid

was used as an internal standard, and a response factor was calculated for each fatty acid in order to perform quantitative analyses. Lipid classes were studied as described by Fernández Reiriz et al. (1993).

RESULTS

The two zones studied are located in the north area of the ría de Sada (Galicia, NW Spain) (Fig. 1), 2 miles from each other. During the period studied, winter rainy weather, temperatures were similar (12.83 ± 0.64°C at Lorbé and 12.92 ± 0.62°C at Arnela).

Concerning chlorophyll *a*, the change during the period studied is shown in Figure 2. The mean values at each station are 17.10 ± 11.63 mg/m^3 for Arnela and 10.66 ± 8.15 mg/m^3 for Lorbé.

At the beginning of the experience, the mean size of the mussel seed was 27.61 ± 7.28 mm. At the end, the average size reached by the Arnela mussel was 40.73 ± 0.53 mm, whereas the one cultured at Lorbé attained 34.89 ± 0.44 mm. The evolution of the condition index (Table 1) under size groups at both locations shows significant differences from size group 50 on.

Allometric relations ($y = ax^b$) weight/size from these data respond to the following equation:

Arnela $y = 0.00264 \times^{3.062}$ ($r = 0.996$, $p < 0.0001$, $n = 10$)
Lorbé $y = 0.00668 \times^{2.782}$ ($r = 0.998$, $p < 0.0001$, $n = 10$)

The slopes of the allometric relation between locations were compared by means of a covariance analysis, after logarithmic transformation. The analysis shows significant differences ($F1,16 = 6.12$, $p < 0.025$) between slopes, depending on the zone of culture. The highest performances in dry weight flesh were attained at Arnela.

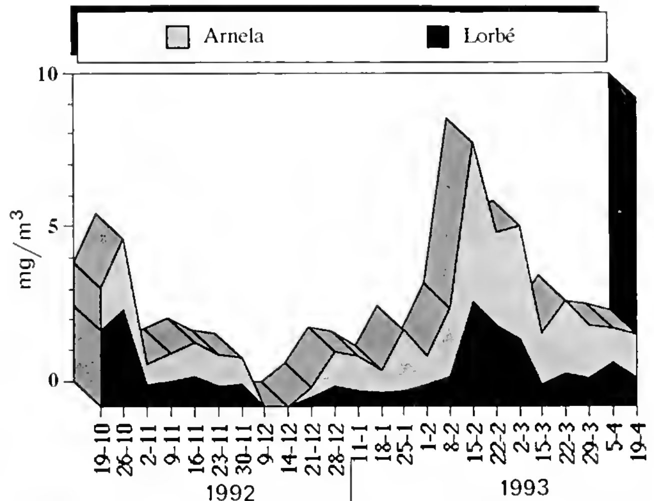

Figure 2. Evolution of chlorophyll *a* (mg/m^3) during the period studied.

TABLE 1.

Condition index of size groups at both locations.

Size of Class	Arnela	Lorbé
15	10.0	8.7
20	12.0	12.2
25	11.9	12.3
30	14.7	15.8
35	17.3	17.3
40	18.9	18.6
45	20.4	16.9
50	22.3	19.7
55	23.6	19.6
60	25.8	18.5

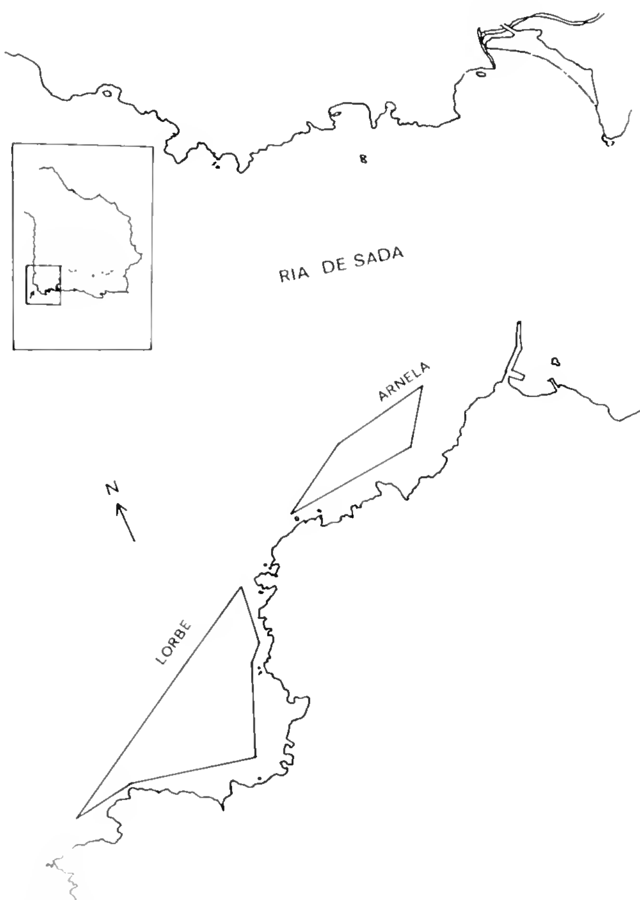

Figure 1. Location in the ría de Sada (Galicia, NW Spain) of the two zones studied.

Chemical Composition (Protein, Carbohydrates, Glycogen, Total Lipids, Lipid Classes, and Fatty Acids)

The relations among the different chemical parameters (y, milligrams) and the size (x, millimeters) in both locations are described by means of the following equations:

Protein:
Arnela $y = 0.00276 \times^{2.897}$ ($r = 0.986$, $p < 0.0001$, $n = 10$)
Lorbé $y = 0.00340 \times^{2.830}$ ($r = 0.998$, $p < 0.0001$, $n = 10$)

Carbohydrates:
Arnela $y = 0.00009 \times^{3.495}$ ($r = 0.973$, $p < 0.0001$, $n = 10$)
Lorbé $y = 0.00019 \times^{3.236}$ ($r = 0.988$, $p < 0.0001$, $n = 10$)

Glycogen:
Arnela $y = 0.00008 \times^{3.253}$ ($r = 0.946$, $p < 0.0001$, $n = 10$)
Lorbé $y = 0.00005 \times^{3.247}$ ($r = 0.958$, $p < 0.0001$, $n = 10$)

Lipids:
Arnela $y = 0.00021 \times^{3.206}$ ($r = 0.980$, $p < 0.0001$, $n = 10$)
Lorbé $y = 0.00050 \times^{2.967}$ ($r = 0.994$, $p < 0.0001$, $n = 10$)

The slopes of the allometric relations between the different

locations were compared by a covariance analysis. According to it, there are no significant differences ($p > 0.05$) between slopes. Regarding intercept, there are only significant differences in glycogen ($F_{1,16} = 14.17$, $p < 0.0025$). These results confirm that the relationship of glycogen content/size was the same for both Arnela and Lorbé mussels, although glycogen content at Arnela is nearly double that for Lorbé mussels.

If the initial size (27.61 mm) and the standard final one (stated as 40 mm) are inserted in the allometric equations of the initial sampling and in the final (organic component/size) one, several changes in the different components studied can be observed (Table 2). Regarding the initial mussel, total increments in protein, carbohydrates, glycogen, and lipids are, in general, higher in the mussel presenting a greater growth rate, so these increments depend on the global growth of mussel. Worth noting is that protein continues to be the main component in both locations and that Arnela mussels have twice as much glycogen as those from Lorbé.

Lipid Classes

Relations among the different lipid classes (y, milligrams) and size (x, millimeters) are described by the following regressions:

Phospholipids:

Arnela $y = 0.00007 \times^{3.353}$ ($r = 0.998$, $p < 0.0001$, $n = 10$)
Lorbé $y = 0.00008 \times^{3.301}$ ($r = 0.999$, $p < 0.0001$, $n = 10$)

Triacylglycerols:

Arnela $y = 0.00008 \times^{2.882}$ ($r = 0.842$, $p < 0.0014$, $n = 10$)
Lorbé $y = 0.00021 \times^{2.671}$ ($r = 0.889$, $p < 0.0003$, $n = 10$)

Sterols:

Arnela $y = 0.00001 \times^{3.015}$ ($r = 0.989$, $p < 0.0001$, $n = 10$)
Lorbé $y = 0.00005 \times^{2.640}$ ($r = 0.998$, $p < 0.0001$, $n = 10$)

Covariance analyses identified only significant differences between slopes for sterols ($F_{1,16} = 5.45$, $p < 0.05$). At the end of the research, phospholipids became the main lipid class (Table 2). There are no significant differences in phospholipidsand tryglicerides between the locations.

Fatty Acids

Fatty acids content (y, milligrams) varies with size (x, millimeters), according to the following allometric equations:

Saturated:

Arnela $y = 0.000009 \times^{3.576}$ ($r = 0.991$, $p < 0.0001$, $n = 10$)
Lorbé $y = 0.000065 \times^{3.127}$ ($r = 0.998$, $p < 0.0001$, $n = 10$)

Monounsaturated:

Arnela $y = 0.000013 \times^{3.282}$ ($r = 0.993$, $p < 0.0001$, $n = 10$)
Lorbé $y = 0.000037 \times^{3.097}$ ($r = 0.984$, $p < 0.0001$, $n = 10$)

Polyunsaturated:

Arnela $y = 0.000018 \times^{3.436}$ ($r = 0.988$, $p < 0.0001$, $n = 10$)
Lorbé $y = 0.000042 \times^{3.236}$ ($r = 0.996$, $p < 0.0001$, $n = 10$)

$\Sigma\omega$-6:

Arnela $y = 0.000006 \times^{3.165}$ ($r = 0.991$, $p < 0.0001$, $n = 10$)
Lorbé $y = 0.000008 \times^{3.095}$ ($r = 0.985$, $p < 0.0001$, $n = 10$)

$\Sigma\omega$-7:

Arnela $y = 0.000002 \times^{3.490}$ ($r = 0.964$, $p < 0.0001$, $n = 10$)
Lorbé $y = 0.000002 \times^{3.491}$ ($r = 0.982$, $p < 0.0001$, $n = 10$)

$\Sigma\omega$-9

Arnela $y = 0.000011 \times^{3.223}$ ($r = 0.996$, $p < 0.0001$, $n = 10$)
Lorbé $y = 0.000037 \times^{2.933}$ ($r = 0.996$, $p < 0.0001$, $n = 10$)

ω-3PUFA:

Arnela $y = 0.000018 \times^{3.395}$ ($r = 0.981$, $p < 0.0001$, $n = 10$)
Lorbé $y = 0.000021 \times^{3.342}$ ($r = 0.996$, $p < 0.0001$, $n = 10$)

Nonmethylene-interrupted dienoic (NMID):

Arnela $y = 0.000011 \times^{2.637}$ ($r = 0.983$, $p < 0.0001$, $n = 10$)
Lorbé $y = 0.000024 \times^{2.599}$ ($r = 0.995$, $p < 0.0001$, $n = 10$)

Total fatty acids:

Arnela $y = 0.000041 \times^{3.445}$ ($r = 0.989$, $p < 0.0001$, $n = 10$)
Lorbé $y = 0.000140 \times^{3.163}$ ($r = 0.997$, $p < 0.0001$, $n = 10$)

Relation ω-3/ω-6:

Arnela $y = 1.59 \times^{0.415}$ ($r = 0.931$, $p < 0.001$, $n = 10$)
Lorbé $y = 1.31 \times^{0.432}$ ($r = 0.980$, $p < 0.0001$, $n = 10$)

The covariance analysis carried out to compare the slopes of the allometric relations between locations shows that there are only significant differences between slopes for PUFA ($F_{1,16} = 12.51$, $p < 0.005$), for $\Sigma\omega$-9 ($F_{1,16} = 5.51$, $p < 0.05$), and for the total of fatty acids ($F_{1,16} = 18.9$, $p < 0.001$). As for intercepts, differences were only detected for monounsaturated fatty acids ($F_{1,16} = 34.82$, $p < 0.0005$), NMID ($F_{1,16} = 18.9$, $p < 0.001$), and the ω-3/ω-6 relation ($F_{1,16} = 11.96$, $p < 0.005$). Therefore, the relation between content of monounsaturated and NMID fatty acids and size was the same for both Arnela and Lorbe mussels, although the content in those fatty acids was higher at Lorbé.

Saturated and polyunsaturated (PUFAs) fatty acids are the main groups. Monounsaturated ones form a smaller group (Table 2). In our study, we have found that *M. galloprovincialis* has high contents of essential ω-3 PUFAs and low levels of NMID.

TABLE 2.

Biochemical changes (expressed as milligrams per individual) during the culture of mussel (*M. galloprovincialis* Lmk).

		Final	
Component	**Initial**	**Arnela**	**Lorbé**
Protein	39.78	120.80	116.22
Carbohydrate	6.23	35.75	28.69
Glycogen	3.93	13.34	7.64
Lipids	5.87	28.78	28.05
Lipid classes			
Phospholipids	1.18	15.92	16.19
Triacylglycerols	1.25	3.34	4.06
Sterols	1.35	0.80	0.87
Fatty acids			
Saturated	0.77	5.00	6.61
Monounsaturated	0.34	2.34	3.35
PUFAs	0.57	5.75	6.39
ω-3PUFAs	0.31	4.97	4.75
ω-6PUFAs	0.10	2.11	0.73
NMID	0.05	0.19	0.35

DISCUSSION

The reserve cycles of mussel indicate a complex interaction between food and temperature and between growth and the gametogenic cycle. The association between gonadal development and the reserve accumulation cycles is well known (Gabbott 1983).

Growth and Biochemical Composition

In our study with similar temperatures in both locations, Arnela presents a significantly higher ($p < 0.05$) concentration of chlorophyll *a* than does Lorbé, so the slower growth shown by Lorbé

mussels can be explained by the smaller availability of food and chlorophyll *a* at this location. These differences in primary production offer different growth rates between mussels at both localities.

Pérez Camacho and Román (1979), Dickie et al. (1984), and Mallet and Carver (1989) clearly state the influence that the zone of culture has on *Mytilus edulis* growth. Rodhouse et al. (1984) indicate that in many situations, food availability is the most important individual factor acting on mussel growth. Following this trend, Page and Hubbard (1987) detect a clear relation between the concentration of chlorophyll *a* and the growth in length of *M. edulis*. They did not find any connection between this growth and water temperature.

While studying the gametogenic cycle of mussels in the bays of Galicia, Villalba (1995) observed a slowed down gonadal development in Lorbé mussels, together with small growth rates, with regard to other bays of Galicia. Gabbot (1976) observed that the use of glycogen in *M. edulis* is closely related to its gametogenic cycle. Therefore, the highly significant contents of glycogen of Arnela mussels found in this study could be related to the gametogenic cycle, which is directly related to the available food in each locality. Further study of the gametogenic cycle of mussels from both areas could confirm this hypothesis.

Lipid Classes

The highest contents of phospholipids and triacylglycerols were observed in April in both locations. Beninger and Lucas (1984) observed in *Tapes decussatus* and in *Tapes philippinarum* a similar variation for phospholipids and triglicerides, with the highest values in spring (April). For those authors, these values are in relation to the active gametogenesis period. In our study, we only have data at the beginning (November) and at the end (April) and it only can be imputed to available food cycles in the "rías" and energetic reserve utilization for the mussels but without evidence of seasonal changes. There is little information on sterol changes, apart from their having an unespecified role in gonadal development (Gabbott 1976).

Fatty Acids

It is worth noting the huge contribution of ω-3PUFA (20:5ω-3 and 22:6ω-3) to the total of fatty acids, representing about 29 and 38% of them for Lorbé and Arnela, respectively. A fact also described by Gabbott (1976). Chu et al. (1990) say that the variations observed in ω-3PUFAs can be due both to the diet and to the reproductive cycle. Pollero et al. (1979) in *Crassostrea tehuelcha* and Trider and Castell (1980) in *Crassostrea virginica* studied the role of ω-3PUFA and observed that the level of 20:5ω-3 increased before the hatching and decreased afterwards.

With regard to NMID, and although their function is not clearly discerned, it seems that their biosynthesis is regulated both functionally and physiologically. Actually, big amounts of these acids were observed in the membranes of sponges (Morales and Lichfield 1986) and molluscs (Ackman and Hooper 1973), which would indicate a functional and structural role in the biological system. Klingensmith (1982) clearly indicates a competitive incorporation between NMID and PUFAs, especially 20:5ω-3 and 22:6ω-3. Fang et al. (1993), working with deep-water mussels in the Gulf of Mexico, found that they had a high NMID content, but not essential PUFAs. In our study, we have found *M. galloprovincialis* to have high contents of essential PUFAs and low levels of NMID. With regard to the increase of saturated and monounsaturated fatty acids, Waldock and Holland (1979), suggest that this increase is the result of the synthesis *de novo* from glycogen.

Dietetic Value

Fatty acid composition in marine organisms is a topic of great interest these days, because of the beneficial effects ω-3PUFA seem to have in order to reduce deaths caused by cardiovascular illnesses. Although the ideal amount a human diet must take in order to prevent them is not yet known, the effect its consumption produces has been already stated. Ackman (1990), following Weber, shows the existence of a positive correlation between deaths by coronary illnesses and a high relation of ω-6/ω-3 (between 12 and 50), so the effect that lipid composition has on the quality of a product destined for human consumption is important. Mussels studied here have a high content of ω-3PUFA, between 29 and 38% of the total of fatty acids (1 and 2% dry weight), and a ω-6/ω-3 relation of 0.2 in all size groups studied, a much lower one than those cited as harmful in the literature.

CONCLUSIONS

In big firms of mussel culture, management departments call for detailed knowledge about culture areas, their environmental factors, biological cycles, and the final characteristics of the products they are to obtain, in order to make the most of production and marketing. This work has been done taking all of these demands into account, and the following conclusions have been drawn:

1. Mussels cultured at Arnela present higher growth rates and also a higher condition index than those cultured at Lorbé; this is clearly related to food availability and the density of culture in each area.
2. The chemical composition of the mussels of the two zones showed a similar pattern, although some significant differences were observed in the contents of certain components: Arnela mussels present the highest contents of glycogen, as well as the lowest ones in saturated, monounsaturated, and NMID fatty acids and sterols. These facts show an influence of the habitat in the biochemical components of mussels, beyond those probably conditioned by the gametogenic cycle.
3. As far as the dietetic value of this product is concerned, it is important to note the high content of ω-3PUFA (between 1 and 2% dry weight) found in the mussels of both locations and the low ω-6/ω-3 relation (about 0.2).

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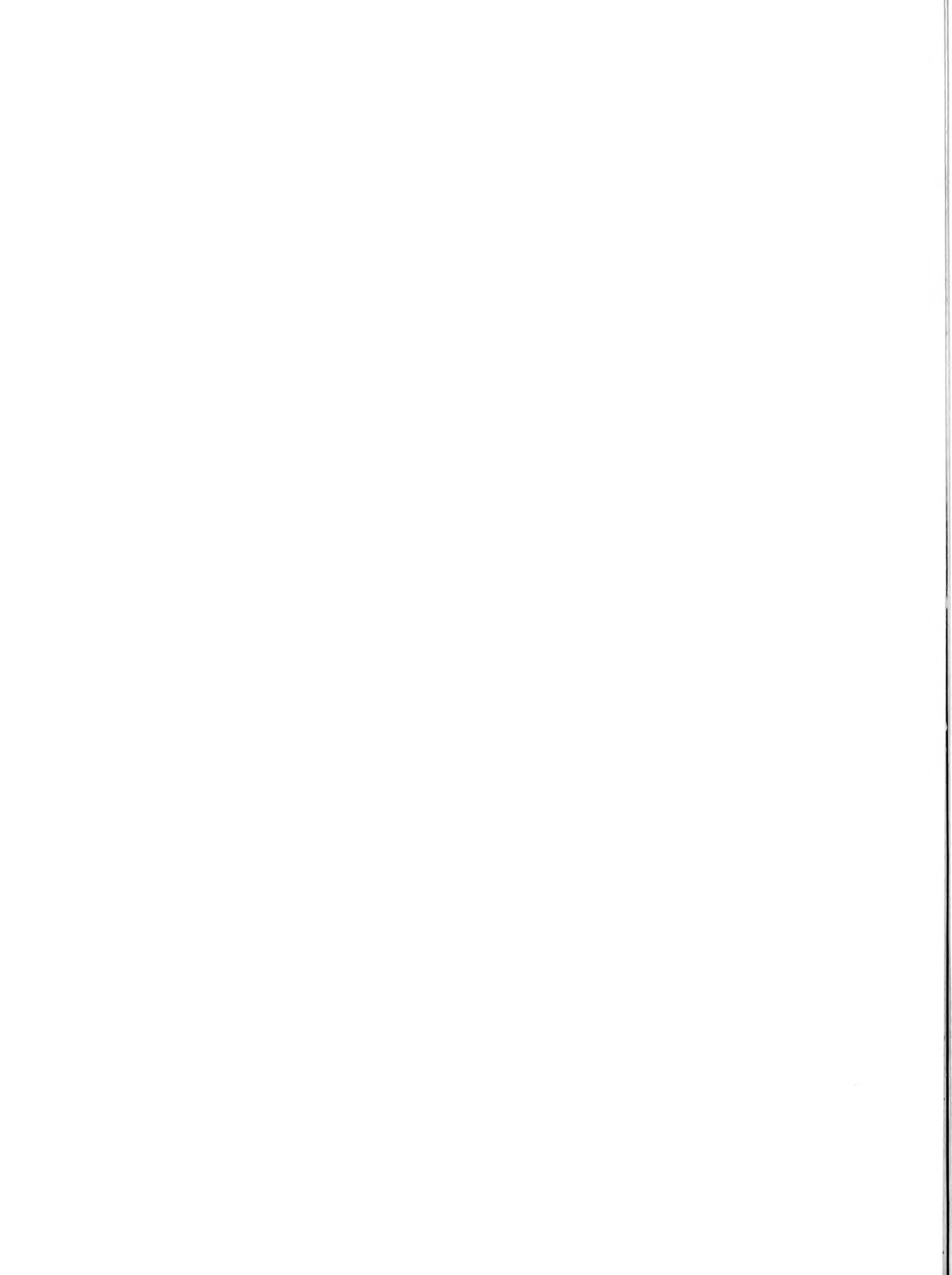

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SIZE-DEPENDENT SURVIVORSHIP OF THE BIVALVE *YOLDIA NOTABILIS* (YOKOYAMA, 1920): THE EFFECT OF CRAB PREDATION

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ABSTRACT Age-specific survivorship of the bivalve *Yoldia notabilis* was estimated by quantitative field samplings, and the possible effect of predation by the crab *Paradorippe granulata* on its survivorship pattern was examined by laboratory experiments. The annual mortality rate in the field was high (>86%) in the two youngest year classes, whereas mortality was lower (<40%) in the classes older than 3 y, leading to Deevey's type 3 survivorship curve. Field mortality was size dependent; bivalves smaller than 10 mm in shell length suffered high mortality (>68%), whereas those larger than 10 mm did not (<40%), regardless of actual age. Burrowing depth of *Y. notabilis* increased with shell length, although considerable variation was found among larger individuals. The laboratory experiment revealed that crabs preferred smaller bivalves. The resultant size-related mortality pattern in the experiment was similar to that in the field, suggesting that size-selective predation by *P. granulata* contributes to the size-specific survivorship pattern of *Y. notabilis*. Size-selective predation by this crab did not depend on the size-dependent burrowing ability of the bivalve, because small sizes were preferred even when bivalves of all sizes were constrained to live in shallow sediments.

KEY WORDS: *Yoldia*, survivorship, crab, size-selective predation, burrowing depth, laboratory experiment

INTRODUCTION

For understanding the population dynamics of organisms with a long lifespan, it is essential to estimate age- or size-specific changes in survivorship. Age-specific survivorship has been determined for several marine bivalves, including some long-lived species such as *Chlamys islandica* (Vahl 1981), *Macoma balthica* (Green 1973), *Mercenaria mercenaria* (Kennish 1980), *Mya arenaria* (Brousseau 1978, Goshima 1982), *Mytilus edulis* (Thompson 1984), and *Ostrea edulis* (Walne 1961). Most of these animals show high mortality as juveniles and low mortality as adults, which leads to an negative exponential survivorship curve, or the type 3 curve of Deevey (1947). Size-dependent mortality factors are often responsible for producing this survivorship pattern. Size-dependent mortality may result from the relation of size to predation (Paine 1976, Seed 1993) or competition (Bell and Coull 1980) or differences in vulnerability to physical stress or disturbance (Levinton and Bambach 1969, Hughes 1970, Rhoads and Young 1970, Woodin and Marinelli 1991).

Size-selective predation by predators such as benthic fish, crabs, and sea birds has been well established as setting the survivorship patterns of marine bivalve populations (Paine 1976, Seed and Brown 1978, Holland et al. 1980, Peterson 1982a, Peterson 1982b, Arnold 1984, Sanchez-Salazar et al. 1987, Zwarts and Blomert 1992b, Seed 1993). For infaunal bivalves, the effect of predation is often affected by the burrowing depth of individuals (Hughes 1970, Blundon and Kennedy 1982, Möller and Rosenberg 1983, Elmgren et al. 1986, Haddon et al. 1987, Zwarts and Blomert 1992a). Field observations and experiments have been undertaken to test the possible effects of predators on intertidal and shallow subtidal bivalve populations, but rarely have such studies been conducted at water depths deeper than 10 m, mainly because of difficulties in estimating field mortality, as well as logistical constraints in carrying out field experiments.

Yoldia notabilis is a deposit-feeding bivalve that lives in muddy-sand bottoms of the northwestern coast of the Pacific. It occurs at high densities (ca. 200 m^{-2}) at depths of 10–15 m in Otsuchi Bay, northeastern Japan. Age is easily determined by counting the number of external and internal shell growth rings (Nakaoka 1992a, Nakaoka and Matsui 1994). It is therefore possible to quantify its age-specific survivorship by monitoring the density of each year class over time. *Yoldia* spp. are often consumed by benthic predators such as fish and crabs (Tyler 1972, Jewett and Feder 1980). In Otsuchi Bay, the distribution of *Y. notabilis* overlaps that of potential epibenthic predators such as the crabs, *Paradorippe granulata* and *Ovalipes punctatus* and the flounders *Paralichtys olivaceus* and *Kareius bicoloratus*. *P. granulata*, in particular, is known to be an active bivalve feeder (Sasaki 1993, Goshima personal communication). The predation pressure from these predators may affect the age- and size-specific survivorship of *Y. notabilis*.

The main objectives of this article are (1) to test whether the survivorship of the field population of *Y. notabilis* is dependent on size, and (2) to evaluate the potential for size-selective predation by *P. granulata* to contribute to the observed survivorship pattern of *Y. notabilis*. I carried out a field census to quantify densities of the bivalve and the crab and also laboratory predation experiments using these animals. I also tested whether size-selective predation by the crab depends on the burrowing depth of the bivalve.

MATERIALS AND METHODS

Estimation of Field Densities

A field census was carried out between June 1990 and June 1991 at two stations (Stn YA, 10 m in depth; Stn YD, 14 m) established at the inner part of Otsuchi Bay (see map of sites in Nakaoka 1992b). Four to 10 quantitative sediment samples were taken monthly at each station with a 0.1-m^2 Smith-McIntyre grab sampler. The collected sediments were sieved through a 1-mm-mesh sieve, and individuals of *Y. notabilis* and *P. granulata* retained on the sieve were sorted out. The age of each individual *Y. notabilis* was determined by counting the annual growth rings on the external shell surfaces. A previous study has shown that these rings are formed annually during winter (Nakaoka 1992a). Individuals were classified into one of seven year classes between

Class 1983 (8 y old in 1991) and Class 1990 (1 y old) or a compound class that consisted of individuals that were recruited before 1983 (Class <1983; >8 y old). Nakaoka (1992a) has shown that the youngest year class (Class 1991; 0 y old) cannot be collected by this method because the individuals are so small that they pass through the 1-mm-mesh. To estimate the density of this class, three to four subsamples each of 0.01 m^2 were taken from the grab samples (one subsample from each grab sample) monthly at Stn YD (except March 1991) and on six occasions (June, July, and November 1990 and April, May, and June 1991) at Stn YA, and they were sieved through a 0.5-mm-mesh sieve to collect 0-y-old individuals. For all of the bivalves, shell length was measured with a caliper to the nearest 0.1 mm and the density of each year class was determined for each month and station. The number of *P. granulata* collected by the same grab sampler was also recorded. More detailed information on the study sites and sampling procedure is given in Nakaoka (1992b).

Burrowing Depth

The *in situ* burrowing depth of *Y. notabilis* was measured from grab samples at Stn YD on January 22, 1990. Six 8-cm-deep sediment cores (each 0.01 m^2 in area) were taken from three grab samples, and the cores were immediately divided on shipboard into 2-cm sections. The sliced sediments were sieved through a 1-mm-mesh. Individuals of *Y. notabilis* in each section were counted, and the shell length of each individual was measured.

Because the sediments deeper than 8 cm were not taken quantitatively, the burrowing depth was also observed in the laboratory. Individuals were collected at the same date as above and kept alive in the laboratory in two aquaria (each 0.01 m^2 in area). Each aquarium contained 15 individuals of sizes ranging between 4.2 and 38.4 mm in shell length. Aquaria were filled to a depth of 10 cm, with sediment collected at Stn YD, and supplied with running seawater. Four days later, the sediments in the aquaria were taken out at 2-cm intervals to determine the burrowing depth of each individual.

Experiment 1: Size-Specific Crab Predation on Bivalves

A laboratory experiment testing for the size-selective predation by *P. granulata* on *Y. notabilis* was carried out from July 11 to August 8, 1992. Individuals of *Y. notabilis* and *P. granulata* were collected with the grab sampler and a biological dredge with a 50-cm mouth opening at Stn YA and Stn YD a day before the experiment. Eighteen individuals of *Y. notabilis* (2.9–39.1 mm in shell length) were held in each of six aquaria (each 0.025 m^2 in area). The size distribution of the bivalve was similar in all aquaria. Four aquaria had one crab (16.7–20.6 mm in carapace length) added to each of them (experimentals), and the remaining two did not (controls). The resulting densities of *Y. notabilis* and *P. granulata* (720 and 40 m^{-2}, respectively) were greater than those observed in the field (179 and 206 m^{-2} at Stn YA and YD, respectively, for *Y. notabilis;* 1.8 and 1.7 m^{-2} for *P. granulata;* see Results; see also Nakaoka 1992b) because of the limitation of experimental space. The sediment used for the experiment was taken from Stn YA and Stn YD, sieved through a 1-mm-mesh sieve, mixed, and dried under sunlight for more than 3 d to remove any live animals. The aquaria were filled with this sediment to a depth of 8 cm, and running seawater was supplied to each aquarium. Water temperature in the aquaria varied no more than 2°C from that of the natural environment (15.9–19.0°C during the experimental period; Takagi et al. 1992). I first introduced bivalves to the aquaria and then crabs on the following day, after all bivalves had burrowed under the sediment surface. No alternative food was supplied to the crabs during the experiment. At the end of the experiment, sediments were removed from each aquarium at 2-cm intervals from the surface, and the sizes of all live and dead specimens of *Y. notabilis* in each layer were recorded.

Observations of the feeding behavior of *P. granulata* in the laboratory revealed that crabs find *Y. notabilis* by scooping the sediment surface (at least to more than 1 cm deep) with their chelipeds. Crabs feed on the soft parts of the bivalve by inserting the chelipeds through a gape between two valves of the prey. In most cases, the shells are broken by this activity. Even if the shells sometimes remain unbroken, the mortality due to crab predation is distinguishable from other mortality causes in the laboratory because soft tissue is completely removed from the shells by predation. I classified the fate of each bivalve into three categories: (1) alive, (2) predated (broken shell and unbroken empty shell), and (3) mortality due to other causes (unbroken shell with soft parts remaining).

Experiment 2: The Effect of Burial Depth on Crab Predation

To test the effect of the burrowing depth of *Y. notabilis* on susceptibility to *P. granulata* predation, I changed the sediment depth in the aquaria in the second laboratory experiment (July 8–August 14, 1993). I used a methodology and experimental protocol similar to those in Experiment 1, but with a different experimental design. Eighteen individuals of *Y. notabilis* (4.1–36.3 mm in shell length) and a crab (19.0–23.0 mm in caparace length) were held in each six aquaria, three containing an 8-cm layer of sediment and another three with a 2-cm layer of sediment. A higher predation rate is expected with decreased sediment depth if crab predation is related to the burrowing depth of the prey (see Blundon and Kennedy 1982, Elmgren et al. 1986 for similar experimental designs). No controls (aquaria without the crab) were prepared because of an insufficient number of bivalves. Two crabs (one in each treatment) died during the experiment. The data form these two aquaria were excluded from the analysis.

Data Analyses

A survivorship curve for each year class of *Y. notabilis* at each station was obtained by transforming monthly mean density x to $\log(x + 1)$ and by regressing it against date t. The slope of the regression equation expresses the rate of decrease in density due to mortality. I tested the heterogeneity of slopes between year classes and stations using a general linear model expressed as follows:

$$y_{ijk} = \alpha + \alpha_{1i} + \alpha_{2j} + (\alpha_1\alpha_2)_{ij} + \beta t_{ijk} + \beta_{1i}t_{ijk} + \beta_{2j}t_{ijk} + (\text{gkb}\ _1\beta_2)_{ij}t_{ijk} \quad (1)$$

where y_{ijk} is the log-transformed density of year class i at station j at date t_{ijk}; α and β are average regression coefficients (intercept and slope, respectively); α_{1i}, α_{2j}, β_{1i} and β_{2j} are treatment effect coefficients; and $(\alpha_1\alpha_2)_{ij}$ and $(\beta_1\beta_2)_{ij}$ are effects of interaction between year class and station. The F-test on the terms $\beta_{1i}t_{ijk}$ and $\beta_{2j}t_{ijk}$ shows whether the slope differs significantly among age classes and between stations, respectively, and the test on the term $(\beta_1\beta_2)_{ij}t_{ijk}$ examines the age*station interaction effect on the slope. This test assumes that the estimate of density for each month is independent of that for other months, which may not be true if one samples the population repeatedly at the same site.

During my research, however, I tried not to collect sediments exactly from the same spot. The density estimates, therefore, can be regarded as independent.

To test the between-site differences in age- and size-specific survivorship, the slopes of the regression equations were also compared between the stations for each year class and for each size class, the latter by classifying each year class into six different size categories (0–5, 10–15, 15–20, 20–25, 25–30, and >30 mm in shell length) according to the mean shell length of each year class (Nakaoka 1992b). The general linear model for this analysis was reduced to:

$$y_{ijk} = \alpha + \alpha_{1i} + \beta t_{ijk} + \beta_{1i} t_{ijk}. \qquad (2)$$

Year class 1989 and size class 5–10 mm were not analyzed because these classes did not occur at Stn YA. The annual mortality rate was calculated from the difference in predicted densities on June 1, 1990 ($t = 0$) and June 1, 1991 ($t = 365$), which were estimated by use of the regression equations.

The relationship between bivalve size and burrowing depth was tested by the use of Fisher's exact test of independence after categorizing data into three size classes (0–10, 10–20, and 30–40 mm in shell length) and three depth layers (0–2, 2–4, and >4 cm in depth). The effect of the crab on the bivalve size-burrowing depth relationship was tested for the data of Experiment 1 by the use of a three-factor log-linear model, expressed as follows:

$$\ln f_{ijk} = \mu + \alpha_i + \beta_j + \gamma_k + \alpha_i\beta_j + \alpha_i\gamma_k + \beta_j\gamma_k + \alpha_i\beta_j\gamma_k \qquad (3)$$

where f is the expected frequency of a three-way contingency table; μ is the mean of the logarithm of the expected frequencies; α_i, β_j, and γ_k are the effects of categories i, j, and k of three factors (treatment, size, burrowing depth); and $\alpha_i\beta_j$, $\alpha_i\gamma_k$, $\beta_j\gamma_k$, and $\alpha_i\beta_j\gamma_k$ are the interaction terms expressing the dependency of two or three factors. Individual bivalves that survived until the end of the experiment were classified according to treatment (Experimental and Control), shell length (0–10, 10–20, 20–30, and 30–40 mm), and burrowing depth (0–2, 2–4, and >4 cm).

I did not test the size dependency of the crab predation rate or bivalve mortality rate in the two experiments by analysis of variance or other parametric methods because the number of replicates for each treatment (n = 2–4) and the number of individuals in some size classes (see Figs. 3 and 4) were too small to obtain enough statistical power. Instead, I tested the differences in the size-frequency distribution of predated or dead bivalves using the Kolmogorov-Smirnov two-sample test after pooling data for each treatment. The underlying assumption of this analysis is that individuals within each aquarium behaved independently so that individuals instead of aquarium can be treated as a unit of replicates (see Peterson 1982b for similar analysis). This assumption may be violated in cases where larger bivalves can escape better from predators, which affects the predation rates on smaller individuals. However, no observation on their behavior was made after they burrowed in the sediments. In Experiment 1, the size-frequency distributions of predated or dead bivalves in the experimental treatment were compared with that predicted under size-independent predation or mortality, i.e., the initial size distribution before the experiment. In Experiment 2, the difference in size-frequency distribution was tested between the two treatments. The bivalves were classified into seven size classes (0–5, 5–10, 10–15, 15–20, 20–25, 25–30, and >30 mm) in Experiment 1, but into only six size classes (4–10, 10–15, 15–20, 20–25, 25–30, and >30 mm) in Experiment 2 because of an insufficient number of the smallest individuals (<5 mm). All statistical analyses were performed with SAS (SAS Institute 1987).

RESULTS

Age-Specific Survivorship of the Bivalve

The density of *Y. notabilis* remained steady between June 1990 and June 1991 for most year classes recruited before 1988, whereas declines in density were detected in Class 1990 at Stn YA and Classes <1983 and 1987–1990 at Stn YD (Figs. 1 and 2). No individuals belonging to Class 1989 were collected at Stn YA during the research period. The slope of the regression lines expressing survivorship curves was negative in all classes except Class 1984 at Stn YD, although the level of significance was often low (Table 1). At the 5% significance level, the slope differed significantly from 0 in five year classes at Stn YD (Classes <1983 and 1987–1990) and in one year class (Class 1990) at Stn YA (Table 1). Younger year classes tended to have more negative values in the slope of the survivorship curve. The test of heterogeneity of the slope revealed that the slope differed significantly between age (Table 2).

The annual mortality rate was high (>86%) in Classes 1989 and 1990 and lower (<40%) in the year classes recruited before 1988 at both stations (Table 3). A large between-site difference in annual mortality was found in Class 1988, in which the annual mortality rate was 20% at Stn YA but 68% at Stn YD. A between-site comparison of slopes of the survivorship curves showed that slopes differed significantly for this year class (Table 4). The significant differences in slope were not detected in other year classes, although the estimated mortality was somewhat higher at Stn YD than at Stn YA in Classes <1983 and 1983 (Table 3).

A comparison of annual mortality with mean shell length of each year class showed that annual mortality was more than 68%

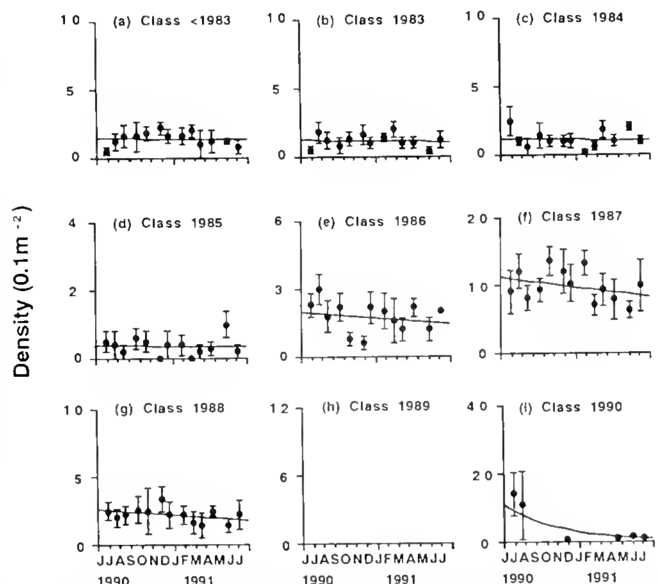

Figure 1. Seasonal changes in mean density (±SE) of nine year classes of *Y. notabilis* at Stn YA. The individuals of Class 1989 at Stn YA did not occur during the research period. The line indicates the survivorship curve fitted using the parameters in Table 1. Original data and sample sizes are shown in Nakaoka (1992b).

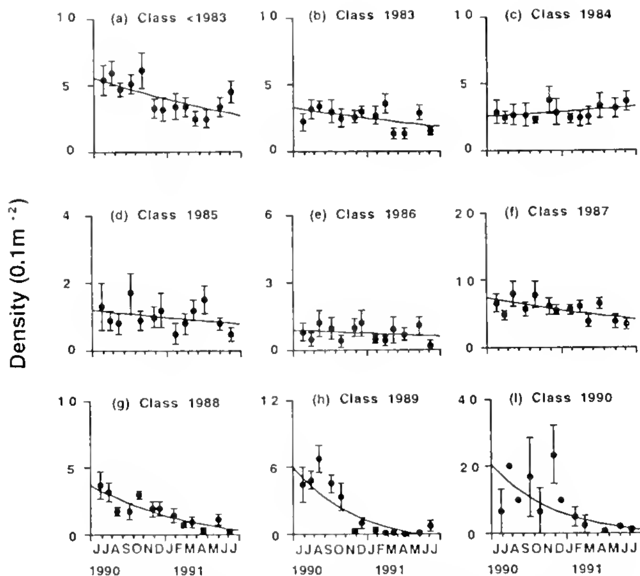

Figure 2. Seasonal changes in mean density (±SE) of nine year classes of *Y. notabilis* at Stn YD. See legend to Figure 1 for explanation.

for classes smaller than 10 mm in shell length and lower than 40% for those larger than 10 mm (Table 3). The test of the heterogeneity of slopes of survivorship curves showed that the slopes did not differ significantly between stations when compared on the basis of size (Table 4).

TABLE 1.

Parameters of the survivorship curve for each year class of *Y. notabilis* at the two stations.

Year class	n	a ($\times 10^{-4}$)	b	r	p
		Parameters of Survivorship Curve			
Stn YA					
<1983	13	−0.443	0.382	−0.057	0.852
1983	13	−0.660	0.340	−0.081	0.792
1984	13	−0.639	0.332	−0.062	0.841
1985	13	−0.414	0.135	−0.060	0.846
1986	13	−2.17	0.474	−0.227	0.456
1987	13	−2.84	1.08	−0.362	0.224
1988	13	−2.65	0.554	−0.424	0.149
1989	—	—	—	—	—
1990	6	−27.4	1.03	−0.838	0.037
Stn YD					
<1983	13	−5.92	0.816	−0.668	0.013
1983	13	−4.29	0.627	−0.492	0.088
1984	13	1.91	0.548	0.421	0.152
1985	13	−2.08	0.338	−0.321	0.285
1986	13	−1.81	0.275	−0.251	0.410
1987	13	−5.02	0.918	−0.617	0.025
1988	13	−13.6	0.667	−0.878	<0.001
1989	13	−24.2	0.847	−0.854	<0.001
1990	12	−24.7	1.33	−0.784	0.003

The survivorship curve was obtained with regression analysis and is expressed as $\log(x + 1) = at + b$, where x is the mean density for each month, t is the cumulative date (in days; $t = 0$ on June 1, 1990), a is the slope, and b is the intercept of the regression equation. r is the regression coefficient, and p is the probability at which the slope (a) did not differ from zero. The survivorship curve was not obtained for Class 1989 at Stn YA because no individuals occurred at this station.

TABLE 2.

The results of the general linear model for testing the heterogeneity of slopes of the survivorship curves in Table 1 between age classes and stations.

Factor	df	F	p
Age	8	16.48	0.0001
Station	1	2.35	0.1271
Age*station	1	1.13	0.3490

Field Density of the Crab

During the study, I quantitatively collected seven *P. granulata* at Stn YA and nine at Stn YD, giving density estimates (average density over the research period) of 1.8 and 1.7 m^{-2}, respectively. The data were too few to examine for seasonal changes in density. The size range of crabs collected at Stn YA varied between 15.1 and 28.1 mm in carapace length (mean, 20.5 ± 2.1 mm [SE]) and between 5.1 and 28.1 mm (mean, 18.1 ± 2.4 mm [SE]) at Stn YD. Crab sizes were not significantly different between the two stations (t test; $p = 0.476$).

Burrowing Depth of the Bivalve

Larger individuals of *Y. notabilis* tended to be found deeper in the sediments both in the field and in the laboratory (Table 5). Individuals smaller than 10 mm in shell length were generally restricted to the upper-most 2-cm layer. On the other hand, individuals larger than 30 mm were distributed throughout the sediment, ranging between 0 and 8 cm deep. More than 50% of the individuals larger than 30 mm were found in the 2- to 4-cm layer in the laboratory and in the 4- to 6-cm layer in the field. No individuals were collected from the deepest layer of the sediments (6–8 cm in the field and 8–10 cm in the laboratory). The results of Fisher's exact test revealed that the relationship between size and burrowing depth for the field data was almost significant at the 5% level ($p = 0.070$), but the small sample size ($n = 14$) probably

TABLE 3.

Annual mortality rate (% y^{-1}) of each year class of *Y. notabilis* at two stations.

Year Class	Age in 1991	Annual Mortality Rate (Mean Shell Length in mm) Stn YA	Stn YD
<1983	>8+	3.7 (34.1)	39.2 (35.6)
1983	8+	5.4 (31.0)	30.3 (30.8)
1984	7+	5.2 (28.6)	0.0* (26.8)
1985	6+	3.4 (24.7)	16.0 (21.6)
1986	5+	16.7 (23.8)	14.1 (18.9)
1987	4+	21.2 (19.2)	34.4 (13.0)
1988	3+	20.0 (10.7)	68.1 (7.0)
1989	2+	—	86.9 (2.1)
1990	1+	90.0 (1.0)	87.5 (0.9)

Mortality rates were estimated from the differences in predicted densities on June 1, 1990 ($t = 0$), and June 1, 1991 ($t = 365$), which were estimated by use of the regression equations in Table 1.
* The mortality was assumed to be 0 because the predicted density increased during the period (Table 1).

TABLE 4.

The results of the general linear model for testing the heterogeneity of slopes of survivorship curves between stations for each year class and each size class.

Factor	df	F	*p*
Year class			
<1983	1	3.20	0.0872
1983	1	1.18	0.2898
1984	1	0.58	0.4550
1985	1	0.36	0.5555
1986	1	0.01	0.9175
1987	1	0.55	0.4657
1988	1	15.24	0.0008
1990	1	0.07	0.7995
Size class (mm)			
0–5	1	0.05	0.8304
10–15	1	0.85	0.3663
15–20	1	0.12	0.7371
20–25	1	0.03	0.8691
25–30	1	0.58	0.4550
>30	1	3.02	0.0885

reduced the power of this test. The relationship was highly significant ($p < 0.001$) for the laboratory data.

Experiment 1: Size-Specific Crab Predation on the Bivalve

Experiment 1 clearly demonstrated that smaller individuals of *Y. notabilis* suffered higher mortality from crab predation (Fig. 3). Mortality was as high as 80% in the size class 0–5 mm in shell length. It decreased with increasing size and was no more than 20% in the classes larger than 15 mm. Individuals smaller than 10 mm were killed solely by crabs, whereas mortality due to other causes was also observed for those larger than 10 mm. The size-frequency distributions of the bivalve that had been eaten and those that were dead were significantly different from those expected from size-independent predation and mortality, i.e., the initial size-frequency distribution before the experiment (Kolmogorov-Smirnov two-sample test; $D = 0.443$, $p = 0.001$ and $D = 0.331$, $p = 0.020$, respectively).

The burrowing depth of live specimens of *Y. notabilis*, measured at the end of this experiment, increased with size (Table 6). The results are in agreement with the field and laboratory observations described above (Table 5). The three-factor log-linear model shows a significant interaction only between size and depth ($p = 0.049$; Table 7). The nonsignificance in the three-factor interaction among treatment, size, and depth ($p = 0.271$; Table 7) suggests that the size-burrowing depth relationship was not affected by the presence or absence of the predator.

Experiment 2: The Effect of Burial Depth on Crab Predation

The size-frequency distributions of *Y. notabilis* in Experiment 2 showed a pattern similar to that in Experiment 1 (Fig. 4). Predation occurred most severely on the smallest size classes (4–10 mm in shell length), even when the burrowing depth was restricted to a depth of 2 cm. The size-frequency distributions of the predated and dead bivalves were not significantly different between the two treatments (Kolmogorov-Smirnov two-sample test; $D = 0.179$, $p = 0.971$ and $D = 0.133$, $p = 0.999$, respectively).

DISCUSSION

The annual mortality rate of *Y. notabilis* in the field was estimated to be high (>86%) in the year classes younger than 3 y. In contrast, mortality was lower in most bivalves older than 3 y (Table 3). This leads to the type 3 survivorship curve of Deevey (1947), which is typical for marine invertebrates, including several long-lived bivalves such as *M. arenaria* (Brousseau 1978, Goshima 1982), *M. edulis* (Thompson 1984), *O. edulis* (Walne 1961), and *Scrobicularia plana* (Hughes 1970).

The only significant between-site difference in the slope of survivorship curves was found in Class 1988 (Table 4), resulting in the large difference in the estimated mortality between stations (Table 3). However, the slopes did not differ between stations when they were compared on the basis of size (Table 4). The relationship between shell length and mortality shows that individuals suffered high mortality when they were smaller than 10 mm in shell length, but this was reduced when shell length exceeded 10 mm (Table 3). The mortality of *Y. notabilis* thus appeared to be more dependent on size than age.

Size-dependent mortality has been reported in a variety of marine bivalves, and in most cases, this has been attributed to size-selective predation (Paine 1976, Holland et al. 1980, Arnold 1984, Sanchez-Salazar et al. 1987, Peterson 1990, Seed 1993). In this case, the size-specific mortality pattern in the field corresponded well with that obtained from the predation experiment in the laboratory (Figs. 3 and 4). This suggests that predation by the crab, *P. granulata*, could contribute to the size-dependent survivorship pattern of *Y. notabilis*. If one assumes that the feeding rate of *P. granulata* in the field is similar to that determined in Experiment 1 (six bivalves mo^{-1} per crab), this crab would consume about 130 bivalves $m^{-2}y^{-1}$. This estimate represents 70 and 60% of the natural population at Stn YA and YD, respectively, suggesting that crab predation may have a considerable effect on the survivorship pattern of *Y. notabilis* in the field.

Such estimates, however, must be interpreted with caution, especially when one applies the results of laboratory experiments to a field situation. In this study, the relationship between prey density and crab predation rate was not investigated experimentally because of difficulties in collecting sufficient numbers of

TABLE 5.

Depth distribution of *Y. natabilis* in the field and in the laboratory.

Depth (cm)	No. of Individuals — Shell Length (mm) 0–10	10–20	20–30*	30–40
Field				
0–2	4	1	0	1
2–4	1	2	0	1
4–6	0	0	0	4
6–8	0	0	0	0
Laboratory				
0–2	11	5	0	0
2–4	0	2	0	7
4–6	0	1	0	3
6–8	0	0	0	1
8–10	0	0	0	0

* Individuals with shell length between 20 and 30 mm did not occur during the study period (January 1990) because of the heterogeneous age structure of the population (Nakaoka 1993).

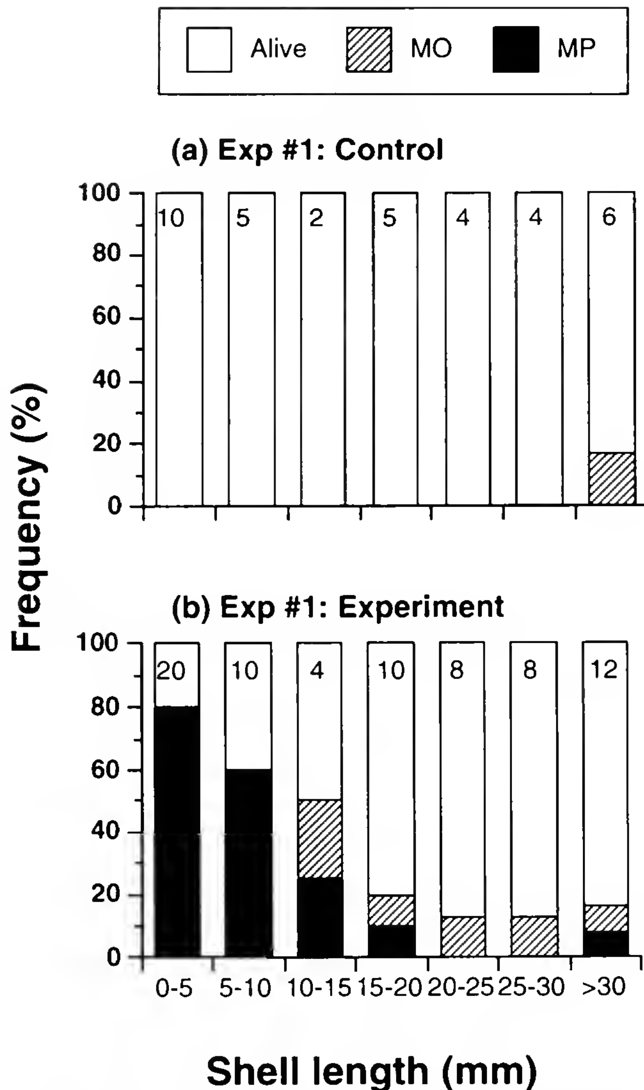

Figure 3. Proportion of live and dead individuals of *Y. notabilis*, maintained for 28 d in (a) aquaria without predators (Control) and (b) aquaria with the predator *P. granulata* (Experiment) in Experiment 1. Bivalves were classified into three categories; (1) alive, (2) mortality due to predation (MP; broken shell and unbroken empty shell), and (3) mortality due to other causes (MO; unbroken shell with soft parts remaining). The numeral in each column indicates sample size.

crabs and bivalves. Predation rates usually change with prey density, and their relationship (i.e., functional response) may vary from one environment to another, for example, with sediment type (Lipcius and Hines 1986, Eggleston et al. 1992). The prey densities used in this experiment were higher than field densities, and this may change predation rates considerably. Furthermore, the two stations compared in this study differed with respect to the size distribution of *Y. notabilis*, the composition and abundance of alternative prey, and the sediment composition (Nakaoka 1992b). These differences may shift the prey-preference of foraging activity of the crab, thus influencing feeding rates at the two stations.

Predators other than *P. granulata* may also contribute to the observed survivorship pattern of *Y. notabilis* in the field. Other potential epibenthic predators include another crab, *O. punctatus*, and the flounders *P. olivaceus* and *K. bicoloratus*, which were collected by dredging and trawling in the study area. However, the densities of these predators could not be estimated by the grab sampler because of their greater mobility compared with *P. granulata*. Infaunal predators include the naticid snail, *Neverita didyma*. This snail feeds on *Y. notabilis* by boring into shell, but its effect is probably minimal, given the low proportion of bored shells in the field (less than 5%; Nakaoka unpubl. data).

Size-related difference in vulnerability to physical stress or disturbance is another possible factor producing the size-dependent mortality pattern. Levinton and Bambach (1969) estimated the size/age-specific mortality of *Y. limatula* from size distribution of undamaged dead shells and found that individuals smaller than 11 mm suffered higher mortality than larger individuals in an area with unstable substrate, whereas the mortality was constant over the whole size range in another area with firmer sediments. They considered that the unstable and turbid medium is unfavorable for juveniles, resulting in a size-related change in mortality only in the former site. In Otsuchi Bay, the physical disturbance of bottom sediment is the most severe in the winter, when the northeastern monsoon predominates (Kutsuwada et al. 1988). This may also be related to higher mortality in younger shallow-burrowing individuals. Seasonal changes in survivorship, however, were not obvious because of the variation in monthly estimates of density.

In infaunal bivalves, burrowing depth in the sediment is often limited by size, and this is often thought to be responsible for size-dependent mortality (Hughes 1970, Blundon and Kennedy 1982, Goshima 1982). Although the burrowing depth of *Y. nota-*

TABLE 6.

Depth distribution of *Y. notabilis* at the end of Experiment 1.

Depth (cm)	No. of Individuals			
	Shell Length (mm)			
	0–10	10–20	20–30	30–40
Control				
0–2	13	2	0	0
2–4	2	5	3	3
4–6	0	0	4	2
6–8	0	0	1	0
Experiment				
0–2	8	4	2	0
2–4	0	6	8	5
4–6	0	0	4	5
6–8	0	0	0	0

TABLE 7.

The result of the three-factor log-linear model testing the dependence among treatment (with or without predator), shell length, and burrowing depth of live specimens of *Y. notabilis* at the end of Experiment 1.

Source	df	χ^2	*p*
Treatment	1	0.53	0.468
Size	3	6.25	0.100
Depth	2	2.98	0.225
Treatment*size	3	3.33	0.343
Treatment*depth	2	1.83	0.401
Size*depth	3	7.86	0.049
Treatment*size*depth	1	1.21	0.271

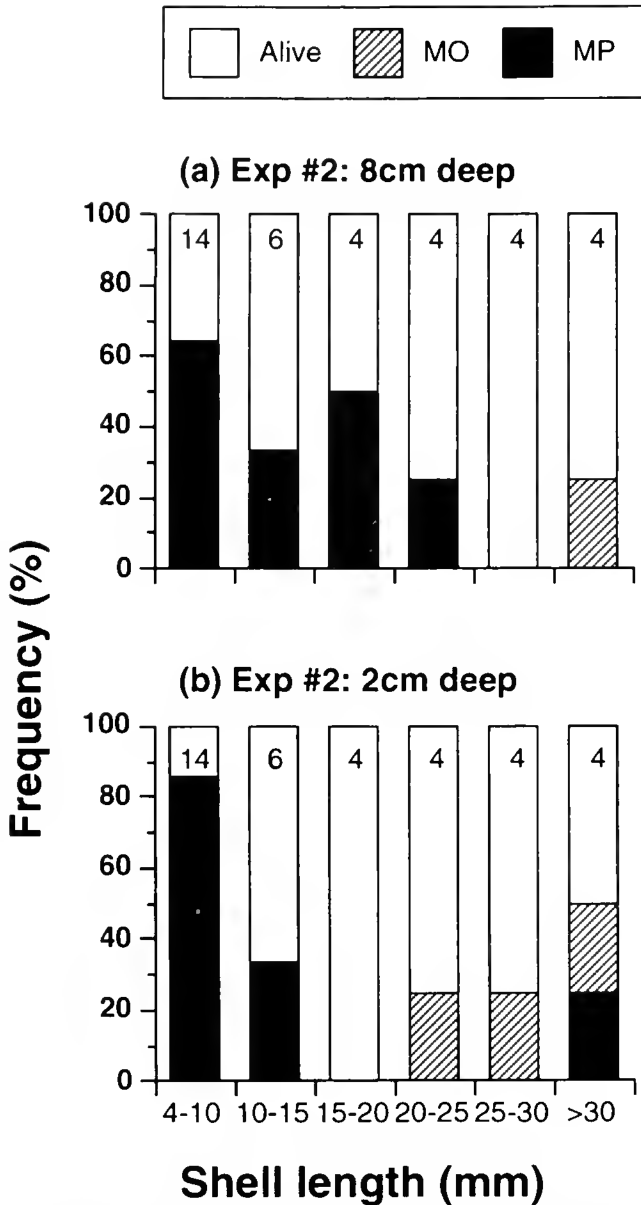

Figure 4. Proportion of live and dead individuals of *Y. notabilis*, maintained for 37 d in aquaria with the predator *P. granulata* and sediment of (a) 8 cm or (b) 2 cm deep in Experiment 2. See legend to Figure 3 for explanation.

bilis, an active burrower, was correlated with size (Tables 5 and 6), this relationship was weak when compared with immobile infaunal bivalves such as *M. arenaria* (Goshima 1982). Some large individuals of *Y. notabilis* were found in shallow layers of the sediment, even in the presence of crabs (Table 6). The results of Experiment 1 demonstrate that the size-burrowing depth relationship did not differ significantly between control and experimental treatments (Table 7), indicating that *Y. notabilis* did not change its burrowing depth in the presence of the predator and that crab predation was not selectively removing shallow burrowers. Furthermore, Experiment 2 demonstrates that the size-frequency distribution of *Y. notabilis* eaten by crabs did not differ when the burrowing depth of the bivalve was changed. These findings suggest that the size-limited predation is not a consequence of size-dependent burrowing depth in *Y. notabilis*. The result conflicts with that of similar experiments demonstrating that the predation rate increases when burrowing depth was limited to shallow sediments in other bivalves such as *M. arenaria* (Blundon and Kennedy 1982), *M. balthica* (Elmgren et al. 1986), and *Paphies ventricosa* (Haddon et al. 1987). In contrast, Peterson (1990) reported that the presence or absence of sediment did not affect the size preference of the crab *Callinectes sapidus* for the bivalve *M. mercenaria*. In a review of studies of decapod crustacean predation on molluscs, Juanes (1992) found that predatory decapods tend to prefer small-sized prey even when they are capable of feeding on larger prey. Preference for smaller prey is expected when the rates of energy intake decline with size, or when the mechanical or physiological costs of attacking larger prey are too high (Juanes and Hartwick 1990, Juanes 1992).

In conclusion, I have shown that the survivorship pattern of *Y. notabilis* is size dependent. In addition, laboratory experiments suggest that size-selective predation by the crab *P. granulata* may be one of the major factors responsible for the observed size-dependent survivorship pattern. In previous work, I have found that spatial variation in food availability controls the variation in the growth rate of *Y. notabilis* (Nakaoka 1992b). This study suggests that variation in growth rates, in turn, leads to variation in survivorship through size-dependent mortality, at least in part driven by crab predation.

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BROWN RING DISEASE AND PARASITES IN CLAMS (*RUDITAPES DECUSSATUS* AND *R. PHILIPPINARUM*) FROM SPAIN AND PORTUGAL

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ABSTRACT Signs of brown ring disease (BRD) have been detected in *Ruditapes decussatus* and *Ruditapes philippinarum* cultured in Galicia (NW of Spain) and Aveiro (W of Portugal). Clams found on the surface had higher BRD prevalences than did burrowing ones. BRD signs were also detected in *R. philippinarum* imported from Goro (Italy) into a depuration plant located in the Ría de Vigo. Histopathological studies revealed the presence of several potential pathogens, the most prevalent being (maximum prevalences given) a chlamydia-like organism (86%), a haplosporidian (100%), and a *Perkinsus*-like organism (60%). Bacterial growth was also found in some of the histologically examined samples. A positive relation between the parasitic load and BRD signs was found.

KEY WORDS: Brown ring disease, clams, *Ruditapes decussatus*, *Ruditapes philippinarum*, parasites, acquaculture

INTRODUCTION

Brown ring disease (BRD) was described as the cause of a mass mortality (more than 80%) of cultured clams (*Ruditapes philippinarum*) on the French Atlantic Coast by Paillard et al. (1989). The first sign of the disease consisted of the appearance of small brown spots surrounded by a pale brown halo that, in more advanced stages, developed into an abnormal organic brown-black deposit along the pallial line and at the inner edge of the shell. Although Paillard and Maes (1990, 1994) have demonstrated the transmissibility of the disease by injecting a bacteria, named *Vibrio* P1, isolated from diseased clams into healthy clams, there is still some controversy on the etiology of these signs. Some authors have reported that the abnormal deposit of conchiolin in the shell could be a result of the influence of several factors such as contaminants (Alzieu et al. 1981), nutritional deficiencies (Goulletquer et al. 1989), fungi (Alderman & Gareth Jones 1971), and parasites (Farley 1968, Farley et al. 1988). Juvenile oyster disease, a disease that shares with BRD the appearance of brown deposits on the interior of the shell, has recently been described in hatchery-reared juvenile oysters *Crassostrea virginica* (Bricelj et al. 1992) and bacteria were also considered a possible aetiological agent.

The production of clams in Galicia (NW Spain) is about 2,000 tons per year (2,300 tons in 1991; C.I.P.E.M. 1992) with a relatively high economic value (1,500 pesetas per kilogram, 1 US$ equals 100 Spanish pesetas). Since 1989–1990, repeated mortalities have been detected in several clam beds from this area, but their etiology has not yet been established. Previous clam (*Ruditapes decussatus*) mortalities in the area have been associated with the presence of *Perkinsus*- and haplosporidian-like organisms (Figueras et al. 1992). BRD has been reported in Spain on the coast of Cádiz (south of Spain) (Castro et al. 1990, Castro et al. 1992).

In this work, we assess the prevalence of BRD in clams taken from several natural beds from northwest Spain and west Portugal. The presence of other potential pathogens in the same sampling locations was established by histology, and the relation between the presence of parasites and BRD signs was also studied.

MATERIALS AND METHODS

Study Area

The epizootiological studies were conducted in natural clam beds in Santa Cristina, Moaña, and Punta Cabalo (Ría de Vigo) and in "storage" clam beds in Carril (Ría de Arousa) and Leis (Ría de Camariñas) in Spain and Gafanhas (Ría de Aveiro) in Portugal (Fig. 1). Samples of carpet-shell clams (*R. decussatus*) and manila clams (*R. philippinarum*) were taken between April and December 1993. According to the location of the clams in the sediment, two groups were distinguishable: (i) burrowing clams (individuals found burrowed in the sand) and (ii) surfacing clams (individuals found on the surface of the sand). A sample of clams, *Ruditapes pullastra*, from Moaña (Ría de Vigo, Spain) and another sample of *R. philippinarum* imported from Goro (Italy) for marketing in Spain were also examined.

Assessment of the Signs of BRD

In each sample location, 100 clams were taken for BRD studies. Clams were opened, cutting the adductor muscles with a scalpel, trying to avoid any damage to the shell. The inner side of the valves were examined at 25× with a binocular microscope (Nikon) for the appearance of BRD signs. The BRD developmental stage was assessed by the method of Paillard and Maes (1994). This takes into account the spread and thickness of the brown organic deposit in the interior of the shell and the number of affected valves, scoring from stage 1 (BRD signs visible only under a dissection microscope) to stage 7 (BRD signs visible to the naked eye with both valves affected).

Histopathological Studies

Thirty animals from each sampling location were used for histological studies. These animals were fixed whole, after shucking, in Davidson's fixative (Shaw and Battle 1957) for 24 h, and oblique transverse sections, approximately 5 mm thick, were taken from each specimen so that mantle, gonad, digestive gland, gills, kidney, and foot tissues were included. Tissue samples were embedded in paraffin and, 5-μm sections were stained with haematoxylin-eosin. The Macchiavello stain for rickettsia was also used (Culling 1974). Clam histological sections were examined for the presence of parasites with the aid of a microscope at 400–1,000× magnification (Nikon Optiphot).

Statistical Analysis

A *G*-test of independence with contingency tables (Sokal and Rohlf, 1981) was used to study the relationship of the presence of

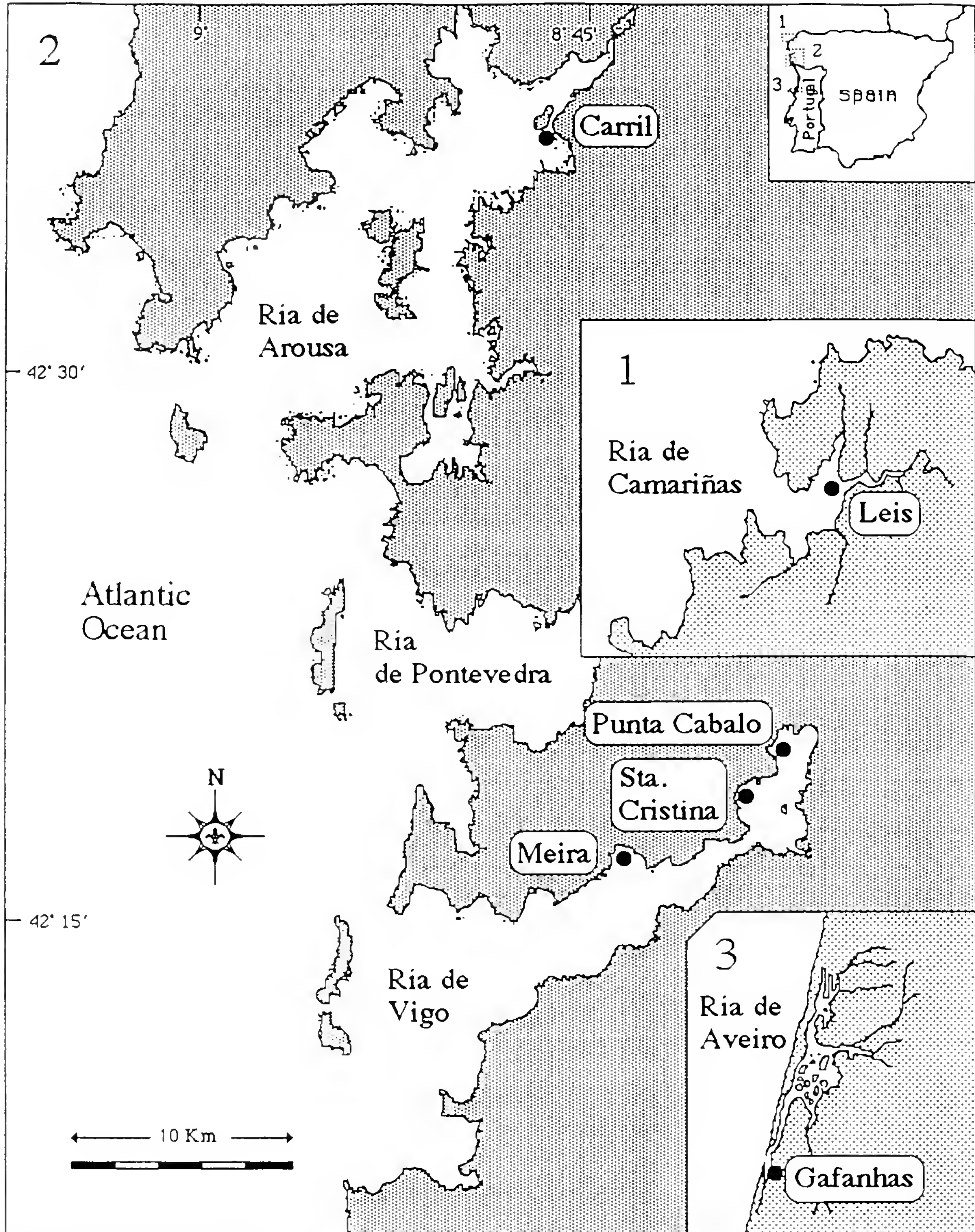

Figure 1. Sampling areas with the particular locations where the epidemiological studies were conducted. Map 1 shows Ría de Camariñas (Galicia, Spain). Map 2 shows Ría de Arousa and Ría de Vigo (Galicia, Spain). Map 3 shows Ría de Aveiro (Portugal).

the disease with the sampling site and with clam location (burrowing, surfacing) in the sediment. The statistical significance of the relation is expressed as follows: NS, significance ($p > 0.05$); significance, *$0.05 < p < 0.01$; **$0.01 < p < 0.005$; ***$p < 0.005$. To investigate the relation between the different pathogens and the presence of BRD signs, a Pearson correlation matrix with Bonferroni adjusted probability was calculated for each species (Systat).

RESULTS

BRD Signs

BRD was detected in *R. decussatus* and *R. philippinarum* cultured from all sampled sites in Galicia (Spain) and Gafanhas (Portugal). In severely affected clams, the signs consisted of an abnormally thick deposit of dark brown organic material along the pallial line and at the inner edge of the shell. The percentage of clams affected by BRD varied depending on the sampling site, clam species, and clam location (burrowing, surfacing) (Table 1).

The maximum prevalence of the disease was found in Carril, where almost all examined carpet-shell clams (*R. decussatus*) found on the sediment surface were affected. Manila clams (*R. philippinarum*) taken from the same area showed considerably lower values, and these differences among clam species were statistically significant (df = 1 in each case, n = 336, G = 45.55***).

In Camariñas, surfacing manila clams were the species most affected by the disease (84.21%). The presence of BRD was dependent on the clam species in the burrowing clams (df = 1 in each case, *R. decussatus* and *R. philippinarum*, n = 349, G = 3.97*) and independent in the surfacing ones (df = 1 in each case, *R. decussatus* and *R. philippinarum*, n = 173, G = 2.85 NS).

In Ría de Vigo, the prevalence of the disease signs was quite low in all of the sampled clam beds, with 13% being the maximum detected. It is important to point out that BRD signs were also detected in *R. pullastra*, although with very low prevalence (2.22%). In Gafanhas (Portugal), there was a time-related increase in BRD prevalence, mainly in the carpet-shell clams, from March (11%) to November (37%).

The most frequent BRD stages detected in all clam species, localities, and situation in the sediment were the second, third, and fourth. Only in the *R. decussatus* found on the surface of the sediment in Carril (prevalence, 93 and 77%) were detected all disease stages (1–7) detected.

Histopathological Studies

The prevalences of all of the potential pathogens detected are indicated in Table 2. No rickettsiae were observed, despite the use of the Macchiavelo staining method. Chlamydia-like organisms (CLO) consisted of small spherical inclusion bodies (mean diameter, 8.3 μm; SD = 1.32; n = 35) found in the cells of the digestive tubules and in the connective tissue. Host cells containing the CLO colonies appeared hypertrophied with a compression of the host cell nucleus against the basal membrane. Although the prevalence was high (up to 86%) and it was found in all sampled

TABLE 1.

Percentages of BRD in clams from Spain and Portugal.

Locality	Date	Clam Species	Type	No. of clams	Mean Length (mm) (±SD)	BRD (%)
Ría de Camariñas (Galicia, Spain)						
Leis	6/5/93	*R. decussatus****	S	111	23.0 (±15.7)	62.1
	6/5/93		B	106	25.0 (±4.9)	22.6
	6/5/93	*R. philippinarum****	S	19	44.1 (±5.7)	84.2
	6/5/93		B	99	35.4 (±8.1)	13.1
	16/12/93	*R. decussatus****	S	13	38.1 (±6.5)	69.2
	16/12/93		B	109	38.7 (±3.5)	17.4
	16/12/93	*R. philippinarum****	S	29	43.0 (±5.4)	68.9
	16/12/93		B	35	45.8 (±4.1)	8.5
Ría de Arousa (Galicia, Spain)						
Carril	22/4/93	*R. decussatus****	S	75	39.1 (±4.0)	93.3
	22/4/93		B	98	38.2 (±6.7)	67.3
	22/4/93	*R. philippinarum*	B	63	49 (±6.4)	19.1
	21/7/93	*R. decussatus****	S	63	35.5 (±5.4)	77.7
	21/7/93		B	99	37.5 (±3.6)	6.7
	21/7/93	*R. philippinarum*	B	106	40.7 (±3.6)	6.6
Ría de Vigo (Galicia, Spain)						
Sta. Cristina	23/4/93	*R. decussatus*	B	103	38.3 (±5.5)	0.9
Sta. Cristina	23/4/93	*R. philippinarum*	Italy	72	44.2 (±3.3)	26.1
Sta. Cristina	24/8/93	*R. decussatus*	B	105	43.0 (±3.4)	12.3
P. Cabalo	1/12/93	*R. decussatus*	B	99	41.7 (±2.4)	9.1
Moaña	1/12/93	*R. decussatus*	B	105	45.1 (±4.5)	3.8
Moaña	1/12/93	*V. pullastra*	B	45	39.8 (±2.6)	2.2
Ría de Aveiro (Portugal)						
Gafanhas	3/6/93	*R. decussatus* NS	S	41	37.2 (±2.5)	12.2
	3/6/93		B	90	38.0 (±2.5)	11.1
	3/6/93	*R. philippinarum*	B	90	26.4 (±2.2)	3.3
	28/11/93	*R. decussatus*	B	81	40.3 (±5.7)	37.0
	28/11/93	*R. philippinarum*	B	101	38.6 (±2.3)	4.9

S, surfacing; B, burrowing; SD, standard deviation; NS and ***, not significant and significant ($p < 0.005$), respectively, when the type of location (surfacing and burrowing) was compared.

TABLE 2.

Prevalence of pathogenic agents found in clams from Spain and Portugal.

Locality	Date	Clam Species	Type	Pathogens (%)					
				CLO	BC	HAP	PER	TRE	CIL
Ría de Camariñas (Galicia, Spain)									
Leis	6/5/93	*R. decussatus*	S	16.6	0	100	0	33.3	10.0
	6/5/93		B	20.0	0	96.6	0	40.0	3.3
	6/5/93	*R. philippinarum*	S				ND		
	6/5/93		B	30.0	0	0	0	3.3	0
	16/12/93	*R. decussatus*	S*	50.0	0	66.6	0	41.0	0
	16/12/93		B	23.3	26.6	83.3	0	56.6	0
	16/12/93	*R. philippinarum*	S	11.5	53.8	19.2	0	19.2	3.8
	16/12/93		B	36.6	30	6.6	0	10.0	0
Ría de Arousa (Galicia, Spain)									
Carril	22/4/93	*R. decussatus*	S	10.0	0	55.1	6.89	3.4	27.5
	22/4/93		B	86.2	0	60.0	60.0	40.0	40.0
	22/4/93	*R. philippinarum*	B				ND		
	21/7/93	*R. decussatus*	S	66.6	0	16.6	0	0	16.6
	21/7/93		B	40.0	0	0	6.6	6.6	3.3
	21/7/93	*R. philippinarum*	B	41.6	0	0	8.3	0	0
Rí de Vigo (Galicia, Spain)									
Sta. Cristina	23/4/93	*R. decussatus*	B				ND		
Sta. Cristina	23/4/93	*R. philippinarum*	Italy				ND		
Sta. Cristina	24/8/93	*R. decussatus*	B	33.3	0	0	40.0	3.3	6.6
P. Cabalo	1/12/93	*R. decussatus*	B	26.6	6.6	86.6	23.3	3.3	20.0
Moaña	1/12/93	*R. decussatus*	B	13.3	93.3	3.33	10.0	0	0
Moaña	1/12/93	*V. pullastra*	B	17.4	20.9	0	0	3.4	3.4
Ría de Aveiro (Portugal)									
Gafanhas	3/6/93	*R. decussatus*	S	30.0	0	20.0	0	0	6.6
	3/6/93		B	46.6	0	16.6	0	0	3.3
	3/6/93	*R. philippinarum*	B	10.0	0	0	0	0	3.3
	28/11/93	*R. decussatus*	B	71.4	0	0	0	0	3.5
	28/11/93	*R. philippinarum*	B	16.6	0	0	0	0	0

BC, bacterial colony; HAP, haploposporidian; PER, *Perkinsus*-like organism; TRE, tramatode; CIL, ciliate; ND, not done. See Table 1 footnote for other abbreviations.

* In the sample of *R. decussatus* from Leis (Ría de Camariñas) in December 1993, only 13 clams were analyzed for histological examination. In the rest of the samples, 30 individuals were processed.

locations, the intensity was low (≤5 CLO per section). No host reaction was detected.

Bacteria were found in both *R. decussatus* and *R. philippinarum*. This condition was often detected in the gills, connective tissue, and foot muscle. Bacteria were found in pockets scattered throughout tissues.

The haplosporidian (mean diameter, 10.75 μm, SD = 4.6; n = 30) was found intracellularly in the epithelium of the stomach and intestine, in the cells of the primary digestive tubules, and in the gills. The intensity was very variable. No spores were found. No host response was detected. In Camariñas, *R. decussatus* showed the highest haplosporidian prevalence, with a year-round value higher than 65%. The clams found on the surface had, in the month of May 1993, a prevalence of 100%.

Perkinsus-like organisms were found in the connective tissues of the digestive gland and foot and in the gills of *R. decussatus* and *R. philippinarum*, but the first species always had the higher prevalences. The mean diameter of the Perkinsus developmental stages detected varied between 3 and 14 μm. A strong host response consisting of a hemocyte infiltration surrounding the parasite cells was often found. This pathogen was found only in the Ría de Arosa and Ría de Vigo.

Trematodes were often found in the foot and in the connective tissue of all of the sampled species in all of the sampled locations, with the exception of the Ría de Aveiro (Portugal). These parasites often disrupted the foot muscle, occasionally eliciting a strong host response. Arrested gonadal development was observed in a few individuals.

Ciliates were detected at low prevalence in the gill epithelium, and again, no damage or host reaction was observed in association with these organisms. No species identification was attempted. Other potential pathogens detected, with much lower prevalences, were *Marteilia*-like organisms, turbellarians and a gregarine resembling *Nematopsis* sp.

In Carril, *R. decussatus* had the highest prevalence of all of the pathogens, whereas *R. philippinarum* showed a very low prevalence of all of the detected pathogens. In the Ría de Vigo, *Perkinsus*-like organisms and CLO were the pathogens with the highest prevalence in *R. decussatus*. *R. pullastra* was also parasitized, but with lower prevalences than *R. decussatus*. In the clams from Gafanhas (Portugal), the haplosporidian and CLO also reached high prevalences (20 and 70%, respectively).

The sampling locations and the clam species with higher BRD prevalence were also the ones with a higher prevalence of para-

sitism, suggesting a possible relation between both conditions. No statistically significant correlation was found between the presence of BRD signs in the shells of individual clams and the prevalence of the different pathogens found in the histological slides.

DISCUSSION

BRD is reported for the first time in Camariñas. Vigo (Galicia, NW of Spain), and Gafanhas (Portugal). BRD was not noted in previous studies on the health status of several clam populations in Galicia (Figueras et al. 1992). The BRD signs detected in *R. decussatus* and *R. philippinarum* sampled from several clam beds in Galicia (Spain) and in Gafanhas (Portugal) were identical to those previously described in France and in the south of Spain (Paillard et al. 1989, Castro et al. 1992).

The etiology of this disease in European clams has been proved by several authors who were able to reproduce the disease by injecting *Vibrio* P1 into healthy clams (Paillard et al. 1989, Paillard and Maes 1990). Although a synergism between the presence of this bacteria in high numbers and other factors such as poor water quality may exist, it is accepted that this *Vibrio* plays an important role in the development of the disease in the European clam species (Paillard and Maes 1994). Maes and Paillard (1992) studied the effect of *Vibrio* P1 in different species of bivalve molluscs and found that *Vibrio* P1 is more virulent for *R. philippinarum* than for *R. decussatus*: however, Oubella et al. (1993) showed that *Vibrio* P1 induced an increased density of circulating hemocytes in both clam species.

The highest prevalence of BRD was found in Camariñas and Carril. In these areas, clams are kept at a high population density for at least 1 month before shipment to the market. As a consequence of these high densities, the food availability is low. In the Ría de Vigo, where the prevalence of BRD was low compared with that in the other sampled sites, the population density was also low. Plana and Le Pennec (1991) demonstrated in laboratory experiments that the mortality attributable to *Vibrio* is decreased by 40% in fed animals, and they suggested that good nutrition will diminish the detrimental effect of potentially pathogenic bacteria.

The frequency of BRD signs was higher in surfacing clams than in burrowing ones in both species. As mentioned by Paillard and Maes (1990), the BRD-affected animals would rise to the surface of the sand before dying, explaining the higher BRD prevalence in surfacing clams.

The distribution of BRD stages in our samples suggests that the evolution of the disease seems to be quite fast in the first steps, with either a subsequent recovery or death of the affected animals thereafter. These results could be explained on the basis of two hypotheses. The first hypothesis stems from the fact that if BRD causes the death of the clam, the animals with advanced disease are likely to die, making it difficult to detect advanced BRD stages in a sample. The second hypothesis is based on the ability of the clam to repair the shell. Paillard and Maes (1994) established that after reaching stage 2 of the disease, clams could recover and only the weaker individuals would develop more advanced stages of the disease. As has been observed in France, the temporal differences on BRD prevalences found in this study in several sampled locations suggest a seasonal pattern of the disease (Paillard et al. 1994).

Although no correlation was found for individual clams, Camariñas, the area where the prevalence of the BRD was highest, also reached the highest prevalences of all detected pathogens, mainly the haplosporidian. The same is true for Carril, but in this case, the *Perkinsus*-like organisms also showed high prevalences. *Perkinsus* and haplosporidian species often cause mortalities in cultured molluscs. *Perkinsus marinus* and *Haplosporidium nelsoni* (MSX) are the major diseases of eastern oysters, *C. virginica*, from the Atlantic Coast of the United States (Andrews 1988, Haskin and Andrews 1988). *P. marinus* also occurs throughout the Gulf of México. In a previous study on the health status of clams from Galicia, Figueras et al. (1992) associated the presence of a *Perkinsus*-like organism with an abnormally high mortality in *R. decussatus* imported from Portugal to a depuration plant in Ría de Vigo. The energetic burden that these pathogens place on their hosts could be used to explain, at least partially, the presence of BRD, because of their weakened situation. Signs similar to those of BRD have been associated with the presence of trematodes in *Donax vittarus*, *Venerupis pullastra*, and *Ruditapes aureus* (Dollfus 1912, Johannessen 1973, Bartoli 1974), fungus (Alderman and Gareth Jones 1971), and mortalities of unknown etiology (Marin and Dauphin 1991). During this study, trematodes were detected in some clams showing BRD signs. As Goulletquer et al. (1989) pointed out, the calcification abnormalities that constitute the major signs of BRD could result from a "disturbance of protein metabolism, due to mechanical or chemical agents, at the level of amino acid biosynthesis or genetic transcription." The high prevalence of several potential pathogens detected could easily explain this disturbance in the metabolism. Moreover, the isolated strains of *Vibrio* P1 and closely related bacteria (Novoa et al. unpub. obs.) may act synergistically with the parasites (i.e., depleting the host energy reserves, disturbing several metabolic pathways) to produce the high prevalence of BRD detected in several sampled places. The experimental infections that we are conducting with several Galician *Vibrio* P1 strains and cultured *Perkinsus atlanticus* may clarify whether there is any increased susceptibility to BRD in the clams and the events (metabolic, defense mechanisms) that take place in the background when the two pathogens are simultaneously present.

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ISOLATION OF A NATIVE BACTERIAL STRAIN FROM THE SCALLOP *ARGOPECTEN PURPURATUS* WITH INHIBITORY EFFECTS AGAINST PATHOGENIC VIBRIOS

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ABSTRACT The mass culture of scallops *Argopecten purpuratus* (Lamarck, 1819) faces serious problems because of high larval mortalities. The main cause of mortality is the presence of pathogenic bacteria belonging to the genus *Vibrio*. In this study, the potential inhibitory activity against vibrios was examined for a native bacterial strain identified as *Alteromonas haloplanktis*. This strain clearly suppressed the growth of *Vibrio alginolyticus* and *Vibrio anguillarum*, two strains that cause severe mortalities in larval cultures of *A. purpuratus*. The active inhibitory components were found to be produced during the stationary phase of the culture, and they appear to be sensitive to heat. The inhibitory metabolites were precipitated by ammonium sulphate, and they possibly contained a proteinaceous compound. This is the first report of the isolation of *Alteromonas* species associated with bivalve culture producing antibiotic substances. This strain is potentially useful as a probiotic in the culture of *A. purpuratus*.

KEY WORDS: *Alteromonas haloplanktis, Argopecten purpuratus*, larvae culture, inhibition, probiotic

INTRODUCTION

In the last few years, efforts have been made to develop aquaculture technology for the farming of *Argopecten purpuratus* in Chile. At this time, there are several aquaculture centers dedicated to the farming of this species along the Chilean coast (Aiken 1993). However, the intensive farming of *A. purpuratus* in Chile has been hindered by high larval mortalities. These mortalities have been attributed to the presence of pathogenic bacteria (Navarro et al. 1991). Recently, the species of bacteria that cause damage to larvae have been identified as *Vibrio anguillarum* (VAR) and *Vibrio alginolyticus* (Pazos et al. 1993, Riquelme et al. 1995b).

Antibiotics have been used for the prevention of the establishment of bacterial pathogens and for the treatment of aquaculture species that harbor pathogenic bacteria. The use of antibiotics in aquaculture activities may, however, pose several environmental risks (McPhearson et al. 1991, Spanggard et al. 1993). Research aimed at developing alternative methods for the control of pathogenic bacteria, such as probiotics that are used in terrestrial animals (Conway 1989), is poorly developed for marine organisms. Very few reports on the use of probiotics in aquaculture have been published (Westerdahl et al. 1991, Austin et al. 1995, Bergh 1995). In addition, dried spray of *Tetraselmis suezica* has been used as a prophylactic measure (Austin and Day 1990). The development of such biological control measures is urgently needed because of problems associated with the increased use of antibiotics in the aquaculture industry (Hansen 1993). A suitable probiotic organism should derive from the autochthonous bacteria at the site of application.

Riquelme et al. (1994) demonstrated the permanent presence of bacteria in the reproductive organs of *A. purpuratus* and also reported evidence for the vertical transmission of the bacteria. Several bacteria associated with *A. purpuratus* broodstock have not shown pathogenic effects on larvae (Riquelme et al. 1995a), suggesting that some of these bacteria may be potential beneficial, or probiotic for larvae.

In our laboratory, the reproductive organs of several hundred *A. purpuratus* broodstock have been bacteriologically examined. We detected inhibitory zones produced by bacterial isolates from these molluscs against vibrios such as *V. alginolyticus*. In this study, we investigate the potential activity of one of these bacterial strains, found to be associated with *A. purpuratus* in culture, against pathogenic vibrios.

MATERIALS AND METHODS

Bacterial Isolation

Bacteriological analysis of gonads of *A. purpuratus* broodstock was described by Riquelme et al. (1995b). Briefly, the gonads were extracted and washed externally with 1% benzalkonium chloride. A small incision was made through the surface of the gonads with a heat-sterilized scalpel, and the contents were spread on tryptone soya agar (TSA) (Oxoid) supplemented with NaCl. In a preliminary screening of inhibitory activity against pathogenic vibrios of *A. purpuratus* larvae, performed following the experimental outline provided by Brock et al. (1987), one of six strains with a broad inhibitory spectrum was selected. Hereafter, the selected strain is referred to as INH.

Identification of INH Strain

The INH strain was identified according to methods presented by Sakata (1989) and Austin (1991), as well as by the use of a

TABLE 1.

Source, code, and sensitivity to *A. haloplanktis* of the different bacteria strains used.

Source/Strain	Code	Sensitivity*
Isolation from larval cultures		
V. alginolyticus	A32	S
V. alginolyticus	A84	S
V. anguillarum	VAR	S
A. hydrophila	C	S
Culture collection		
V. anguillarum	775	S
V. anguillarum	IFO 13266	S
V. alginolyticus	ATCC 17749	S
Vibrio parahaemolyticus	ATCC 17802	S
Vibrio damsella	ATCC 33539	S
Vibrio ordalii	ATCC 33504	S
Pseudomonas fluorescens	IFO 3903	S
Shewanella putrefaciens	IFO 3908	S
Micrococcus luteus	IFO 3333	S
Salmonella typhimurium	LT-2	S
E. coli	k-12	S
S. aureus	IFO 13276	S
Achromobacter sp.	IFO 13495	S
Morganella morganii	ATCC 25830	S
Proteus vulgaria	IFO 3851	S
A. haloplanktis	INH	R

* S, sensitive; R, resistant.

miniaturized multitest system API 20E (Analytab). Furthermore, the 16S ribosomal RNA (rRNA) sequence was used. Primers that anneal to the 16S rRNA genes of almost all bacteria were used. Bact27F (5'-AGAGTTTGATCATGGCTCAGA-3') and Bact530r (5'-GCCAGCAGCCGCGGTAATAC-3') were expected to amplify a 500-base-pair section (Lane et al. 1985). These oligodeoxynucleotide primers were synthesized with the DNA Synthesizer (Gene Assembler Plus; Pharmacia LKB Biotech, Sweden) and purified with the oligonucleotide Purification Cartridge (Applied Biosystem, Inc., Foster City, CA). Polymerase chain reaction (PCR) amplifications were carried out in buffer containing 2.0 mM $MgCl_2$, 200 mM each dNTPS, 1.0 mM primers, 25 μ/ml Taq DNA polymerase (Kurabo, Japan), and 500 ng/ml of chromosomal DNA. This mixture was subjected to 25 cycles of 1 min at 94°C, 2 min at 50°C, and 3 min at 72°C. Chromosomal DNA was prepared from cells cultured overnight. Cells were suspended in 200 μl of a lysis solution (10 mM Tris-HCl, 1 nM EDTA, 1% Triton X-100; pH 8), heated for 5 min at 100°C, and then transferred on ice. After a single chloroform extraction, 5 μl of supernatant was used in a PCR. Amplified DNA was isolated by agarose gel electrophoresis and purified with Geneclean II (Funakoshi Co., Tokyo, Japan). The amplified purified DNA was sequenced with the DNA Sequencer 373A (Applied Biosystems, Inc.) with the Taq DyeDeoxy™ Terminator Cycle Sequencing Kit (Applied Biosystems, Inc.). Computer analyses of the sequences were performed with DNASIS-Mac-0300 software (Hitachi Software Engineering Co., Yokohama, Japan).

Growth of INH Strain

In order to determine the optimal range of temperature for growth of the INH strain, the bacterium was inoculated in tubes of tryptone soya broth (TSB) medium (Oxoid) and incubated for 90 h in quadruplicates at 14, 20, 26, and 37°C. Growth was recorded by measuring optical density at 540 nm in a Spectronic 20D spectrophotometer (Milton Roy Company).

Screening of Inhibitory Activity

The bactericidal effect of the INH strain was examined by the double-layer method of Dopazo et al. (1988). TSA plates supplemented with 2% NaCl were spot inoculated with 10 μl of overnight cultures of INH strain. After incubation for 24 h at 20°C, the colonies were killed with chloroform vapor (45 min). Thereafter, 100 μl of a 10-fold dilution of an overnight culture of a tested bacteria in 6 ml of TSB supplemented with 2% NaCl and 0.9% agar was spread over the plates. The tested bacteria included culture collection strains and three strains pathogenic for *A. purpuratus*—*V. anguillarum* (VAR) (Riquelme et al. 1995a), *V. alginolyticus*, and *Aeromonas hydrophila* (Riquelme et al. 1996). Inhibition of growth of the tested bacteria (Table I) around and/or over the macrocolony with a zone of inhibition greater than 5 mm was considered a positive result.

INH Cell Treatments

Cells of INH harvested from plates of TSA medium were divided into three aliquots and subjected to the following treatments. One aliquot was placed in the bottom of a petri dish and exposed to chloroform vapors for 45 min. These were designated dead bacteria; the lack of viability was checked by inoculating the treated bacteria in fresh medium. The second aliquot of the harvested bacteria was subject to a temperature regime of 60°C for 30 min, and the third aliquot was sonicated for two periods of 10 min in an ultrasonic 50-W series 4710 sonicater (Cole Parmer Instrument Co.). Subsequent to these treatments, the cell suspensions of the INH cells were tested for their ability to inhibit the test bacteria by the method described above.

Ammonium Sulphate Fractionation

Sonicated cells of INH prepared as described above were subjected to fractionation with ammonium sulphate following the method described by Scopes (1987). The precipitated materials and supernatant were dialysed overnight in tubings of 12,000 MW. The dialysed materials were tested for bacterial inhibition by the method described above. The inhibition of vibrios in liquid

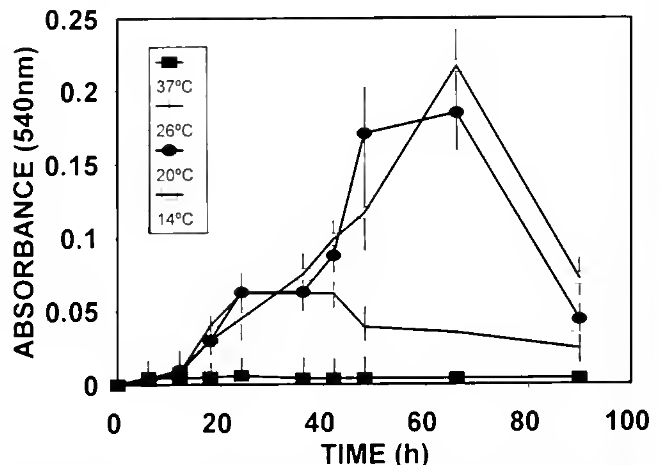

Figure 1. Growth of *A. haloplanktis* at different incubation temperatures. Vertical lines show standard deviation from four replicates.

TABLE 2.

Growth inhibition ability of different INH cell treatments against three pathogenic vibrios of marine organisms.

Strain/Treatments	Live Cells	Dead Cells	Cells T° 60°C	Sonicated Cells
V. ordalii (ATCC 33504)	+ + +	+	+	+ + +
V. anguillarum (ATCC 775)	+ + +	+	+	+ + +
V. anguillarum (VAR)	+ + +	+	+	+ + +

+ + +, halo larger than 10 mm; +, halo smaller than 10 mm; −, no inhibition.

medium (TSB) was also tested. A 50-ml aliquot of the dialysed product of INH was added to test tubes with 5 ml of medium. The inoculum of the test bacteria was 50 μl of the overnight culture (5 × 10^6 cells). Growth was recorded by measuring the optical density at 540 nm. The experiment was carried out in quadruplicate.

Effect of Supernatant of INH on the Growth of Pathogenic Vibrios

To ascertain the extracellular production of inhibitory products, the culture of INH in TSB medium at times of approximately 18, 36, and 66 h, early, middle, and stationary phases respectively, was centrifuged at 3,840× g for 10 min and the supernatant was recovered. The supernatant was filtered through 0.2-μm-pore-size filter membranes (Millipore), and the filtrate was added to TSB medium in 100, 50, and 20% v/v dilutions. After inoculation of the test vibrios in the medium, the growth was recorded as described above.

Effect of INH on Larval Survival

Experiments were carried out with healthy *A. purpuratus* larvae obtained from a commercial hatchery located in the north of Chile (23°, 25′S–78°38′W). Larvae were not subject to antibiotic treatments. The larvae were conditioned with a stationary-phase inoculum of INH strain (cell densities, ca. 5 × 10^6 cells/ml) for 1 and 24 h in a 1-l volume of sterile seawater filtered through 0.2-μm-pore-size membranes (Millipore), at a density of two to three organisms permilliliter in Duran Schott bottles. After this incubation time, the larvae were netted with a mesh (Nytal; previously treated by 30 min with ultraviolet light), washed several times with sterile seawater, and challenged with the pathogen *V. anguillarum* (VAR) from pure culture. This pathogen was isolated from an epizootic of *A. purpuratus* larval culture (Riquelme et al. 1995a). Unconditioned controls were subject to the same procedure as conditioned larvae but without the addition of the INH strain. The assays were performed in triplicate, according to the method described by Riquelme et al. (1996). Briefly, *A. purpuratus* larvae were added (two organisms per milliliter) to sterile seawater filtered through 0.2-μm-pore-size membranes (Millipore), contained in cell culture plates of 15-ml capacity (30 larvae per treatment). Overnight cultures of the pathogenic strain of *V. anguillarum* (VAR) in TSB were washed by centrifugation (3,840× g for 15 min) and suspended in marine saline solution. Approximately 10^3 and 10^6 cells/ml were added to separate plates. Unconditioned larvae were also distributed in culture plates as controls. The bioassays were carried out at 20°C for 24 h. Survival was quantified in an Olympus stereoscopic microscope. Larvae without apparent motion, showing closed valve and velar inactivity, were considered dead. The Tukey test was used to determine the statistically significant effects of treatments (Zar 1984).

RESULTS

The phenotypical identification of the INH strain revealed that it is Gram-negative and oxidase positive, did not metabolize glucose, and required NaCl for growth. This bacterium was tentatively identified as *Pseudomonas* spp. or *Alteromonas* spp. and by the technique of 16 sRNA was confirmed as *Alteromonas haloplanktis*. The results of the growth experiments of the strain INH showed that the strain grew well at 14 and 20°C (Fig. 1), whereas it exhibited poor growth at 26°C and did not grow at 37°C.

The screening of the inhibition of several bacteria species revealed that the INH strain inhibited the growth of all bacteria tested (Table 1). Autoinhibition was not found. Inhibitory assays with the cell suspensions of strain INH subsequent to different treatments showed that the sonicated pellet of INH maintained the inhibitory activity. The whole-cell pellet contained the inhibitory properties, but the temperature treatment (60°C for 30 min) reduced the inhibitory effect (Table 2).

The growth of *V. anguillarum* (VAR) and *V. alginolyticus* was delayed by the components in the first and second fractions of the ammonium sulphate—precipitated INH cells, although a different inhibition pattern of the two strains was found (Fig. 2). The TSB culture supernatant of INH stationary-phase cells delayed the

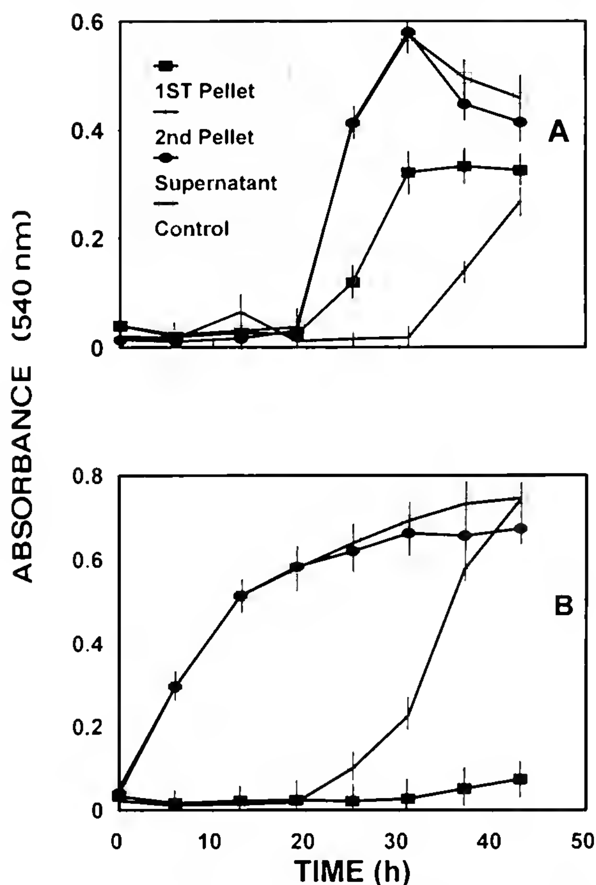

Figure 2. Growth of *V. anguillarum* (VAR) (A) and *V. alginolyticus* (B) in TSB medium with different fractions of ammonium sulphate treatments. Vertical lines show standard deviation from four replicates.

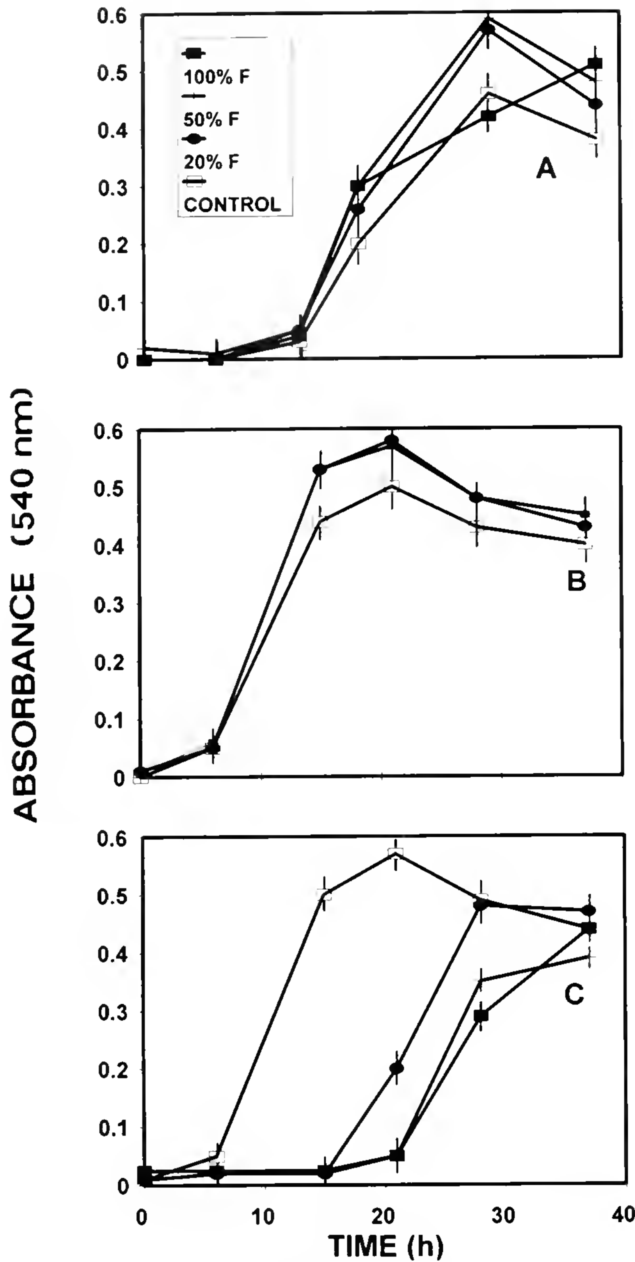

Figure 3. Growth of *V. anguillarum* (VAR) in the supernatant of the early (A), middle (B), and stationary (C) growth phases of *A. haloplanktis*. Vertical lines show standard deviation from four replicates.

growth of pathogenic vibrios. The supernatant of INH from early and middle log phase growth stages, however, did not negatively affect the growth of the vibrios (Figs. 3 and 4).

The larval survival experiments showed that the preconditioning of larvae with the INH strain for a short time (1 h) was effective for larval protection against pathogenic vibrios (Fig. 5) ($P < 0.05$). Preincubation for 24 h resulted in no significant differences from the control ($P > 0.05$). The best result was found with the 1-h bath and with the addition of the pathogen at 10^3 cells/ml. In that case, significant differences were observed between treatment and control groups exposed to the pathogens ($P < 0.05$), but not unexposed controls groups.

DISCUSSION

The search for and use of beneficial microorganisms to improve the production of terrestrial animals are common procedures 11(Stavric et al. 1992, Conway 1989). However, in aquaculture, there are very few studies that attempt to focus on bacteria that prevent the growth of pathogenic organisms (Westerdahl et al. 1991, Olsson et al. 1992, Nogami and Maeda 1992, Bergh 1995, Austin et al. 1995). In recent years, increased interest has focused on the search for suitable probiotics (Douillet and Langdon 1994, Austin et al. 1995). A suitable probiotic organism should derive from the autochthonous bacteria at the site of application. This condition is fulfilled for the INH strain, in that it was isolated from gonads of *A. purpuratus*.

The INH strain is tentatively identified as *A. haloplanktis*. It is a psychrophilic and autochthonous marine strain, on the basis of its temperature dependence for growth and the strict requirement of NaCl for growth. It has been reported that *Alteromonas* species produce antimicrobial substances from marine samples (Barja et al. 1989, Gauthier and Flateau 1976, McCarthy et al. 1985). How-

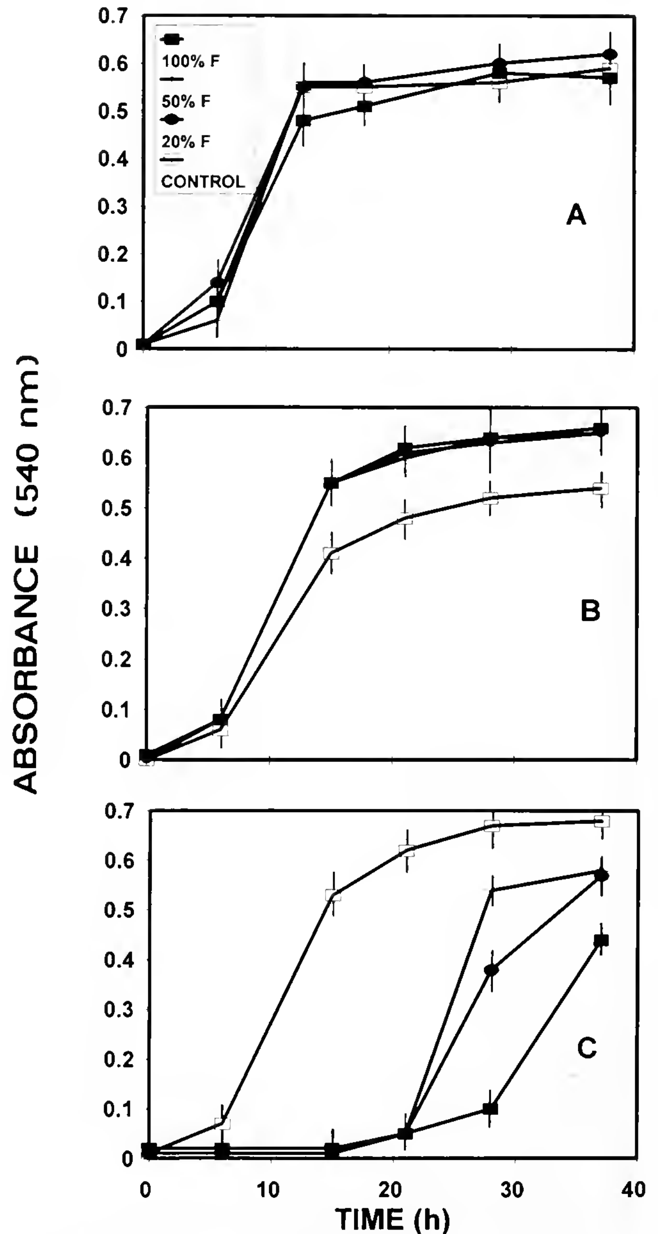

Figure 4. Growth of *V. alginolyticus* in the supernatant of the early (A), middle (B), and stationary (C) growth phases of *A. haloplanktis*. Vertical lines show standard deviation from four replicates.

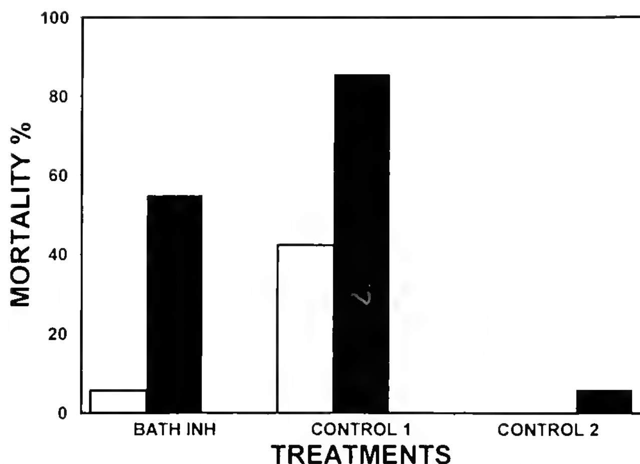

Figure 5. Survival of *A. purpuratus* larvae challenged with pathogenic *V. anguillarum* (VAR) in concentrations of 10^3 (□) and 10^6 cells/ml (■), after the preconditioning of larvae during 1 h with *A. haloplanktis* (INH). The controls are (1) unconditioned larvae challenged with the pathogen and (2) unconditioned larvae without the pathogen.

ever, there is no information about the presence of *Alteromonas* species associated with bivalve molluscs producing antibiotic substances against pathogens of marine organisms. The genus *Alteromonas* also has been reported to produce other bioactive substances such as metamorphosis inducers (Weiner et al. 1988, Leitz and Wagner 1993).

The screening of the inhibition of several bacterial species revealed that *A. haloplanktis* has a broad inhibitory spectrum, inhibiting Gram-positive as well as Gram-negative bacteria. Autoinhibition in *A. haloplanktis* was not found, although this phenomenon has been reported in other *Alteromonas* species producing antibiotic substances (Gauthier and Flateau 1976, McCarthy et al. 1985). The inhibition of larval scallop pathogenes *V. anguillarum* (VAR), *V. alginolyticus* (strains A32 and A84), and *A. hydrophila* (strain C) by *A. haloplanktis* is remarkable because these pathogenic strains are resistant to several antibiotics (Riquelme et al. 1995b, Riquelme et al. 1996). The clinical strains *Escherichia coli* K12 and *Staphylococcus aureus* (IFO 13276) were also found to be sensitive to exposure to *A. haloplanktis*. The active inhibitory compound(s) is produced or excreted by living cells and appear to be contained intracellularly. Furthermore, the inhibitory substance(s) appears to be proteinaceous in nature, because the activity was lost subsequent to temperature treatment and the active compound(s) was precipitated with ammonium sulphate. While examining the inhibition pattern of sulphate ammonium fractions, it was observed that probably more than one active inhibitory component exist. The bactericidal components produced by *A. haloplanktis* are secondary metabolites excreted only in the stationary phase (Figs. 3 and 4). The delayed growth of *V. anguillarum* (VAR) and *V. alginolyticus* in approximately 20 h after the addition of *A. haloplanktis* supernatant was similar to that observed by Olsson et al. (1992). Those authors reported that supernatant from fish mucus bacterial isolates produced a lag period up to 8 h longer than that of the control. Austin et al. (1995) also reported that *V. anguillarum* was less sensitive to the supernatant of a probiotic strain of *V. alginolyticus*, as compared with another pathogen (*Vibrio ordalii*) that had a rapid decline in the numbers of culturable cells after exposure to the probiotic supernatant.

In the experiment of larvae exposure to pathogenic bacteria, it was found that 1 h of preincubation with *A. haloplanktis* was more protective than 24 h of preincubation. The results indicate that *A. haloplanktis* was able to protect the larvae from the infection with *V. anguillarum* (VAR) in a concentration of 10^3 cells/ml. In a high concentration of 10^6 cells/ml, the protection was reduced. In fish, a short 10-min bath with a probiotic bacteria reduced mortality by *V. anguillarum* from 90 (control) to 74% (Austin et al. 1995). Considering that in the hatchery the pathogens at the beginning of the epizootic are not found in a high concentration, such as 10^6 cells/ml, the periodic bath (e.g., every day) with *A. haloplanktis* could be a prophylactic measure. The use of *A. haloplanktis*, or equivalent bacteria, as a means of larval protection against pathogenic bacteria could be promising. However, further research is needed to establish the appropriate conditions, such as cell concentrations and incubation times, among others, for practical use as a potential probiotic in scallop aquaculture.

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A COMPARISON OF *CRASSOSTREA GIGAS* AND *CRASSOSTREA VIRGINICA*: EFFECTS OF TEMPERATURE AND SALINITY ON SUSCEPTIBILITY TO THE PROTOZOAN PARASITE, *PERKINSUS MARINUS*

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ABSTRACT The susceptibility of diploid and triploid (2N and 3N) *Crassostrea gigas* to *Perkinsus marinus* was compared, in the laboratory, with that of *Crassostrea virginica* at three test temperatures (10, 15, and 25°C) at 20–22 ppt and at three test salinities (3, 10, and 20 ppt) at a temperature of 19–22°C. Experimental oysters were challenged twice with freshly isolated *P. marinus* meronts, after acclimation to test temperatures and salinities. Although infection prevalence and intensity increased with temperature ($p = 0.0001$) and salinity in *P. marinus*–challenged oysters of both oyster species, they were highest in *C. virginica* groups. Infection intensity was significantly ($p = 0.001$) higher in *P. marinus*–challenged *C. virginica* than *C. gigas* (2N and 3N) at all temperatures; however, infection prevalence was not statistically different at any temperature treatment. In all salinity treatments, prevalence and infection intensity were significantly higher ($p = 0.0001$) in *P. marinus*–challenged *C. virginica* than 2N and 3N *C. gigas*. Because high infection prevalence and intensity were found in non-challenged *C. virginica*, part of the recorded prevalence and intensity in challenged *C. virginica* was probably attributed to latent infection carried over from the field. High mortality occurred in both 2N and 3N *C. gigas* during temperature and salinity adjustment, particularly at 25°C and 3 psu.

KEY WORDS: Pacific oyster, eastern oyster, *Crassostrea gigas*, *Crassostrea virginica*, oyster disease, *Perkinsus marinus*, temperature, salinity

INTRODUCTION

The eastern oyster, *Crassostrea virginica*, has historically supported a major fishery on the East Coast of the United States. Beginning in the late 1950s, severe mortality in oyster populations has been caused by the two endoparasitic pathogens, *Perkinsus marinus* (Dermo) and *Haplosporidium nelsoni* (MSX) in the mid-Atlantic region. The introduction of a non-native species, the Pacific oyster (*Crassostrea gigas*) to the waters of this region has been proposed to revitalize the oyster fishery (Mann et al. 1991). The Pacific oyster has been successfully introduced and cultured along the West Coast of the United States and in Europe. This oyster species is rarely infected by the protozoan parasite, *Bonamia ostreae*, which has caused severe losses of the European oyster (*Ostrea edulis*) industry in Europe and on the West Coast of the United States over the last decade (Grizel 1985, Elston et al. 1987, Grizel et al. 1988). Results from recent laboratory studies also indicate that the Pacific oyster is less susceptible than the eastern oyster to *P. marinus* (Meyers et al. 1991, Barber and Mann 1994).

Pacific oysters usually propagate in habitats of salinities >18 ppt and temperatures ≤15°C, although they can tolerate temperature as high as 35°C and salinity as low as 10 ppt (Mann et al. 1991). Information regarding temperature-salinity tolerance in *C. gigas* is, however, limited, and the definitive temperature and salinity tolerances of this species have not been established in the laboratory. Therefore, the competence of the Pacific oyster against *P. marinus* under different salinity and temperature regimes is of particular concern, before its introduction into the mid-Atlantic region. This study evaluates in the laboratory the competence of triploid and diploid Pacific oysters and eastern oysters against *P. marinus* under different temperature and salinity conditions.

MATERIALS AND METHODS

Experiment 1: Temperature Effect

Eastern oysters, *C. virginia* (shell length [SH], 7–8 cm), were collected on January 8, 1992, from Ross Rock in the Rappanhannock River, a tributary of the lower Chesapeake Bay. Oysters from this area typically have a low prevalence of *P. marinus* infection (Burreson 1992, Ragone Calvo and Burreson 1994, Ragone Calvo and Burreson 1995). The ambient temperature and salinity at the time of collection were 8°C and 10 ppt. Triploid (3N, assayed to be 95%) and diploid (2N) Pacific oysters (age, 16 mo; SH, 6–7 cm) were progenies from a spawning conducted by Dr. Standish Allen (Haskin Shellfish Laboratory, Rutger's University) in late July of 1990. The spawning was produced from second-generation parents of 1989 broodstocks from Washington, and juveniles were raised at the Virginia Institute of Marine Science, in quarantined flumes with flowing raw York River water (YRW ambient temperature, 8°C and salinity, = 20 ppt, at the time of experiment). Before the start of the experiment, initial assessment was performed on a subsample of 20 *C. gigas* and 25 *C. virginica* for *P. marinus* infection by use of the tissue thioglycollate assay (Ray 1952, Ray 1966) described below. All groups tested negative. The remaining *C. virginica* and *C. gigas* were held separately in aerated 55-gallon tanks and gradually adjusted to the three test temperatures (10, 15, and 25°C, 2°C per 2 d) at salinities of 20–22 ppt (1 μm filtered YRW). Before temperature adjustment, *C. virginica* was first acclimated (3 ppt per 2 d) from ambient salinity (i.e., 10 ppt) to the experimental salinity (i.e., 20–22 ppt). After adjustment to the desired test temperatures and YRW salinity, oysters were maintained in aerated 1 μm filtered YRW in 40-l

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aquaria (20–22 oysters per aquarium). Oysters were fed with algal paste (a mixture of Tahitian *Isochrysis galbana* and *Thalassiosira pseudonana*, 0.1 g/oyster) daily, and mortality was recorded throughout the course of the experiment. If oysters died at the beginning of temperature adjustment, they were replaced. Thus, the number of oysters among groups was similar (N = 37–41) when *P. marinus* challenge was initiated. All experimental oysters were challenged twice with freshly isolated *P. marinus* meronts. Twenty-nine days after the initiation of temperature acclimation, oysters were inoculated with 0.1 ml of meront/merozoite suspension (2.5 × 10^5 meronts/oyster) into the shell cavity. Control oysters were inoculated with filtered YRW (0.22-μm-pore-size filter). Forty-one days after the first challenge, challenged oysters were inoculated with a second dose of meronts (7.0 × 10^3 meronts per oyster). Sixty-eight days after the first challenge (27 d after the second challenge), 10 control and 10 challenged oysters from each temperature treatment were sacrificed and rectal tissues were removed to determine infection by use of the tissue thioglycollate assay (Ray 1952, Ray 1966). Eighty-four days after the first challenge, the remaining oysters were sacrificed and the same parameters mentioned above were measured. Data from the two samplings were pooled to determine the disease prevalence and intensity.

Experiment 2: Salinity Effect

The experimental protocol of this experiment was similar to that of the temperature effect experiment. *C. virginica* (7–8 cm) were collected on May 11, 1992, from Ross Rock, Rappahannock River (Ambient temperature, 19°C; salinity, 6 ppt). *C. gigas* (3N and 2N, 6–8 cm) was from the same stock used for the temperature effect experiment. Initial assessment of *P. marinus* infection on 20 *C. gigas* and 25 *C. virginica* showed that, with the exception of a single *P. marinus cell* detected in one of the diploid *C. gigas*, no oysters were infected with *P. marinus*. The ambient temperature and salinity of YRW at the time of the experiment were 19–22°C and 20 ppt respectively. Both *C. virginica* and *C. gigas* were placed in aerated 200-l tanks, and salinities were gradually adjusted (3 ppt per 2 d) to salinities of 3, 10, and 20 ppt, at 19–22°C. After salinity adjustment was completed, oysters were maintained in aerated 40-l aquaria. During the salinity adjustment period, heavy mortality occurred in both diploid and triploid *C. gigas* at 3 ppt. Consequently, the susceptibility of *C. gigas* and *C. virginica* to *P. marinus* was compared only at 10 and 20 ppt. As in experiment 1, test oysters were challenged twice by freshly isolated meronts/merozoites (2.0 × 10^5 cells/oyster, 21 d after the initiation of salinity adjustment and 5.0 × 10^3 cells per oyster 12 d after the first challenge). Again, control oysters were inoculated with filtered YRW. Fifty days after the initial *P. marinus* challenge, the experiment was terminated to determine disease prevalence and intensity.

Preparation of Meront/Merozoite Suspension

Fresh meront/merozoite suspension was prepared according to La Peyre and Chu (1994). Briefly, *P. marinus*–infected oyster tissues were rinsed thoroughly with filtered (0.22 μm) YRW and subsequently homogenized in (0.22 μm) filtered YRW with a blender (Virtis, Model 23) at high speed for 2 min. The suspension was then passed through a series of screens (100, 35, 20, and 15 μm) to remove oyster tissue residues. The number of merozoites in suspension was counted with a hemacytometer and adjusted to the desired concentration.

P. marinus *Assay*

The tissue thioglycollate assay (Ray 1952, Ray 1966) was used for *P. marinus* diagnosis. Rectal tissue was removed from each oyster and incubated in fluid thioglycollate medium for 4–5 d. The intensity of infection was ranked as 0 (negative), 1 (light), 3 (moderate), and 5 (heavy), on the basis of the number of stained *P. marinus* hypnospores contained in the oyster rectal tissue smear.

Statistical Analysis

Logistic regression and log-linear modelling (Agresti 1990) were used to determine differences in infection prevalence between temperature and salinity treatments and between oyster species. Two-factor analysis of variance was used to determine differences in infection intensity between the three groups (i.e., *C. virginica*, *C. gigas* 2N and 3N) of oysters at different temperature or salinity treatments.

RESULTS

Experiment 1

Mortality

Throughout the course of the experiment, a total of 18 *C. virginica*, 38 diploid (2N) *C. gigas*, and 39 triploid (3N) *C. gigas* died. Most of the deaths occurred at 25°C during temperature adjustment (32 triploid *C. gigas*, 7 diploid *C. gigas*, and 4 *C. virginica*) (Table 1). High mortality was also noted at 25°C after oysters were challenged with freshly isolated *P. marinus*, with the

TABLE 1.

Mortality of *C. virginica* and *C. gigas* During Temperature Acclimation and After Challenge with *P. marinus* (Dermo).

	C. virginica			*C. gigas* (2N)			*C. gigas* (3N)		
Mortality	10°C (N = 80)	15°C (N = 80)	25°C (N = 80)	10°C (N = 79)	15°C (N = 81)	25°C (N = 85)	10°C (N = 82)	15°C (N = 82)	25°C (N = 111)
Mortality (no. of deaths) during acclimation	0	0	4	0	2	7	1	1	32
Mortality (no. of deaths) after *P. marinus* exposure	1	3	10	1	7	21	1	3	1
Total mortality (%) during experiment*	1.3	3.8	17.5	1.3	11.1	32.9	2.4	4.9	29.7

* % = no. of dead oysters/initial total number of oysters.

exception of triploid *C. gigas* (heavy mortality occurred only at the time of temperature adjustment). Although 21 diploid *C. gigas* and 10 *C. virginica* died, only one triploid *C. gigas* died at that temperature. Unfortunately, no tissue was able to be recovered from some of these mortalities for *P. marinus* diagnosis. Hence, mortalities with no meat recovered were excluded from prevalence and intensity calculations. However, for those mortalities that had tissues, it was found that one *P. marinus*–challenged *C. virginica* (N = 9) at 25 °C, one control diploid *C. gigas* (N = 4) at 15°C, and one control (N = 8) and three challenged diploid *C. gigas* (N = 7) at 25°C were infected. None of the triploid *C. gigas* (N = 2) that were examined had infections.

Prevalence and Intensity of *P. marinus* Infection

Infection prevalence (percentage of infected oysters = number of infected oysters/total number of oysters at the time of inoculation) significantly increased ($p = 0.0001$) with temperature in all *P. marinus*–challenged oysters (Fig. 1). Prevalence was higher in *C. virginica* than in the two *C. gigas* groups, with the exception of the 10°C treatment. At 10°C, 3N *C. gigas* had a higher prevalence (30%) than both 2N *C. gigas* (24%) and *C. virginica* (25%). The infection prevalences at 15 and 25°C, respectively, were 50 and 60% for *C. virginica*, 36 and 51% for 2N *C. gigas*, and 37 and 56% for 3N *C. gigas*. However, these differences were not statistically different ($p > 0.05$). Infection intensity increased significantly with increase in temperature and was significantly higher ($p = 0.001$) in *C. virginica* than *C. gigas* (2N and 3N) (Fig 2A). At 25°C, 10 (27%) of the infected *C. virginica* had moderate infections and 5 (14%) had heavy infections. There were four (11%) infected 2N *C. gigas* at 25°C and one (3%) at 10°C with moderate infections. None of the infected 3N *C. gigas* developed advanced (i.e., moderate or heavy) infections. Infection intensity expressed as weighted prevalence (= sum of disease code numbers/number of oysters) also significantly increased with increasing temperature ($p = 0.0001$). *C. virginica* had significantly

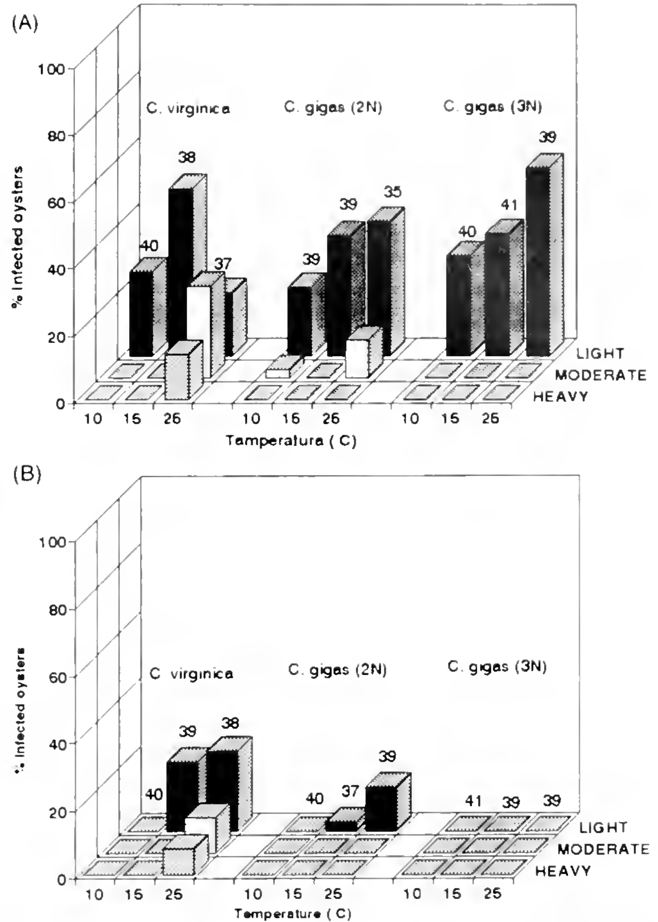

Figure 2. Intensity of *P. marinus* infection in *P. marinus*–challenged (A) and control (B) *C. virginica* and *C. gigas* (2N and 3N) oysters at 10, 15, and 25°C. Numbers above the bars represent total number of oysters in each treatment.

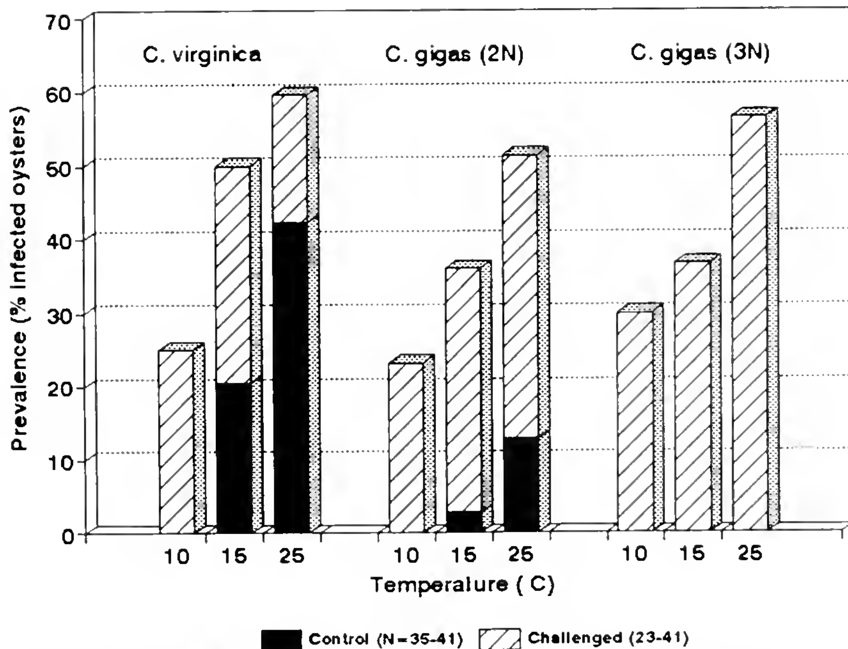

Figure 1. Prevalence of *P. marinus* infection (% infected oysters) in control and *P. marinus*–challenged *C. virginica* and *C. gigas* (2N and 3N) oysters at 10, 15, and 25°C.

higher weighted prevalence (p = 0.0004) than *C. gigas* (2N) and *C. gigas* (3N). Mean weighted prevalences were 0.79, 0.45, and 0.41 in *C. virginica*, *C. gigas* (2N), and *C. gigas* (3N), respectively. Weighted prevalence in 2N and 3N *C. gigas* was not statistically different.

Some of the oysters in the control groups of *C. virginica* and 2N *C. gigas* were infected with *P. marinus* (Fig. 1). Among 2N *C. gigas*, one oyster (3%) at 15°C and five oysters (13%) at 25°C had light infections. Among *C. virginica*, nine (24%), four (11%), and three (8%) oysters had light, moderate, and heavy infections, respectively (Fig. 2B). None of the control 3N *C. gigas* oysters were infected.

Experiment 2

Mortality

During salinity adjustment, high mortality occurred in both diploid (2N) and triploid (3N) *C. gigas*, especially when salinity was adjusted down to 3 ppt (44 of 80 diploid died, 37 of 80 triploid died), but no mortality was noted in the *C. virginica* groups (Table 2). As a result, the *C. gigas* (2N and 3N) at 3 ppt treatments were terminated. When the dead oysters were examined for *P. marinus* infection, one 2N and one 3N *C. gigas* had light infections. After *P. marinus* challenge, mortality in Pacific oysters was consistently high. In total, 2 challenged and 5 control 2N *C. gigas* at 20 ppt, 9 challenged and 11 control 2N *C. gigas* at 10 ppt, 9 challenged and 8 control 3N *C. gigas* at 10 ppt, and 14 control and 3 challenged 3N *C. gigas* oysters at 20 ppt perished. However, only two control and two challenged eastern oysters died after *P. marinus* challenge. None of these dead oysters were found to be infected by *P. marinus*.

Prevalence and Intensity of *P. marinus* Infection

In all salinity treatments, *C. virginica* had the highest prevalence of *P. marinus* infection (p = 0.001) (Fig. 3). In the *P. marinus*–challenged oysters, the prevalence in 2N *C. gigas*, 3N *C. gigas*, and *C. virginica*, respectively, were 25, 35, and 65% at 10 ppt and 25, 31, and 64% at 20 ppt (Fig. 3). Among the control oysters, no Pacific oysters at 10 ppt were infected, but at the same salinity, 7% of the *C. virginica* were infected. At 20 ppt, 5% of the 2N *C. gigas* controls and 13% of the *C. virginica* controls were infected, whereas none of the 3N *C. gigas* were infected. Prevalence was low in *C. virginica* at 3 ppt, 7 and 3%, in challenged and control groups, respectively. All infected oysters in all groups had only light infections, with the exception of one eastern oyster at 20 ppt, which was moderately infected (Fig. 4). Similar to the results

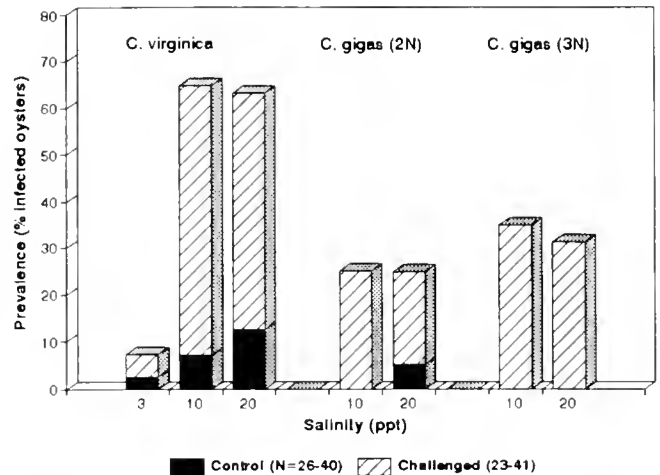

Figure 3. Prevalence of *P. marinus* infection (% infected oysters) in control and *P. marinus*–challenged *C. virginica* and *C. gigas* (2N and 3N) oysters at 3, 10, and 20 psu.

in the temperature experiment, *C. virginica* had significantly higher weighted prevalence than *C. gigas* (2N and 3N) (p = 0.0001). Mean weighted prevalences for *C. virginica*, *C. gigas* (2N), and *C. gigas* (3N) were 0.64, 0.25, and 0.33, respectively. Salinity (10 and 20 ppt) did not significantly affect (p > 0.05) the weighted prevalence. In both oyster species, no differences were observed in pooled infection intensity between salinities (10 and 20 ppt).

DISCUSSION

The results of this study revealed that *C. gigas*, both diploid and triploid, is less susceptible to *P. marinus* than is *C. virginica*. This is consistent with previous findings in experiments comparing *P. marinus* susceptibility, mortality, and growth rates between *C. virginica* and *C. gigas* challenged with the parasite (Meyers et al. 1991, Barber and Mann 1994). At all tested temperature-salinity regimes, *P. marinus*–challenged *C. virginica* suffered higher infection rates than *C. gigas*. Although 27 and 14% of *P. marinus*–challenged *C. virginica* advanced, respectively, to moderate and heavy infections, only 3–11% of moderate infections were detected in *P. marinus*–challenged diploid *C. gigas*. However, because much higher infection rates were found in the control, non-*P. marinus*–challenged *C. virginica*, at any given temperature and salinity treatment, than in non-*P. marinus*–challenged diploid and triploid *C. gigas*, the authors believe that part of the recorded

TABLE 2.

Mortality of *C. virginica* and *C. gigas* During Salinity Acclimation and After Challenge with *P. marinus* (Dermo).

Mortality	*C. virginica* 3 psu (N = 79)	*C. virginica* 10 psu (N = 84)	*C. virginica* 20 psu (N = 81)	*C. gigas* (2N) 3 psu (N = 77)	*C. gigas* (2N) 10 psu (N = 86)	*C. gigas* (2N) 20 psu (N = 95)	*C. gigas* (3N) 3 psu (N = 52)	*C. gigas* (3N) 10 psu (N = 78)	*C. gigas* (3N) 20 psu (N = 81)
Mortality (no. of deaths) during acclimation	0	0	0	44	6	9	37	12	10
Mortality (no. of deaths) after *P. marinus* exposure	4	0	0	—	20	7	—	17	17
Total mortality (%) during experiment*	5	0	0	—	30.2	16.8	—	37.1	33.3

* % = No. of dead oysters/initial total number of oysters, — = treatments were terminated before *P. marinus* exposure due to heavy mortalities.

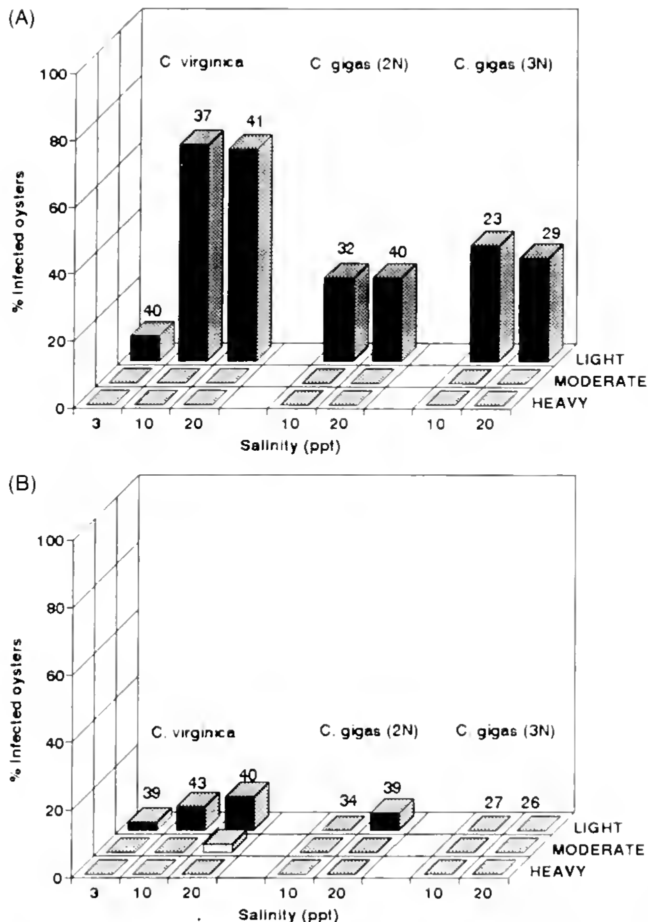

Figure 4. Intensity of *P. marinus* infection in *P. marinus*–challenged (A) control (B) *C. virginica* and *C. gigas* (2N and 3N) oysters at 3, 10, and 20 psu. Numbers above the bars represent total number of oysters in each treatment.

infection in *P. marinus*–challenged *C. virginica* was attributed to the expression of hidden infection carried over from the field. Unfortunately, the thioglycollate tissue assay used in this study for *P. marinus* diagnosis was not sensitive enough to detect cryptic infections, thus restricting the interpretation of the experimental results. However, the nonchallenged *C. virginica* showed substantially lower *P. marinus* infection prevalence and intensity than did *P. marinus*–challenged *C. virginica*. The observed increased disease prevalence and intensity in the challenged *C. virginica* must have been derived from the laboratory challenge. Future studies should use eastern oysters from an area free of *P. marinus* for this kind of study. Also, in oysters collected in winter months, overwintering infections will not develop to detectable levels until 1–2 mo post-exposure to high temperatures (i.e., 25°C). Therefore, to establish baseline information, it would be wise to expose oysters collected during winter to warm temperatures for 1–2 mo before the initial infection assessment.

Although Pacific oysters appear less susceptible than eastern oysters to *P. marinus* infection, heavy non-*P. marinus*-related mortality occurred in both diploid and triploid Pacific oysters at salinities of 10 ppt and below and temperatures higher than 15°C during the acclimation period. This indicates that the Pacific oyster may be less tolerant to high-temperature and low-salinity exposure than eastern oysters. High non-disease-related mortality (70%) was also recorded in Pacific oysters, in conjunction with salinities below 20 ppt, in a study carried out to compare the growth and mortality of *C. gigas* and *C. virginica* challenged with *P. marinus* (Barber and Mann 1994). It seems that low salinity exerts a greater effect on the physiology of this species than does high temperature. All *C. gigas* died when salinity was reduced to 3 ppt. These results suggest that salinities lower than 20 ppt stress *C. gigas*, thus reducing its resistance to *P. marinus*.

In conclusion, the Pacific oyster, *C. gigas*, is less susceptible to *P. marinus* than is the eastern oyster, *C. virginica*. However, they may not survive if introduced into Chesapeake Bay tributaries because they are unable to adapt well to the low-salinity and high-temperature conditions. The mid-Atlantic climate is relatively warm, between temperate and subtropical. The ecosystem of the Chesapeake Bay is complex. The salinity range of oyster habitats in the Chesapeake Bay varies seasonally, from as low as 0 to >20 ppt (Andrews 1988, Ragone Calvo and Burreson 1995). The water temperature of most tributaries along the bay can reach 28–29°C (Andrews 1988) and persist for more than 2 mo during the summer. The oyster pathogen, *P. marinus*, on the other hand, can survive in salinities lower than 5 ppt, and epizootics caused by this parasite increase at high temperatures (Andrews 1988, Burreson and Andrews 1988). Moreover, the shells of *C. gigas* held in water from the lower Chesapeake Bay (i.e., York River, VA) were found to be quite susceptible to invasion by the polychaete, *Polydora* sp. (Burreson and Mann 1994). Further studies are needed to ascertain the competence of *C. gigas* to support a commercial fishery in Chesapeake Bay.

ACKNOWLEDGMENT

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SPATIAL DISTRIBUTION AND INTENSITY OF *PERKINSUS MARINUS* INFECTIONS IN OYSTER RECOVERY AREAS IN MARYLAND

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ABSTRACT The project described here reports on the status and spatial variability of *Perkinsus marinus* in three oyster recovery areas (ORAs) during 1994. The objectives of the study were to examine spatial variability in *P. marinus* infections among oyster bars along single tributaries and within single oyster bars. In addition, the study was conducted to compare estimates of prevalence based on intensive and spatially accurate patent-tong sampling with estimates of prevalence based on traditional dredge sampling of 30 oysters. For comparative purposes, patent-tong and dredge sampling were both conducted in each of four oyster bars, within 35 days, during September–October 1994. In June 1994, a pilot study was conducted to test the use of long range navigation (LORAN) and global positioning system (GPS) for the accurate deployment of patent-tongs and to compare hemolymph and tissue Ray fluid thioglycollate medium assays. In summary, results from this investigation support three conclusions: (1) During fall 1994, the variation of *P. marinus* prevalence, among and within oyster bars in sampled ORA tributaries, was small (<30%) but statistically significant; (2) traditional dredge samples of 30 oysters per bar provided estimates of prevalence remarkably similar (within 5%) to the ones obtained from patent-tong samples of an average of 340 oysters per bar; (3) RFTM assays conducted in the spring showed that hemolymph, rectum, and combined gill and palp samples gave equivalent determinations of prevalence.

KEY WORDS: Spatial distribution, *P. marinus*, variability, oysters, management, Maryland

INTRODUCTION

Perkinsus marinus (Mackin, Owen, and Collier) is a contagious pathogen of oysters *Crassostrea virginica* (Gmelin). The transmission of the parasite occurs directly, from oyster to oyster, through the surrounding water. Studies by Mackin (1962) and Andrews (1988) have indicated that heavy parasite loads in dying oysters spread infections to adjacent oysters. Epizootics are believed to be favored by continuous and densely populated oyster bars in relatively high-temperature and high-salinity environments (Mackin 1962, Ray 1987). According to Andrews (1988), the parasite thrived in beds with high oyster density, and it persisted in public areas with low oyster density. Apparently, disease-induced mortality was highest in areas with high oyster density, relatively isolated areas with low oyster density restricted the spread of the parasite. The spread of this pathogen has also occurred by the movement of infected oysters (Andrews 1988, Ford 1992).

Despite extensive studies on the epizootiology of *P. marinus* reviewed by Andrews (1988), there have been few reports on the geographical distribution and variability of infections in oysters within and between oyster bars. Craig et al. (1989) reported regional foci of infections in the Gulf of Mexico within scales of 300 km or less and 1,500 km or more, and uneven (patchy) distribution of infections with uninfected oysters immediately adjacent to infected ones. In a study of the spatial distribution of *P. marinus* in two oyster reefs in Texas, however, White et al. (1989) reported that infected oysters were evenly distributed on both reefs, even when oysters were unevenly distributed (in patches) in one of the reefs.

Management strategies that could possibly prevent or reduce the effect of diseases on oysters require seasonal and geographical monitoring of disease agents and associated pathologies in oysters. Andrews and Ray (1988) delineated management strategies to control *P. marinus* in the Chesapeake Bay and the Gulf of Mexico. Proposed strategies included isolating protected oyster beds from surrounding infected areas, harvesting early and removing all oysters at harvest, prohibiting the movement of infected oysters, and transferring only disease-free oysters into low-salinity areas, where the acquisition and progression of infections is minimal.

In Maryland, oyster recovery areas (ORAs) have been designed to incorporate some of the *P. marinus*–exclusion strategies proposed by Andrews and Ray (1988). ORAs are part of a plan to balance environmental and commercial interests in oyster rehabilitation (Maryland Oyster Roundtable Action Plan 1993). ORAs have been designated in various tributaries of the Chesapeake Bay including the Chester, Choptank, and Nanticoke Rivers (Fig. 1). Those rivers have been divided into two to three zones along the salinity gradient. Zones A correspond to upstream areas with the lowest salinity suitable for oyster habitat. Oyster harvest and the movement of infected oysters into zones A are prohibited. Zones B are located immediately downstream from zones A. Oyster harvest is permitted within zones B, but the introduction of infected oysters into zones B is not allowed. Zones C, immediately downstream from zones B, extend to the mouth of the tributaries. At this time, there is no restriction on harvest or on the movement of oysters into zones C. However, if quarantine restrictions in zones B prove effective, similar restrictions may be applied to zones C (Maryland Oyster Roundtable Action Plan 1993). The project described here reports on the status and spatial variability of *P. marinus* in three ORAs during 1994. The objectives of this study were to examine spatial variability in *P. marinus* infections among oyster bars along single tributaries and within single oyster bars. In addition, the study was conducted to compare estimates of prevalence based on intensive and spatially accurate patent-tong sampling with estimates of prevalence based on the traditional dredge sampling of 30 oysters.

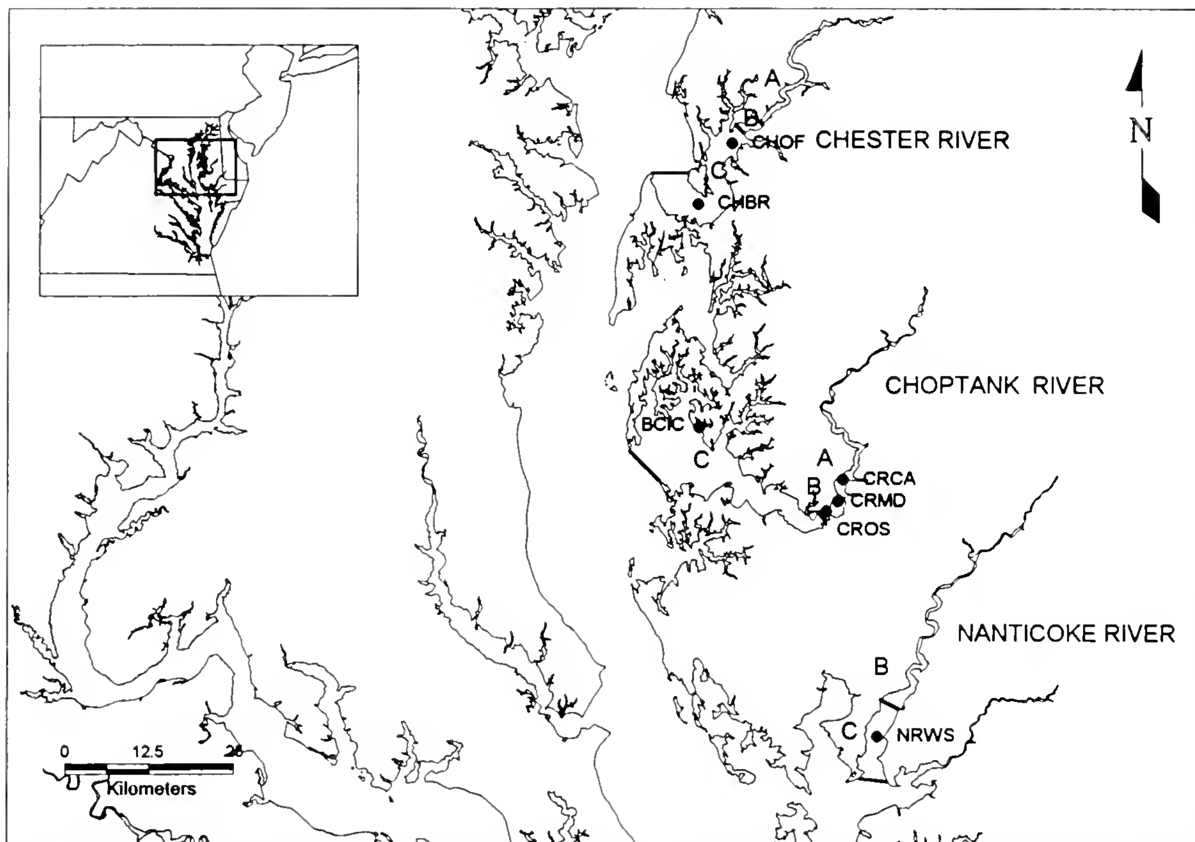

Figure 1. Maryland's portion of the Chesapeake Bay showing ORA zones and oyster bar locations in the Chester, Choptank, and Nanticoke Rivers. A, ORA zone A; B, ORA zone B; C, ORA zone C. Bar code abbreviations: CHOF, Old Field; CHBR, Buoy Rock; CRCA, Cabin Creek; CRMD, Dixon/Mill Dam; CROS, Oyster Shell Point; BCIC, Irish Creek.

METHODS

A pilot study was conducted during June 1994 to test the use of long range navigation (LORAN) and global positioning system (GPS) navigation for the accurate deployment of patent-tongs for the collection of oysters and to compare methods for *P. marinus* diagnosis by the use of hemolymph and tissue assays. The pilot study was restricted to patent-tong sampling on three oyster bars within the Choptank River—Cabin Creek (CRCA), Dixon/Mill Dam (CRMD), and Irish Creek (BCIC) (Fig. 1; Table 1).

In September 1994, the study was expanded to examine the spatial variability of *P. marinus* infections in other oyster bars within the Chester, Choptank, and Nanticoke Rivers, on the Eastern Shore of Maryland (Fig. 1; Table 1). For comparative purposes, patent-tong sampling and dredge sampling were both conducted in each of four oyster bars, within 35 d, during September and October 1994 (Table 2). Conflicting schedules of boat time, gear use, and diagnostic services prevented a more simultaneous occurrence of patent-tong and dredge surveys. Dredge sampling was conducted as part of the Modified Fall Survey (MFS), as described below, which is the standard oyster disease–monitoring method in Maryland.

Sampling

Patent-tong sampling was conducted during June and September 1994, in conjunction with site-specific stock assessment surveys in which bars were divided into a series of stations by superimposing a grid (0.10 min longitude = 146 m, and 0.05 min latitude = 93 m divisions) to bar delineations. A subset of stations within each bar was selected for sampling on the basis of prior surveys (M. Homer, pers. comm.) indicating the presence of oysters within reach of patent-tongs at specific locations. The number of sampling stations per bar ranged from 35 to 155 (Table 1). Oysters were collected with a single patent-tong grab of 1 m^2 per station. The accurate deployment of patent-tongs at each station was accomplished by LORAN and GPS navigation. The accuracy

TABLE 1.

Summary Field Data.

Bar	Stations Sampled (N)	Stations Without Oysters (N)	Stations Without Oysters (%)	Total Oysters (N)
Spring 1994				
CRCA	35	21	60.0	285
CRMD	82	43	52.4	831
BCIC	106	80	75.5	337
Fall 1994				
CHBR	38	26	68.4	27
CHOF	155	111	71.6	430
CRCA	35	11	31.4	547
CRMD	49	21	42.8	641
CROS	55	10	18.2	703
NRWS	103	63	61.2	550

TABLE 2.

Comparison of Prevalence in ORA and MFS Samples.

Bar	Date	Examined (N)	Infected (N)	Prevalence (%)	Temperature (°C)	Salinity (ppt)
ORA samples (patent-tong)						
CHBR	9/08/94	27	4	14.8	23.0	8.0
CHOF	9/08/94	315	57	18.1	23.0	8.0
CROS	9/13/94	528	65	12.3	22.0	6.0
NRWS	9/29/94	487	203	41.7	22.0	13.0
MFS samples (dredge)						
CHBR	10/12/94	30	3	10.0	16.0	8.5
CHOF	10/12/94	30	6	20.0	16.5	7.5
CROS	10/19/94	30	3	10.0	16.0	8.5
NRWS	10/26/94	30	12	40.0	15.0	8.0

of LORAN and GPS was, respectively, ±100 and ±10 m. When fewer than 30 oysters were collected by a grab, all oysters were examined for *P. marinus*. When grabs contained more than 30 oysters, a random sample of 30 oysters was selected from the pool. The target number of oysters to be collected per bar was 500. The average number of oysters collected per bar, during spring and fall, was, respectively, 484 and 483.

Dredge sampling was conducted according to MFS methods established by Smith and Jordan (1993). Following the referred methods, a single sample of 30 oysters was collected per bar with a dredge (91-cm-wide opening). Oysters were randomly selected from an aggregate of five (9-l substrate) samples collected, each with individual dredge-tows. LORAN navigation was used to help in locating the same approximate site, within each bar, where multiple dredge-tows have been conducted over the years. Tow distance varied depending on the density of the bottom material. Five tows covered a minimum distance of 100 m, which was necessary to fill the dredge in areas with the highest density of bottom material. All 30 oysters collected in each bar were examined for *P. marinus*, as described below.

Diagnosis

In the laboratory, oysters were scrubbed of fouling organisms and shell height was determined for each individual. All sampled oysters were examined for *P. marinus* by the use of hemolymph Ray fluid thioglycollate medium (RFTM) assays following the technique used at the Cooperative Oxford Laboratory for the rapid diagnosis of oysters (A. Farley, pers. comm.). The technique is a simplification of the hemolymph assay (Gauthier and Fisher 1990), as described below. To collect hemolymph, a small orifice was drilled into each oyster adjacent to the adductor muscle with a 3.12-mm bit, and a 0.5-ml sample was withdrawn with a 3-ml syringe fitted with a 20-gauge needle. Hemolymph samples were dispensed into 3.4-ml wells of 24-well culture plates (Corning 2582-24) and covered with a 2 ml of RFTM fortified with chloromycetin (0.025 g/ml). A 0.1-ml suspension of mycostatin (500,000 U in 125 ml of distilled water) was then added to each sample. After incubation at room temperature for 7 d, the top layer containing approximately 1 ml of RFTM was carefully aspirated with a transfer pipette and one to two drops of Lugol's iodine solution (6 g of potassium iodide and 4 g of iodine in 100 ml of distilled water) were added to the remaining sample containing oyster hemocytes and parasite cells. Stained hypnospores (prezoosporangia) were counted on an inverted microscope. When cell abundance was low (<250 cells), all cells in the well were counted. When cell abundance was higher, cells were counted in five replicate fields of view and the total cell number per well was estimated by multiplying the average cell number per field by the number of fields in the well. If necessary, when cell abundance was very high (>1 million), samples were diluted 10-fold to facilitate counting. The intensity of infection was then ranked, on the basis of cell abundance estimates per 1 ml of hemolymph, into seven categories with 10-fold increments from stage I (1–10 cells)

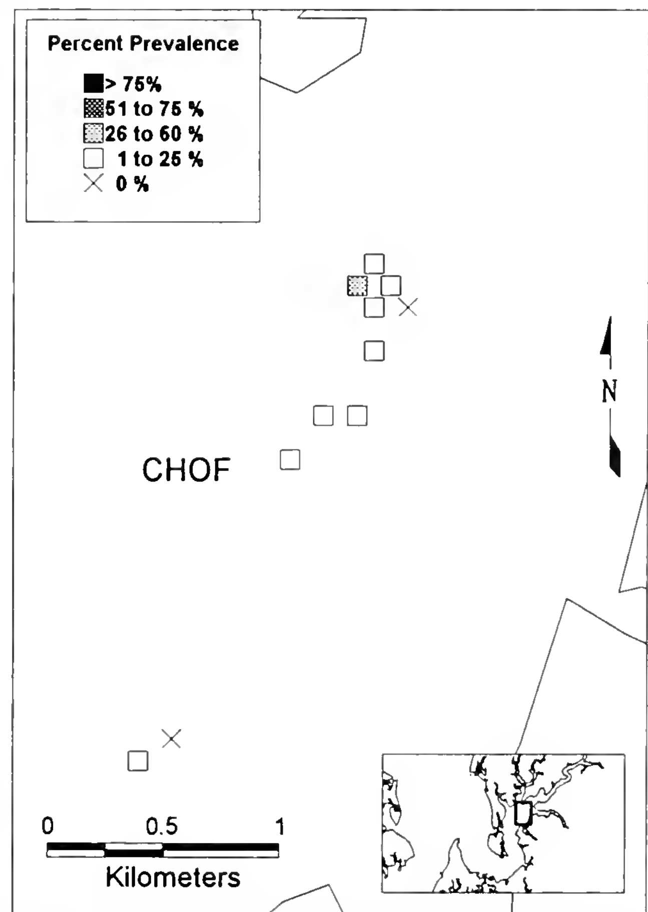

Figure 2. Prevalence of *P. marinus* in Chester River–Old Field (CHOF). Fall 1994. Only stations with 10–30 oysters are shown.

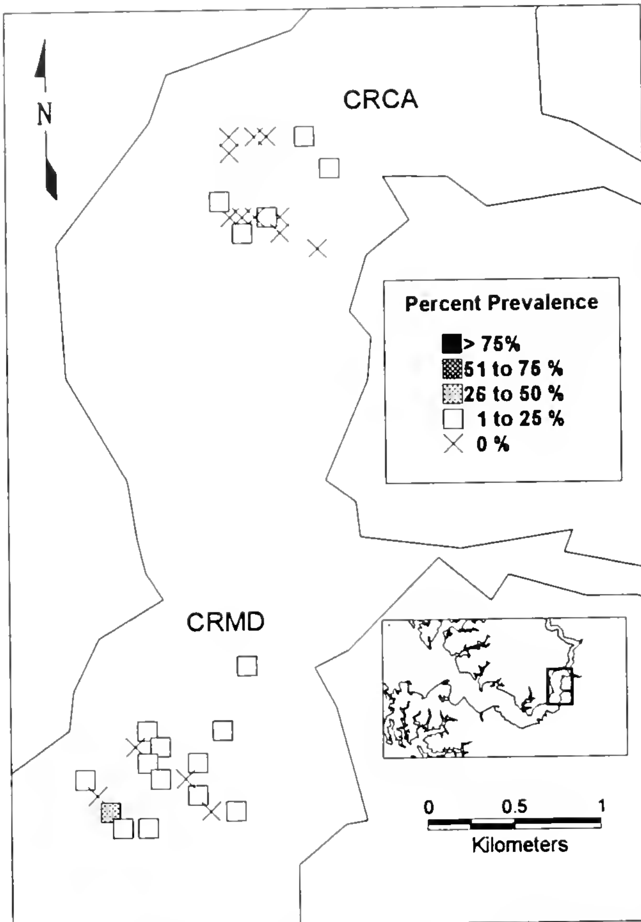

Figure 3. Prevalence of *P. marinus* in the Choptank River–Cabin Creek (CRCA) and Choptank River–Dixon/Mill Dam (CRMD). Fall 1994. Only stations with 10–30 oysters are shown.

to stage VII (>1 million cells). The diagnosis of *P. marinus* was by RFTM assay (Ray 1966, Howard and Smith 1983). Separate RFTM assays were conducted on gill and palp tissue combined and on rectal tissue. Quantitation of low-intensity infections (<250 cells/sample) in combined gill and palp samples and in rectum samples was determined and ranked as in hemolymph samples. Infections of higher intensities (>250 cells/sample) were assigned to categories on the basis of the relative abundance and density of cells, but cell counts were not performed on those samples.

Data Analysis

Records of individual oysters containing shell height, infection stage, date of sample collection, bar and station codes, and latitude and longitude coordinates were entered into a computer data base. Additional information on oyster density and percent mortality, at the time of sampling, was obtained from stock assessment data bases (M. Homer, pers. comm.).

Parasite prevalence was calculated separately for each bar, station, or size class considered. To avoid unrepresentative data arising from stations with small sample size, only stations with 30 oysters were selected for statistical analysis of prevalence. A χ^2 analysis and a Fisher Exact Test (Zar 1984) were conducted to compare the frequency of infected oysters between bars in the Chester and the Choptank Rivers and between stations within bars.

To examine geographical distribution of prevalence, data were imported into a geographical information system (MapInfo 3.0). Because few stations had 30 oysters, stations with 10–30 oysters were used to graphically illustrate the variation of prevalence among stations. Prevalence was divided into categories of 0%, 1–25%, 26–50%, 51–75%, and 76%–100% (Figs. 2–5). To further examine the variation of prevalence within Nanticoke River-Wilson School (NRWS), the oyster bar was divided into two subareas containing contiguous stations. Subarea A contained 15 stations with a total of 145 oysters, and subarea B contained 21 stations and a total of 333 oysters (Fig. 5).

Graphical plots of prevalence versus oyster density were used to examine, for stations with 30 oysters, if the two variables were related. To further investigate if oyster density affected *P. marinus* prevalence, regression analyses were performed (Zar 1984). For regression analysis, prevalence was arcsin transformed to improve normality and homogeneity of variance. A plot of residuals versus predicted values showed no pattern.

Nonparametric statistics were used to examine differences in intensity categories for stations with 1–30 oysters. Kruskal-Wallis tests (Zar 1984) were used to examine differences in intensity categories among: (1) tributaries, (2) bars within single tributaries, and (3) stations within single bars. Spearman's rank correlation analysis (Zar 1984) was used to examine the relationship between oyster size and intensity category. A χ^2 analysis was used to

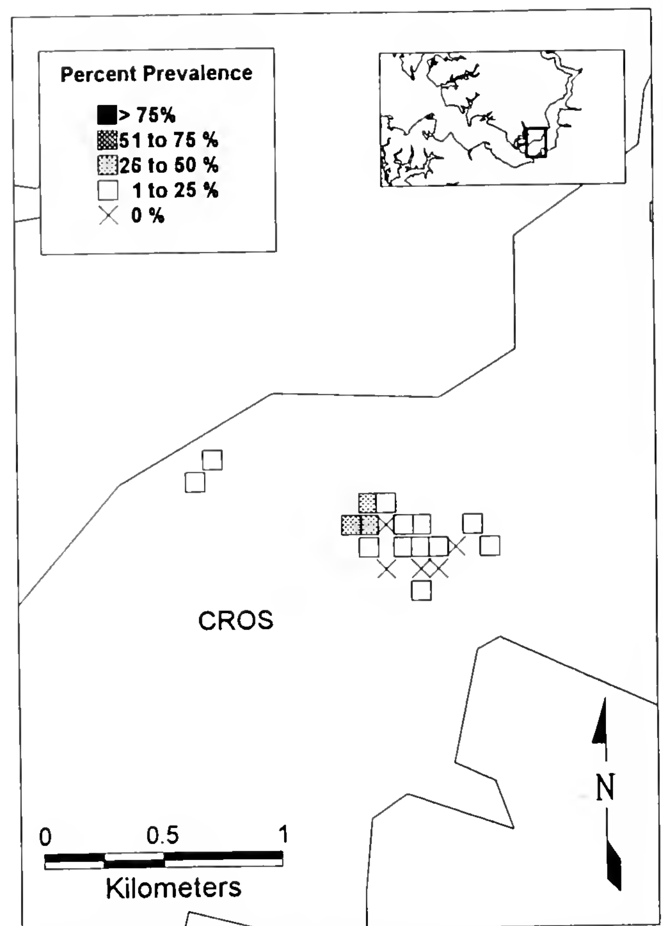

Figure 4. Prevalence of *P. marinus* in the Choptank River–Oyster Shell Point (CROS). Fall 1994. Only stations with 10–30 oysters are shown.

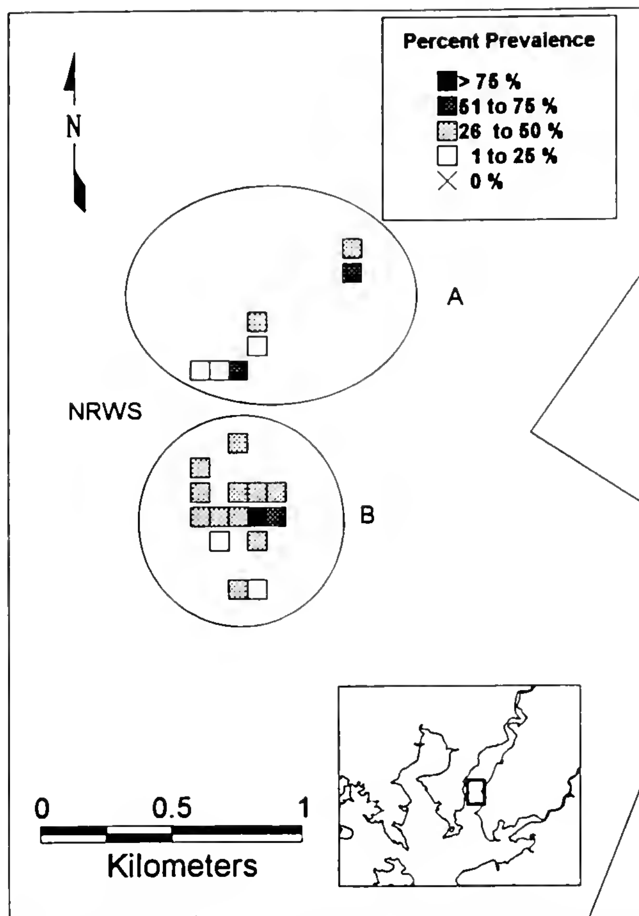

Figure 5. Prevalence of *P. marinus* in Nanticoke River–Wilson Shoal (NRWS). Fall 1994. Only stations with 10–30 oysters are shown. A, subarea with 38.6% prevalence; B, subarea with 72.8% prevalence.

compare the frequency of positive parasite detection by rectum, gill, and palp and hemolymph diagnoses in BCIC oysters.

RESULTS

Spring 1994

The average number of oysters collected from Choptank River bars was 484. Often, no oysters were present in stations, or none were collected by patent-tongs. The frequency of stations where oysters were absent, or beyond the reach of patent-tongs, ranged from 52.4% in CRMD to 75.5% in BCIC (Table 1). At the time of collection, temperature was 26–27°C and salinity ranged from 4 ppt in the upstream bar CRCA to 7 ppt in the downstream bar BCIC. Mean shell height was 59.8 mm in BCIC oysters, 63.0 mm in CRCA oysters, and 70.9 mm in CRMD oysters.

On the basis of hemolymph diagnosis, prevalence was 17.2% in CRCA, 3.3% in CRMD, and 89.7% in BCIC. Prevalence among CRCA stations with 30 oysters (N = 3) ranged from 0 to 37%. No prevalence results are presented for stations within CRMD or BCIC, because none of the stations had 30 oysters. Differences in prevalence among Choptank River bars and among stations within CRCA were significant (χ^2 and Fisher Exact statistics, $p < 0.05$). Most of the infected oysters had light-intensity (stages I and II) infections. Pearson's χ^2 statistic indicated that there was no significant ($p > 0.05$) difference in the prevalence of *P. marinus* by rectum, gill, and palp and hemolymph diagnoses of the same BCIC individuals (N = 96).

Fall 1994

The average number of oysters collected in the Chester, Choptank, and Nanticoke Rivers was 483 oysters per bar. A high proportion of the stations sampled had no oysters, or none were collected by patent-tongs. The percentage of stations with no oysters ranged from 68.4 to 71.6% in Chester River bars; ranged from 18.2 to 42.5% in Choptank River bars; and was 61.2% in NRWS (Table 1). At the time of sampling, temperature was 22–23°C. Salinity was 8 ppt in Chester River bars, 5–6 ppt in Choptank River bars, and 13 ppt in NRWS (Table 2). Mean shell height ranged from 79.0 to 96.0 mm in Chester River oysters, ranged from 57.7 to 72.5 mm in Choptank River oysters, and was 80.4 mm in NRWS oysters (Table 3).

Prevalence among bars, within single tributaries, ranged from 14.8 to 18.1% in the Chester River and from 2.0 to 12.3% in the Choptank River. Prevalence among stations with 30 oysters (within individual bars) ranged from 7.0 to 13.0% in Chester River-Old Field (CHOF), 0 to 10% in CRCA, 0 to 27% in CRMD, 0 to 30% in CROS, and 50 to 80% in NRWS. Differences in prevalence among Choptank River bars and among stations in CRCA, Choptank River-Oyster Shell Point (CROS), CRMD, and NRWS were significant (χ^2 and Fisher Exact statistics, $p < 0.05$); differences in prevalence among Chester River bars and among CHOF stations were not significant (χ^2 and Fisher Exact statistics, $p > 0.05$).

Distribution of prevalence among stations, with 10–30 oysters examined, was as follows. Prevalence in most stations located within bars in the Chester and Choptank was 1–25% (Figs. 2–4). Prevalence in most stations within the Nanticoke River bar (NRWS) ranged from 26 to 75% (Fig. 5). However, the difference in prevalence among stations with 30 oysters examined was at most 30%. A pattern of contiguous stations with similar prevalence ranges was observed in CROS and NRWS (Figs. 4 and 5). Prevalence in NRWS subareas A and B was 38.6 and 72.8%, respectively.

Estimates of prevalence derived from intensive patent-tong sampling were remarkably close, within 5%, of estimates of prevalence based on less intensive dredge sampling (Table 2). On the basis of patent-tong sampling, prevalence for CHBR, CHOF, CROS, and NRWS, was, respectively, 14.8, 18.1, 12.3, and 41.7%. On the basis of dredge sampling, prevalence for the same bars was, respectively, 10.0, 20.0, 10.0, and 40.0%.

TABLE 3.

Prevalence of *P. marinus*, Oyster Density, Size, and Mortality, Fall 1994.

Bar	Prevalence (%)	Mortality (%)	Mean Oyster Density (l/m²)	Mean Shell Height ± SD (mm)
CHBR	14.8	37.2	0.7	96.0 (17.2)
CHOF	18.2	13.5	2.8	79.0 (26.4)
CRCA	2.0	23.5	15.6	57.6 (18.2)
CRMD	8.2	16.7	13.4	66.5 (14.0)
CROS	12.3	18.4	11.3	72.5 (16.6)
NRWS	41.7	6.8	5.4	80.4 (15.8)

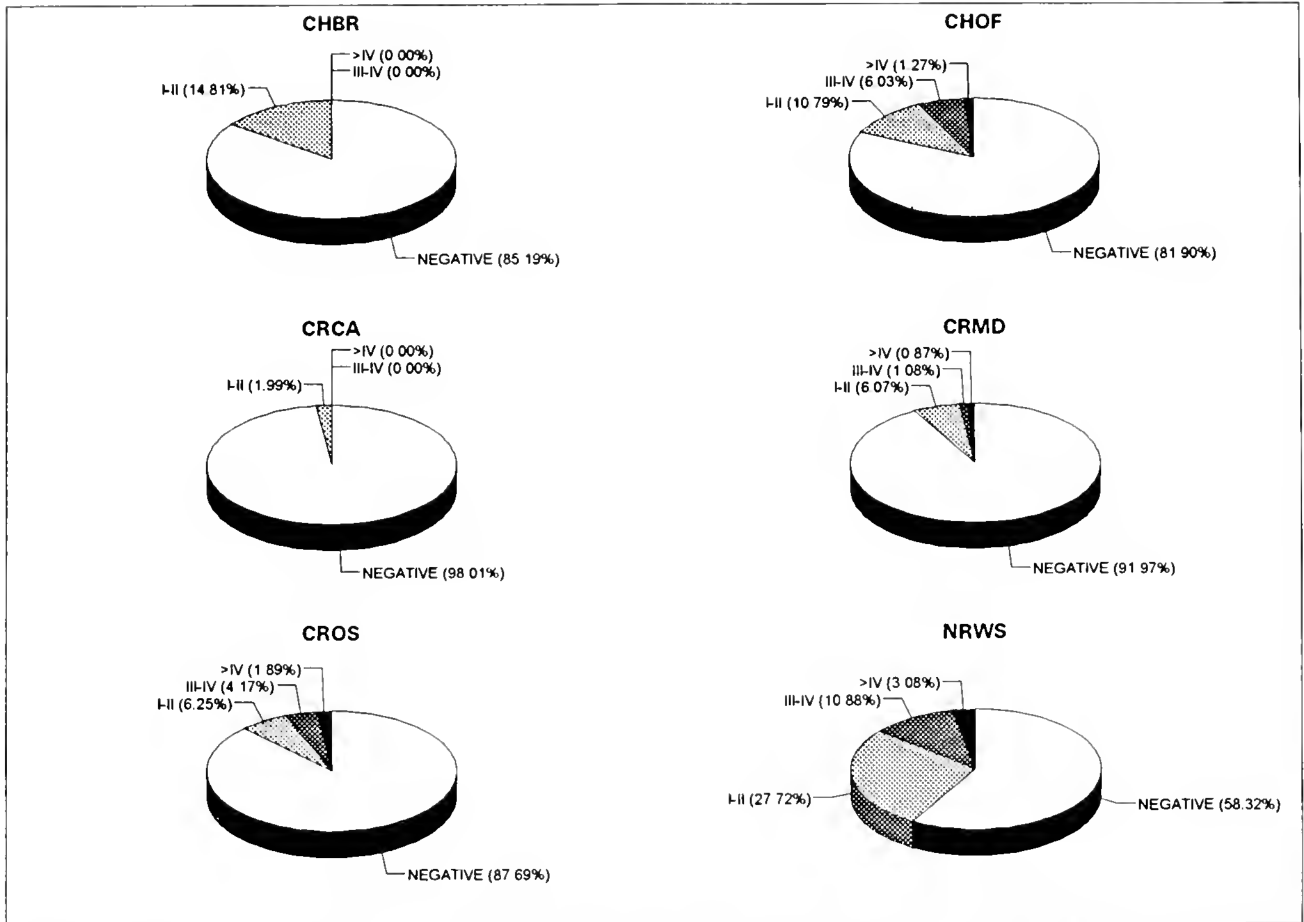

Figure 6. Intensity of *P. marinus* infections in sampled bars in the Chester, Choptank, and Nanticoke Rivers. Fall 1994. Bar code abbreviations: CHOF, Old Field; CHBR, Buoy Rock; CRCA, Cabin Creek; CRMD, Dixon/Mill Dam; CROS, Oyster Shell Point; BCIC, Irish Creek. Pie charts show percentage of cases in a given category.

In general, infection intensities were light. The largest proportion of the cases were categorized as I (1–100 cells/ml of hemolymph) and II (100–1000 cells/ml of hemolymph) (Fig. 6). Significant differences (Kruskal-Wallis statistic, $p < 0.05$) in the distribution of intensity categories were found among tributaries; among bars within the Choptank River; and among stations within CHOF, CRMD, CROS, and NRWS bars. Differences among bars in the Chester River and among stations within CHBR and CRCA were not significant (Kruskal-Wallis statistic, $p > 0.05$) (Table 4).

Overall, most of the infected oysters collected in the fall were large (50–200 mm). Prevalence in small (20–50 mm) oysters was 9.9% (38 infected in 383 examined), and prevalence in large oysters was 20.8% (456 infected in 2,187 examined). Thus, the ratio of the percentage of small infected oysters to large infected oysters was roughly 1:2. Analysis of data for individual bars indicated that the referred 1:2 ratio held for bars with low salinity (e.g., CHOF) and high salinity (e.g., NRWS).

The relationship between oyster size and infection intensity was also investigated with the whole fall data set (N = 2,624 observations) and subsets of data (N = 27–528 observations) for individual bars. The following results apply to both the whole data set and individual subsets of data. A plot of size versus infection stage revealed that stages 4 and above were less frequent in small (20- to 50-mm) oysters and very large (100- to 200-mm) oysters than in medium (50- to 100-mm) oysters. The correlation between

TABLE 4.

Comparison of *P. marinus* Infections Among ORA Tributaries, Bars, and Stations.

Comparison	K-S Statistic	DF	p Value	Significance
Among tributaries	287.05	2	0.0001	S
Among bars within Chester River	0.27	1	0.5999	NS
Among bars within Choptank River	34.80	2	0.0001	S
Among stations within CHBR	15.78	13	0.2613	NS
Among stations within CHOF	75.45	42	0.0012	S
Among stations within CRCA	22.71	25	0.5946	NS
Among stations within CRMD	52.36	27	0.0024	S
Among stations within CROS	92.17	42	0.0001	S
Among stations within NRWS	77.51	36	0.0001	S

Fall 1994. Results of Kruskal-Wallis (K-S) tests on oyster hemolymph infection stages. At $\alpha = 0.05$; S, significant; NS, not significant.

oyster size and infection stage, however, was not significant (Spearman's rank correlation statistic, $p > 0.05$).

Percent oyster mortality ranged from 13.5 to 37.2% in Chester River bars; ranged from 16.6 to 23.5% in Choptank River bars; and was 6.7% in NRWS. Mean oyster density ranged from 0.7 to 2.8 oysters/m^2 in Chester River bars; ranged from 11.3 to 15.6 oysters/m^2 in Choptank River bars; and was 5.4 oysters/m^2 in NRWS. Mean oyster density in NRWS subareas A and B was 11.5 and 18.1 oysters/m^2, respectively. Prevalence, percent mortality, and mean oyster density did not show corresponding patterns among oyster bars (Table 3). Prevalence was not affected by density (R^2 0.011, $p < 0.05$).

DISCUSSION

In agreement with MFS results, this study showed that *P. marinus* infections in Maryland ORAs were of low prevalence and intensity during 1994. MFS results indicated that the prevalence of *P. marinus*, for most bars sampled in fall 1994, was 30% below corresponding values for 1993 and 1992. *Haplosporidium nelsoni* was absent from most samples examined during the MFS conducted in 1994 (Krantz 1995). The low prevalence and intensity of *P. marinus* infections, and the near absence of *H. nelsoni* infections, during 1994 can be attributed to extreme low-salinity conditions prevailing in the Chesapeake Bay during June to September 1994, when mean streamflow into the bay was above the long-term (>60 y) average (U.S. Geological Survey 1994, Krantz 1995).

This study showed that there was no significant difference (Pearson's χ^2, $p > 0.05$) in the frequency of *P. marinus* detection (prevalence) by rectum, gill, and palp and hemolymph diagnoses of the same individual oysters. Similarly, Bushek et al. (1994) reported no significant differences in *P. marinus* prevalence as determined by hemolymph and rectum assays.

Seasonal, spring to fall, comparison of *P. marinus* prevalence in Choptank River bars showed an increase from 3.3 to 8.2% in CRMD but a decrease from 17.7 to 2.0% in CRCA. The seasonal increase in prevalence, in CRMD, was expected on the basis of the characteristic seasonal cycle of disease progression with increasing temperatures from spring to fall (Andrews 1988). The unexpected seasonal decrease in prevalence in CRCA may have been related to a long duration (3 mo) of low salinity (4–5 ppt, at the time of spring-fall sampling at that location). Reduction of infection prevalence during a period of low salinity and warm temperature in CRCA may be attributed to a disproportionally high mortality of infected oysters stressed by extreme low-salinity conditions, as described above. Extreme low-salinity conditions during 1994 may have been conducive to relatively high oyster mortality in CRCA (23.5%) and CHBR (37.2%). On the basis of very limited mortality data, restricted to once-yearly box counts conducted in the fall, we speculate that the benefit afforded to oysters by low-salinity environments as a refuge from the presence and virulence of parasites may have been offset in some ORAs during 1994 by the adverse effects of extreme low salinity on oyster survival.

As expected, the geographic distribution of prevalence in sampled ORAs corresponded with the salinity regime of the particular area. Oyster bars located in areas with low salinity had low parasite prevalence, and oyster bars located in areas with high salinity had high parasite prevalence. In most cases, variation in prevalence among oyster bars within sampled ORA tributaries and among stations within oyster bars was statistically significant but small (<30%). In the spring, however, prevalence between the upstream and the downstream Choptank River bars (CRCA and BCIC) varied by as much as 87% in an area separated by 40 km of distance and with salinity within 3 ppt. Unfortunately, it was not possible to determine if spring prevalence in BCIC (89.7%) was maintained in the fall, because BCIC was substituted with CROS during fall sampling. The difference in fall prevalence between CRCA and CROS was only 10%, but CRCA and CROS had salinity within 1 ppt and were only 7 km apart. Ray (1987) reported that very large differences in prevalence (96%) were maintained for 1 y between monthly samples collected from two oyster reefs within 10 km and subjected to similar environmental conditions. Ray's study continued for an additional year, when infections intensified and the referred difference was eventually reduced to a minimum of 4%. The existence of relatively large differences in *P. marinus* infections among oyster bars located in nearby areas and experiencing similar environmental conditions may be related to the close proximity of oysters required for the transmission of *P. marinus* (Andrews 1965, Andrews 1988, Ray 1987). Mackin (1962) suggested that large doses of *P. marinus* cells, mostly originating from decomposing tissues in dead oysters, are the main source of new infections for surrounding oysters. The role of water flow in transmission dynamics (Mackin 1962, Ray 1987) remains unclear. However, water currents may concentrate or disperse parasites and favor or discourage the establishment of foci of infections, depending on the pattern of the flow. Investigations on water flow patterns around oyster bars may be necessary to better explain parasite dispersal and the spatial distribution of infections.

Prevalence variability among stations with 30 oysters, within a bar, was low (<30%). However, this variability increased when sample size was reduced to include 10–30 oysters per station, even though the total number of oysters and stations available for comparison increased. For instance, prevalence in NRWS stations (N = 22) with 10–30 oysters (N = 404 oysters) varied by as much as 72%, and prevalence in NRWS stations (N = 3) with 30 oysters (N = 90 oysters) varied by 30%. Because there were few stations with 30 oysters, it was necessary to include stations with 10–30 oysters to examine the spatial variation distribution of prevalence. However, a sample of 30 or more oysters is required to statistically detect a minimum of 10% prevalence in a population of 100,000 oysters (Amos 1985). Stations with 30 oysters in NRWS, however, may have been located in areas having higher prevalence than stations with 10 oysters because the overall prevalence within NRWS was 41.7% (N = 487 oysters) and the prevalence for stations with 30 oysters (N = 90 oysters) was 63.3%. By partitioning NRWS into subareas containing a series of contiguous stations, it was possible to determine that prevalence in the subarea containing stations with 30 oysters (N = 333 oysters) was 72.8% and prevalence in the other subarea (N = 145 oysters) was 38.6% (Fig. 5).

Differences in prevalence among stations, within most bars, were significant. This result appears to contradict Craig et al. (1989), who did not find significant differences (binomial test, $p > 0.05$) in prevalence within bars, after comparing 49 bars with three stations in each bar. In this study, there were significant differences (χ^2 and Fisher Exact Tests, $p < 0.05$) in prevalence among stations (N = 35–155) in four of five of the oyster bars examined. Comparison of our results with those of Craig et al. is complicated, however, because the referred authors did not specify the type of gear used in a particular location other than indicating that hand, tong, or dredge was used, depending on water

depth. White et al. (1989) reported that, in general, infected oysters were negatively autocorrelated (evenly) distributed in two reefs in Texas, even when oysters were positively correlated (distributed in patches) in one of the reefs. However, White et al. also indicated that infected oysters were distributed in patches at spatial scales >60 cm at both reefs sampled. White et al. used a 50-cm-wide by ≥50-cm-long rectangle (with its length adjusted according to oyster density) to sample 60 oysters per reef. By examination of correlograms with test statistic plotted versus distances between one to seven clumps of oysters (12–84 cm), White et al. were able to detect patches of infected oysters at the scale of 60–84 cm, but not at <60 cm. By the use of different methods, this study showed that groups of stations with similar parasite prevalence occurred (in patches) over areas at scales of 100–500 m (Fig. 5). It appears that the distribution of *P. marinus* within bars was more sporadic in oyster bars with low prevalence (e.g., CRCA and CRMD) than in oyster bars with higher prevalence (NRWS). Craig et al. (1989) examined the regional distribution of *P. marinus* in Gulf Coast oyster beds and found that prevalence was positively autocorrelated (an indication of patchiness) at scales of ≤300 and ≥1,500 km. Comparing the distribution of infected oysters at different scales, however, may not be valid. Often in aquatic ecosystems, the scale at which heterogeneity manifests itself varies widely and changes of scale can transform a heterogeneous pattern into a homogeneous one and vice versa (Dutilleul 1993).

Prevalence estimates based on the patent-tong collection of an average of 340 oysters per bar were in agreement with prevalence estimates based on dredge samples of only 30 oysters per bar (MFS). In most oyster bars examined, estimates of prevalence based on dredge samples taken in September corresponded with slightly (<5%) lower estimates based on patent-tong samples taken in October. Decreasing water temperatures in October, 6°C lower than in September, may have contributed to the small decrease in prevalence. We speculate that estimates of prevalence based on dredge sampling reflect estimates of prevalence based on patent-tong sampling because dredge samples were composed of oysters collected over a relatively large area. Thus, spatial variability in prevalence was masked by dredge sampling, and intense patent-tong sampling was necessary to show significant, albeit small (<30%), differences in prevalence among stations.

As expected, *P. marinus* infections were more prevalent in large (50- to 200-mm) oysters than in small (20- to 50-mm) oysters. It has generally been accepted that *P. marinus* infections in small oysters are less prevalent and intense than those in larger oysters. White et al. (1989) found infections in large (>50-mm) oysters to be three to four times as prevalent as infections in small (<50-mm) oysters. We found infections in large (50- to 200-mm) oysters to be twice as prevalent as infections in small (20- to 50-mm) oysters. Ray (1953) found oysters of increasing age to be increasingly susceptible to infections, and Paynter and Burreson (1991) noted that larger animals tended to be infected sooner and more intensely than smaller ones. Small oysters or spat may be less susceptible to infections, given the limited volume of water they filtered compared with large animals (Paynter and Burreson 1991). Surprisingly, however, oyster size and infection intensity were not correlated in this study. The lack of correlation between oyster size and infection intensity may be attributed to the overall scarcity of higher than light intensity infections as compared with abundant light infections.

Average oyster density and prevalence revealed no corresponding patterns. However, there were only minor differences in mean oyster density among bars. Perhaps, densities within the range examined (0.7–15.6 oysters/m^2) did not affect disease acquisition and/or transmission dynamics. Density varies widely within bars, however, and the difference in prevalence between subareas in NRWS corresponded with a difference in oyster density. A relatively low prevalence (38.6%) corresponded with a relatively low oyster density (11.5 oysters/m^2) in NRWS subarea A, and a relatively high prevalence (72.8%) corresponded with a relatively high oyster density (18.1 oysters/m^2) in NRWS subarea B. It is also interesting to note that the parasite was present even at host densities less than 1 oyster/m^2. Therefore, a very low oyster density may be necessary to avoid disease transmission.

The relationship between prevalence and mortality was not clearly defined. On the basis of very limited mortality data, as previously described, we make the following observations. The pattern of prevalence did not correspond with the pattern of mortality (Table 3). In fact, the bar with the highest prevalence (NRWS with 41.7%) had the lowest mortality (6.8%), the bar with the lowest prevalence (CRCA with 2.0%) had 23.5% mortality, and CHBR, with a prevalence of only 14.8%, had the highest mortality (37.2%). Mortality estimates, however, are based on annual counts of live and dead oysters, which may correspond to lethal infections present during the year before sampling. Thus, high prevalence levels during 1993 (roughly two to four times higher than in 1994) may have resulted in disease-induced mortality during 1994.

In summary, results from this investigation support three conclusions. (1) During fall 1994, variation of *P. marinus* prevalence, among and within oyster bars in sampled ORA tributaries, was small (<30%) but statistically significant. (2) Traditional dredge samples of 30 oysters per bar provided estimates of prevalence remarkably similar to the ones obtained from patent-tong samples of an average of 340 oysters per bar. (3) RFTM assays conducted in the spring showed that hemolymph, rectum, and combined gill and palp samples gave equivalent determinations of prevalence.

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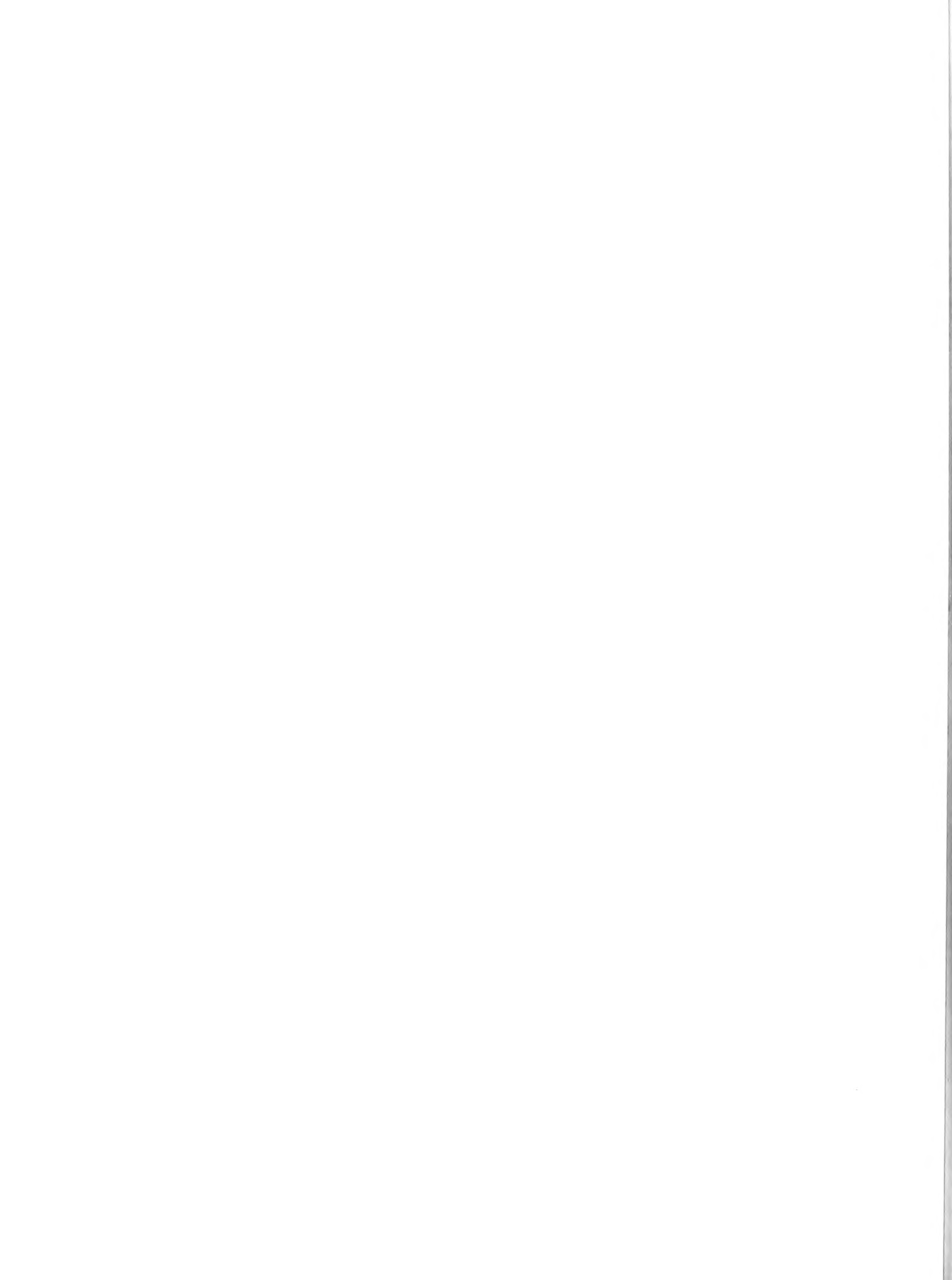

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CORRELATION BETWEEN THE PRESENCE OF LATHYROSE WITH THE ABSENCE OF *HAPLOSPORIDIUM NELSONI* IN *CRASSOSTREA VIRGINICA* FROM TWO SOUTH CAROLINA TRIBUTARIES WHERE *PERKINSUS MARINUS* ALSO INHIBITS HEMOCYTE AGGLUTINATION BY THE *LATHYRUS ODORATUS* LECTIN

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ABSTRACT Hemocytes from *Crassostrea virginica* from two adjacent tributaries in South Carolina were exposed to six dilutions of the *Lathyrus odoratus* lectin. As a result, it has been further confirmed that there is a correlation between the presence of lathyrose, the yet-uncharacterized saccharide that binds to this lectin, and the essential absence of the pathogen *Haplosporidium nelsoni*. Furthermore, it has been demonstrated that there was inhibition of the agglutination of lathyrose-positive hemocytes by the *L. odoratus* lectin when a second protistan pathogen, *Perkinsus marinus*, was present. This confirms that lathyrose (or a functionally very similar molecule) occurs on the surface of *P. marinus*. Comparisons of the *H. nelsoni* infection frequencies in oysters from the two sites suggest that the intertidal marsh separating the two sites was a sufficient barrier to result in shifts in infection frequencies within 12 mo.

KEY WORDS: *Crassostrea virginica, Haplosporidium nelsoni, Perkinsus marinus*, lathyrose, *L. odoratus* lectin

INTRODUCTION

It is known that the oyster pathogen *Haplosporidium nelsoni* occurs in coastal South Carolina (Dougherty et al. 1993). Subsequent surveys by personnel of the South Carolina Marine Resources Research Institute (SCMRRI) (unpubl.) have revealed that ~25% of the oysters, *Crassostrea virginica*, (Gmelin), collected during May through September 1994, from Inlet Creek and Toler's Cove, tributaries of Charleston Harbor and the Atlantic Ocean, respectively, on Sullivan's Island, Charleston County, South Carolina, harbored this parasite. A second protistan parasite, *Perkinsus marinus*, also occurs in oysters at these sites.

Because coastal South Carolina is punctuated by numerous tributaries and minor inlets commonly separated by intertidal marshes, the principal question being posed was whether there is transmission of infections by such protistan parasites as *H. nelsoni* between oysters native to two adjacent tributaries during a season when the separating marsh was essentially dry. An answer to this question could shed some light on the transmission mechanism of *H. nelsoni*, i.e., whether a continuous aquatic milieu is essential for transmission.

Because earlier studies (Cheng et al. 1994a, Cheng et al. 1995) have revealed that there is a correlation between the occurrence of a yet-uncharacterized saccharide designated as lathyrose (Cheng and Dougherty 1994a) on the surface of hemocytes from oysters free of *H. nelsoni*, a second objective of the study being reported here in was to ascertain whether there is a similar correlation between the presence of lathyrose and the absence of *H. nelsoni* in oysters from adjacent tributaries that had revealed a similar incidence of infection with *H. nelsoni*.

Third, because earlier studies (Cheng and Dougherty 1994b, Cheng and Dougherty 1995) had suggested that lathyrose occurs on the surface of intramolluscan stages of *P. marinus* in Chesapeake Bay and Apalachicola Bay oysters, we were interested in obtaining additional evidence to support or reject the commonality of this phenomenon by testing *P. marinus* from oysters from essentially the same location.

MATERIALS AND METHODS

Oysters

A total of 65 oysters were collected from Inlet Creek during March through September 1995. These specimens measured from 69.1 to 111.3 mm in length. Sixty-eight oysters were collected from Toler's Cove during the same period. These measured from 59.9 to 140 mm in length. Inlet Creek and Toler's Cove, separated by approximately 1.5 miles, are connected by an intertidal marsh that is commonly completely devoid of water during a dry season, as during the summer of 1995. The salinity at both collection sites during the collection period was 22–28‰.

Hemolymph Collection

After the external surfaces of the oysters were cleaned, approximately 2 ml of whole hemolymph were collected from the adductor muscle sinus of each oyster by use of a sterile 21-gauge hypodermic needle and a 1-ml tuberculin syringe. The samples were washed three times in isotonic (540 mOsm) saline involving centrifugation at 300g. After the third wash, the cell pellets were gently resuspended in 2 ml of isotonic saline. The final cell counts averaged 1-2 × 10^5/ml.

Lectins

The *Lathyrus odoratus* (sweet pea) lectin was tested against washed oyster hemocytes. The initial lectin solution used was at a concentration of 0.1 mg/ml. It was prepared in phosphate-buffered saline, pH 7.4, and was serially diluted twofold with isotonic saline in microtiter plates to give final dilutions of 1:1 to 1:2,048. It is known that the agglutination of oyster hemocytes by the *L. odoratus* lectin is not inhibited by *N*-acetyl-D-glucosamine, D(+)-glucose, or D(+)-mannose, the known inhibitors of cell aggluti-

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nation mediated by this lectin (Ticha et al. 1980); nevertheless, the possible inhibitory effects of two of these saccharides, *N*-acetyl-D-glucosamine and D(+)-glucose, in the hemocyte-lectin combinations comprising this study were tested.

In addition to the *L. odoratus* lectin, the *Canavalia ensiformis* lectin (Con A, type III, jackbean) was also tested against washed oyster hemocytes. These tests served as positive controls because it is known that Con A will agglutinate hemocytes of *C. virginica* (Yoshino et al. 1979, Cheng et al. 1980, Cheng et al. 1993, Kanaley and Ford 1990). In the tests involving Con A, the concentration of the initial lectin solution was 1.0 mg/ml and *N*-acetyl-D-glucosamine and D(+)-glucose were used as the inhibition saccharides. The Con A solution was prepared in phosphate-buffered saline and was serially diluted twofold with isotonic saline in microtiter plates to give final dilutions of 1:1 to 1:2,042. In negative control tests, isotonic saline instead of lectin was used. None of these resulted in the agglutination of hemocytes. In inhibition tests involving both the *L. odoratus* lectin and Con A, each lectin was diluted serially in 0.2 M solutions of the appropriate inhibition sugar.

Agglutination tests were carried out in 96-well U-bottom plates (Cell Wells, Corning, NY). Fifty microliters of hemocyte suspension was added to each experimental and control well, and the plates were incubated for 24 h at room temperature (25°C).

Earlier studies (Cheng and Dougherty 1994a, Cheng et al. 1993, Cheng et al. 1994, Cheng et al. 1995) had revealed that not all of the hemocytes exposed to the *L. odoratus* lectin or Con A agglutinated. Therefore, the percentages of clumped cells (i.e., number of agglutinated cells/100 cells) were ascertained at the highest concentration of the two lectins tested as well as at dilutions of 1:2, 1:64, 1:512, 1:1,024, and 1:2,048. The counting of agglutinated and single cells was achieved microscopically. When three or more cells were clumped, these were considered to be agglutinated. Pairs of cells were seldom observed.

Detection of Infections

Determinations of the presence or absence of *H. nelsoni* and/or *P. marinus* were carried out in two ways: (1) by histological examination of representative hematoxylin & eosin–stained sections, and (2) by using a modification of the panning technique of Ford et al. (1990), which Cheng and Dougherty (1995) have reported to be as effective qualitatively for the detection of protistan parasites in oysters as the hemolymph assay method of Gauthier and Fisher (1990). Also, as Bushek et al. (1994) have pointed out, the commonly used fluid thioglycollate medium used for the qualitative diagnosis of *P. marinus* in *C. virginica* has major drawbacks. Consequently, we elected to use a combination of the histological and panning techniques in our determination of the possible presence of *P. marinus* and also of *H. nelsoni*. It is possible that by using these methods, we may have overlooked some very light infections with *P. marinus*.

In brief, the panning technique involves placing ~1 ml of whole hemolymph on the bottom of a Petri dish and permitting it to stand for 30 min at 25°C. Because oyster hemocytes portray greater adherence to the substrate, microscopical examination of nonadhering cells permits the identification of *H. nelsoni* and/or *P. marinus*. The tissues prepared for histological examination and the hemolymph samples subjected to panning were from the same oysters from which the hemocytes used for the lectin studies were obtained.

Statistical Analyses

To test for significance between the difference in percentages of agglutinated hemocytes from oysters infected with *H. nelsoni* or *P. marinus* and between uninfected and infected oysters from both collection sites that had been exposed to the dilutions of the *L. odoratus* lectin tested, the two-sample t test (Neter et al. 1983) was used. Furthermore, the results were verified by the use of Bonferroni's correction for multiple t tests (Neter et al. 1983).

RESULTS

Inlet Creek Oysters

Presented in Table 1 are the percentages of agglutinated hemocytes ± standard deviations (SD) from Inlet Creek oysters that were infected with either *H. nelsoni* or *P. marinus*, as well as those of hemocytes from uninfected oysters. All three categories of hemocytes had been exposed to six dilutions of the *L. odoratus* lectin. The results of inhibition tests involving *N*-acetyl-D-glucosamine and *D*(+)-glucose are also presented.

Toler's Cove Oysters

Presented in Table 2 are the percentages of agglutinated hemocytes ± SD from Toler's Cove oysters that were infected with

TABLE 1.

Mean Percentages of Clumped Oyster Hemocytes ± SD From Inlet Creek That Had Been Exposed to Six Dilutions of the *L. odoratus* Lectin.

Group	Lectin Dilutions					
	1:1	1:2	1:64	1:512	1:1,024	1:2,048
Infected with *H. nelsoni* (n = 12)	4.4 ± 0.2	0	0	0	0	0
Infected with *P. marinus* (n = 19)	59.5 ± 5.8	45.0 ± 4.2	43.5 ± 4.4	9.2 ± 2.3	2.3 ± 1.5	1.2 ± 1.1
Uninfected (n = 34)	79.4 ± 8.3	52.3 ± 6.4	48.5 ± 7.1	38.4 ± 6.6	24.3 ± 4.9	13.3 ± 1.7
Inhibition saccharides						
N-Acetyl-D-glucosamine	ninh	ninh	ninh	ninh	ninh	ninh
D(+)-Glucose	ninh	ninh	ninh	ninh	ninh	ninh

The oysters from which the hemocyte samples were collected were infected with either *H. nelsoni* or *P. marinus* or were not infected. The results of inhibition tests involving two saccharides are also presented. ninh, no inhibition.

TABLE 2.

Mean Percentages of Clumped Oyster Hemocytes ± SD From Toler's Cove That Had Been Exposed to Six Dilutions of the *L. odoratus* Lectin.

Origin of Oysters Toler's Cove	Lectin Dilutions					
	1:1	1:2	1:64	1:512	1:1,024	1:2,048
Infected with *H. nelsoni* (n = 19)	6.5 ± 1.2	5.1 ± 0.4	0	0	0	0
Infected with *P. marinus* (n = 25)	78.0 ± 7.2	63.8 ± 5.3	35.1 ± 8.4	27.5 ± 2.4	18.3 ± 5.2	4.1 ± 2.1
Uninfected (n = 24)	84.7 ± 7.3	73.7 ± 6.9	52.2 ± 9.4	42.0 ± 8.3	29.8 ± 9.4	17.0 ± 3.3
Inhibition saccharides						
N-Acetyl-D-glucosamine	ninh	ninh	ninh	ninh	ninh	ninh
D(+)-Glucose	ninh	ninh	ninh	ninh	ninh	ninh

The oysters from which the hemocyte samples were collected were infected with either *H. nelsoni* or *P. marinus* or were not infected. The results of inhibition tests involving two saccharides are also presented. ninh, no inhibition.

either *H. nelsoni* or *P. marinus* and those of uninfected oysters from the same location. All of the hemocytes had been exposed to six dilutions of the *L. odoratus* lectin. The results of inhibition tests involving *N*-acetyl-D-glucosamine and D(+)-glucose are also presented.

Histological Sections Vs. Panning

As stated, the detection of *H. nelsoni* and/or *P. marinus* was carried out in two ways: (1) by histological examination of representative hematoxylin & eosin–stained sections, and (2) by using a modification of the panning technique of Ford et al. (1990). By comparing qualitative results, i.e., the presence or absence of either one or both of the pathogens, exact correspondence was ascertained. The numbers of oysters infected with *H. nelsoni* or *P. marinus* from both collecting sites are presented in Tables 1 and 2. Doubly infected oysters were not found.

DISCUSSION

H. nelsoni *Infections*

As presented in Table 1, hemocytes from Inlet Creek oysters that harbored *H. nelsoni* were not agglutinated at most of the concentrations of the *L. odoratus* lectin tested. The only lectin concentration at which hemocyte agglutination occurred was at the 1:1 dilution. Even then, of the 12 hemocyte samples tested, only 2 revealed very few agglutinated cells, hence, the very low mean percentage (4.4 ± 0.2%) of clumped cells. The difference between 4.4 ± 0.2 and 79.4 ± 8.3% (the mean percentages of agglutinated cells from *H. nelsoni*–infected and uninfected oysters at the 1:1 dilution of the *L. odoratus* lectin) is significant ($P < 0.001$).

As also presented in Table 1, some hemocytes from Inlet Creek oysters infected with *P. marinus* agglutinated when exposed to all six concentrations of the *L. odoratus* lectin. Furthermore, as expected, the mean percentages of agglutinated cells diminished as the dilution of the lectin was increased. Statistical comparisons of the percentages of clumped cells from uninfected oysters with those from *P. marinus*–infected oysters revealed that the lower mean percentages associated with infected oysters was significant ($P < 0.001$) when exposed to 1:1, 1:512, and 1:1,024 dilutions of the *L. odoratus* lectin. Neither *N*-acetyl-D-glucosamine nor D(+)-glucose inhibited the agglutination of hemocytes by the *L. odoratus* lectin (Table 1).

In the case of *H. nelsoni*–infected oysters from Toler's Cove, a small percentage (6.5 ± 1.2 and 5.1 ± 0.4%) of their hemocytes agglutinated when exposed to the 1:1 and 1:2 dilutions of the *L. odoratus* lectin (Table 2). Moreover, among the 19 *H. nelsoni*–infected oysters, only a small number of hemocytes from 3 agglutinated when exposed to the two lowest lectin dilutions tested. The differences between the percentages of clumped cells from uninfected oysters and those from oysters infected with *H. nelsoni* are significant ($P < 0.001$).

Also presented in Table 2 are the percentages of agglutinated hemocytes from oysters with *P. marinus* when exposed to six concentrations of the *L. odoratus* lectin. Statistical comparisons between the mean percentages of clumped cells from *P. marinus*–infected and uninfected oysters revealed that the percentages of clumped cells are significantly lower ($P < 0.001$) when exposed to the 1:2, 1:64, 1:512, and 1:2,048 dilutions of the *L. odoratus* lectin. Neither *N*-acetyl-D-glucosamine nor D(+)-glucose inhibited the agglutination of hemocytes exposed to the lectin (Table 2).

Results with Con A

As expected, in our positive controls, hemocytes from the three categories of oysters (uninfected, infected with *H. nelsoni*, infected with *P. marinus*) that had been exposed to the six dilutions of Con A agglutinated. Moreover, the percentages of clumped cells decreased as the lectin dilution increased (data not shown but essentially identical to those presented by Cheng et al. 1994). The agglutination of hemocytes exposed to Con A at all six dilutions was inhibited by *N*-acetyl-D-glucosamine and D(+)-glucose.

In view of the above, it may be concluded that the earlier observations that there is a correlation between the occurrence of lathyrose on the hemocyte surface of *C. virginica* and the essential absence of *H. nelsoni* appear to be supported, in this case, in oysters from the same region. Furthermore, the statistically insignificant difference between the mean percentages of agglutinated hemocytes in uninfected oysters from Inlet Creek and Toler's Cove (79.4 ± 8.3 and 84.7 ± 7.3%) (Tables 1 and 2) suggests that oysters from these two adjacent tributaries probably belong to the same subpopulation. However, among the oyster samples examined (65 Inlet Creek and 68 Toler's Cove oysters), 12 (18.5%) were infected with *H. nelsoni* and 19 (29.2%) were infected with

P. marinus at Inlet Creek and 19 (27.9%) were infected with *H. nelsoni* and 25 (36.8%) were infected with *P. marinus* at Toler's Cove. These differences in infection frequencies at the two sites suggest that the 1.5 miles of intertidal marsh (which is periodically dry) was sufficient as a barrier to result in the stated differences, i.e., the pathogens are totally or partially invagile.

As stated earlier, personnel of the SCMRRI, as a result of a survey conducted during May through September 1994, reported that ~25% of the oysters from Inlet Creek and Toler's Cove harbored *H. nelsoni*. Thus, it would appear that during a 12-mo period, *H. nelsoni* infection frequencies can be altered as a result of invagility.

Relative to the third question posed, i.e., whether there is inhibition of hemocyte agglutination by the *L. odoratus* lectin by intramolluscan stages of *P. marinus*, the significantly lower percentages of clumped cells from *P. marinus*–infected oysters from both Inlet Creek and Toler's Cove when compared with the percentages of clumped cells in uninfected oysters (Tables 1 and 2) indicate that inhibition did occur. This supports the earlier reports (Cheng and Dougherty 1994b, Cheng and Dougherty 1995) that such occurs and that the basis is possibly the result of the presence of lathyrose on the surface of *P. marinus*. Thus, it is predicted that in areas where *H. nelsoni* and *P. marinus* coexist, one would not expect to find the high percentages of agglutinated hemocytes when exposed to the *L. odoratus* lectin that exist in areas where *P. marinus* does not occur. Furthermore, if lathyrose is indeed in some yet undetermined way associated with the innate resistance of *C. virginica* to *H. nelsoni*, then the presence of *P. marinus* may influence the susceptibility of *C. virginica* to *H. nelsoni*. Finally, our finding of the exact correspondence between the prevalences of *H. nelsoni* and *P. marinus* in oysters from both Inlet Creek and Toler's Cove examined by histological study and panning suggests that panning is a reliable qualitative diagnostic technique.

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PREVALENCE, INTENSITY, AND DETECTION OF *BONAMIA OSTREAE* IN *OSTREA EDULIS* L. IN THE DAMARISCOTTA RIVER AREA, MAINE

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ABSTRACT Oysters, *Ostrea edulis*, collected from three locations in the Damariscotta River area, Maine, were examined for the presence of *Bonamia ostreae* four times between June 1994 and April 1995. Overall prevalence was 5% (13 of 291), with no significant differences between sampling sites or dates. All but one of the infections were classified as "low" intensity. Results obtained with histological preparations and stained blood smears were similar. Results obtained with the immunofluorescence technique were unclear. No fluorescent *B. ostreae* cells were observed with monoclonal antibody (MAB) 20B2. Even though MAB 15C2 was in 75% agreement with results obtained by standard histological preparations, the fluorescence was faint. Condition index was not significantly different between infected and uninfected oysters. Although *B. ostreae* is present in the Damariscotta River area, the development of disease and mortality may be precluded by the low density of oysters in natural beds and the relatively cold winters.

KEY WORDS: Oysters, *Ostrea edulis*, *Bonamia ostreae*, condition index, disease

INTRODUCTION

Bonamiasis, a disease affecting oysters (*Ostrea* spp.), is caused by the protozoan parasite *Bonamia ostreae* (Ascetospora) (Pichot et al. 1980). This parasite affects the flat oyster, *Ostrea edulis* L., in several countries including France, Spain, the Netherlands, Ireland, the United Kingdom, and the United States (Balouet et al. 1983, Elston et al. 1987, Montes and Melendez 1987, Farley et al. 1988, Friedman et al. 1989, McArdle et al. 1991, van Banning 1991, Friedman and Perkins 1994). Mortalities related to bonamiasis can be higher than 80% (Balouet et al. 1983, Montes and Melendez 1987, McArdle et al. 1991). Bonamiasis is characterized by hemocytic infiltration around the stomach, style sac, and intestine, and the parasite is observed as small (2–3 μm) "microcells" within hemocytes (Balouet et al. 1983, Elston et al. 1986, Sindermann 1990). In advanced cases, parasites are also seen in ciliated epithelial cells of the gills (Montes et al. 1994). The parasite is phagocytosed by hemocytes (primarily granulocytes) and localized within a parasitophorous vacuole, where it multiplies and spreads to other tissues via the hemolymph (Balouet et al. 1983, Chagot et al. 1992, Montes et al. 1994).

Transmission of the disease is likely related to the proximity of infected individuals, local currents, density of oysters, and the oyster's susceptibility to disease (Hudson and Hill 1991). Infected material (e.g., mud and organisms) moved from infected places to areas free of *B. ostreae* is another means by which the disease is spread (van Banning 1991). Susceptibility to bonamiasis varies between sites, age classes, and even between different individuals (Grizel et al. 1988, van Banning 1991, Hervio et al. 1995). Factors such as harvesting, replanting, storage, handling, and fluctuation in environmental parameters can also affect the occurrence of the parasite (Grizel et al. 1987, van Banning 1991, Cáceres-Martínez et al. 1995).

The diagnosis of *B. ostreae* is based on time-consuming histopathological techniques. Two alternative methods of diagnosis that promise to be faster, less expensive, and more accurate have been developed: stained heart smears and indirect antibody immunoassay (Mialhe et al. 1988b, Boulo and Mialhe 1989).

B. ostreae has recently been detected in *O. edulis* from the New Meadows River, Quahog Bay, and the Damariscotta River, ME (Barber and Davis 1994, Friedman and Perkins 1994). The Damariscotta River is the center of oyster culture in Maine because of favorable environmental conditions, including high primary productivity (Hidu et al. 1981). Oyster farmers are interested in culturing *O. edulis* but are concerned about the potential effect of *B. ostreae*. Knowledge of the distribution of *B. ostreae* in the Damariscotta River area and its prevalence at various sites and times of year provides an important starting point for making decisions regarding the culture of flat oysters in Maine. The objectives of this study were to determine 1) the seasonal prevalence and intensity of infection of *B. ostreae* in natural oyster beds in the Damariscotta River area, and 2) whether the condition index of oysters is affected by infection. Three diagnostic techniques for detecting *B. ostreae* (histological preparations, blood smears, and indirect immunofluorescence) were compared.

MATERIALS AND METHODS

Oysters were collected from three locations (each more than 6 km apart) within the Damariscotta River watershed: Little Point (Lat. 44°01′N; Lon. 69°32′W); Mears Cove (Lat. 43°57′N; Lon. 69°34′W); and Witch Island, Johns Bay (Lat. 43°52′N; Lon. 69°33′W) (Fig. 1). The density of oysters at each site was estimated by counting the oysters present in 10 (1-m^2) quadrats along a transect. Water temperature was registered throughout the year with temperature-recording devices (Ryan RTM 2000 Thermographs) installed at Little Point and at the Darling Marine Center (near the Mears Cove location). Records of temperature from the Maine Department of Marine Resources, Boothbay Harbor, were considered an approximation of temperature at Witch Island. At all stations, monthly average surface temperature was calculated from records obtained every 6 h.

Scuba divers hand collected samples of 25 adult oysters of similar size (>70 mm in length) from each location on June 10th, August 25th, and November 30th, 1994, and on April 18th, 1995. Fouling organisms were removed before whole weights of each

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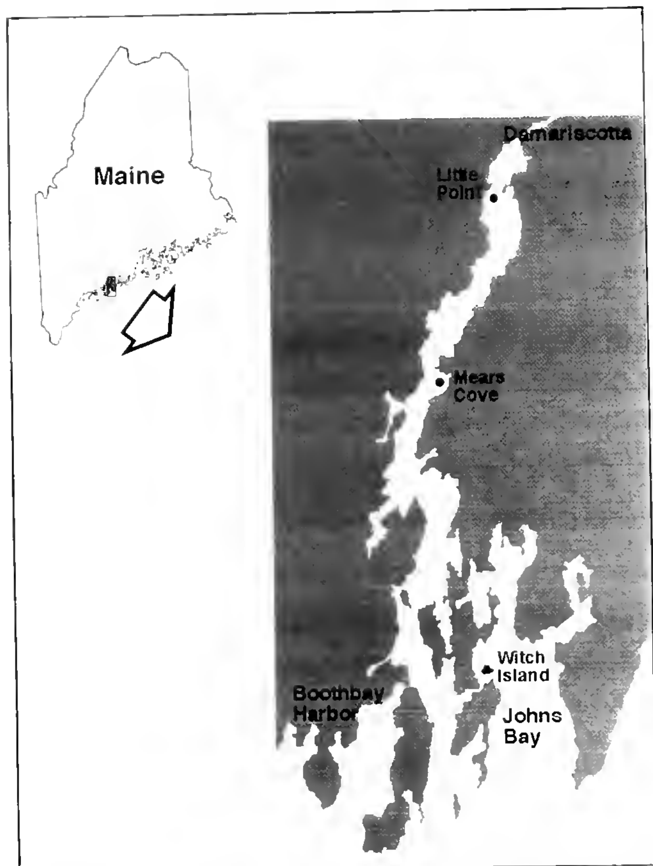

Figure 1. Detail map of the Damariscotta River area, Maine, showing locations of sampling sites: Little Point, Mears Cove, and Witch Island.

oyster were obtained. Oysters were then opened, and a cross-section (4–5 mm) adjacent to the labial palps containing the digestive gland, gonad, and gill tissues was removed and weighed (wet) before fixation for histopathological examination. The wet weight of the remaining tissue was recorded. The remaining tissue was then placed in an individual weighing dish, dried in a drying oven (82°C) to a stable weight, and held at room temperature in a desiccator before dry weight was recorded. Dry shell weight was recorded in a similar fashion. A dry weight:wet weight ratio of the remaining tissue was used to estimate the dry weight of the cross-section; the dry weight of the cross-section and the dry weight of the remaining tissue were added to obtain the dry weight of the entire animal (Barber et al. 1988).

Condition index was determined for each individual as: dry soft tissue weight (g) × 1,000/internal shell cavity capacity (g), where internal shell cavity capacity = the total whole live weight (g), minus the dry shell weight (g) (Hawkins et al. 1987). The condition indices of uninfected oysters were examined with respect to site and sampling date by the use of two-factor analysis of variance without replication (Zar 1984). A Student's *t*-test (Zar 1984) was used to compare condition indices of infected and uninfected oysters collected in November from Little Point and Witch Island, because at Mears Cove and on other sampling dates, the parasite was either absent or was observed at very low prevalence.

Histological Preparations

Tissue cross-sections were fixed in Helly's fixative (June 1994 only) or Dietrichs' fixative (all other samples) (Barszcz and Yevich 1975) and processed for routine paraffin histology. Deparaffinized tissue sections (5 μm) were stained with Shandon's instant hematoxylin and eosin-Y and coverslipped with Flo-texx mounting medium. Stained tissue sections were examined with a Nikon Labophot microscope (100×), and the presence of hemocytic infiltration was recorded. The number of *B. ostreae* cells observed in 10 min (phase contrast, 1,000×) in a randomly selected transect (including mantle, gonad, intestine, and digestive gland) of each histological preparation was recorded. The confidence intervals for Poisson random variables were calculated to detect differences in the prevalence of *B. ostreae* (number of infected oysters) among the three sites (Beyer 1968).

Infection intensity was established by the procedure of Rogan et al. (1991). Oysters were "negative" if no parasites were observed within 10 min. In oysters with "low" infections, fewer than 100 parasites were observed within 10 min. In "heavy" infections, practically all blood cells were parasitized. A Student's *t*-test (Zar 1984) was performed to compare the level of infection (logarithm of the number of parasites per infected oyster) of samples collected in November from Little Point and Witch Island, the only samples where more than one parasite was detected.

Blood Smears

Cardiac tissue was used in the preparation of smears because it provides the most accurate diagnosis of *B. ostreae* infection (Boulo and Mialhe 1989). The heart (ventricle) of each oyster collected in June 1994 was removed, dried on blotting paper, pressed onto a slide, and air dried. Because of the excessive aggregation of hemocytes, oysters collected subsequently were processed as follows. A syringe (3 cm^3) was inserted into the pericardial cavity, and hemolymph (0.5 ml) was drawn into the barrel. The hemolymph was diluted with 2.5 ml of filtered (0.45-μm-pore-size filter) seawater. Aliquots (0.5 ml) of diluted hemolymph were placed on slides, and hemocytes were allowed to settle (15 min). Excess seawater was drained, and the smears were air dried.

Smears were fixed (LeukoStat fixative solution, Fisher Diagnostics), stained with Hemacolor blood stains (EM Diagnostic Systems), and mounted with Flo-texx mounting medium. The slides were observed for 10 min under the microscope (1,000×) and the number of parasites was recorded. Slides were considered positive only when the parasite was unambiguously observed. Slides were considered negative when, after two observations of more than 10 min each, either no parasite was detected or suspicious intrahemocytic inclusions (smaller diameter and without an evident nucleus) could not be positively identified as *B. ostreae* cells.

McNemar's test (Zar 1984) was performed to compare the accuracy of histological preparations and stained blood smears in determining the prevalence of infection. A paired Student's *t*-test (Zar 1984) was used to compare the intensity of infection recorded by the two techniques.

Indirect Immunofluorescence

Air-dried smears were fixed in acetone for 8 min and frozen at −20°C. The smears were overlaid with a monoclonal antibody solution (MAB). Two concentrations of MAB were tested, 50 and 100 μg/ml (in phosphate buffer solution [PBS]: phosphate, 10 mM; NaCl, 150 mM; pH 7.4). After incubation (30 min) at room temperature in a moist chamber, the slides were washed with PBS and then overlaid with fluorescein isothiocyanate–conjugated anti-

Table 1.

Estimated Density of Oysters, *O. edulis*, Substrate Type, and Depth Range (Low Tide) at Each Collection Site.

Location	Density (Oysters/m²)	Substrate	Depth (m)
Little Point	0.5	Shell hash, cobble, sandy-mud	2–5
Mears Cove	0.33	Soft mud	5–8
Witch Island	0.04	Shell hash, sandy mud	2–4

mouse immunoglobulin antiserum (Sigma Immunochemicals) diluted in PBS containing 0.01% Evans-Blue. The slides were again incubated and washed as described. The slides were mounted with glycerin buffer solution and immediately examined for fluorescence with a Nikon Fluophot microscope at 1,000× (Boulo and Mialhe 1989). *B. ostreae* cells were counted over a 10-min period. Two *B. ostreae* MAB, 20B2-1B12 and 15C2-2F2 (IFREMER, Montpellier, France), were tested. The technique was tested with oysters collected in November from the three locations and with oysters collected in April from Little Point and Mears Cove. Results from the histopathological technique were used as the control.

RESULTS

The Little Point and Witch Island sites had similar bottom characteristics, being primarily composed of shell hash and sandy-mud substrate (Table 1). Both sites were fairly shallow, with depth ranging from 2 to 5 m at low tide. In contrast, Mears Cove had a softer substrate (mud) and was deeper at low tide, 5–8 m. The highest estimated density of oysters ($0.5/m^2$) was recorded at Little Point (Table 1).

The monthly average temperature at Little Point was higher than that at the other two sites from May to September, ranging from 13 to 22.5°C (Fig. 2). At Mears Cove and Witch Island, the range for the same period was 9–18°C.

In total, 291 oysters were processed throughout the year of sampling. Hemocytic infiltration was evident in 16% (45 of 291) of the oysters. Only 5% (13 of 291) of all the oysters examined throughout the year were infected with *B. ostreae*. Suspicious intrahemocytic inclusions were seen in another 5% (13 of 291). Of the 13 infected oysters, 31% (4 of 13) had focal inflammation of connective tissue, and *B. ostreae* cells were found only in those foci. Concurrent inflammation and *B. ostreae* infection were seen in 47% (9 of 19), 7% (1 of 13), and 14% (2 of 14) of oysters from Little Point, Mears Cove, and Witch Island, respectively. The parasite was observed in gill tissue in most of the infected oysters, even when not detected in connective tissue around the intestine or digestive gland, and was present in the intestinal wall only in more highly parasitized oysters.

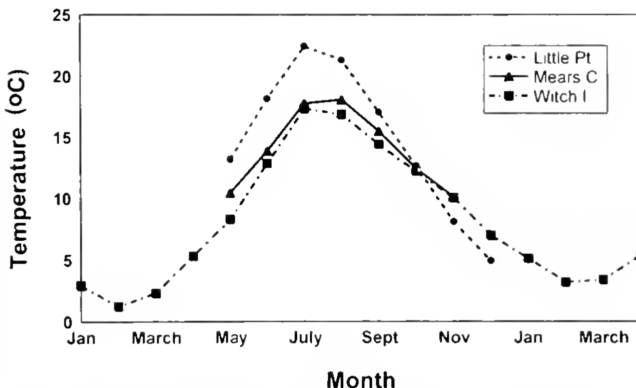

Figure 2. Monthly average water temperatures from the three sample locations (Little Point, Mears Cove, and Witch Island) from January 1994 to April 1995.

TABLE 2.

Number of Oysters, *O. edulis*, Infected with *B. ostrea* (as Determined by Histological Examination), by Site and Sampling Date; Sample Size Indicated in Parentheses.

Location	10 Jun 1994	25 Aug 94	30 Nov 1994	18 Apr 1995
Little Point	0 (25)	0 (23)	8 (25)	1 (25)
Mears Cove	0 (25)	0 (25)	0 (25)	1 (25)
Witch Island	0 (25)	0 (25)	3 (25)	0 (17)

Prevalence and Intensity of B. ostreae

In this study, even though approximately 8.7% (13 of 148) of oysters collected between June and August had suspicious intrahemocytic inclusions, typical *B. ostreae* cells were not observed before November 1994 (Table 2). The confidence interval for Poisson random variable indicated that there were no significant differences ($P > 0.05$) among sites in the prevalence of *B. ostreae* in November 1994 (confidence limits: Little Point, 3.4–15.8; Mears Cove, 0.0–3.7; Witch Island, 0.6–8.8) and April 1995 (Little Point, 0.0–0.2; Mears Cove, 0.0–0.2; Witch Island, 0.0–0.2); there were no significant differences ($P > 0.05$) between Little Point (0.1–0.6) and Witch Island (0.0–0.2) for samples collected in November 1994 and April 1995.

The intensity of *B. ostreae* infection, on the basis of the examination of histological preparations, was "low" in most of the infected oysters (Table 3). Only one individual (Little Point, November 1994) had a "heavy" infection (practically all blood cells parasitized). The intensity of *B. ostreae* infection, however, was not significantly different between Little Point and Witch Island oysters collected in November (*t*-test, $P = 0.46$).

Condition Index

Two-factor analysis of variance without replication revealed no significant differences in condition index (range, 82.2–217.6) associated with either site ($P = 0.28$) or sampling date ($P = 0.20$) (Table 4). Even though infected oysters collected in November

TABLE 3.

Intensity of *B. ostreae* Infection as Determined From Histological Preparations of *O. edulis*.

	10 Jun 1994		25 Aug 1994		30 Nov 1994		18 Apr 1995	
Location	M	R	M	R	M	R	M	R
Little Point	0	—	0	—	9.5	4–334	1	—
Mears Cove	0	—	0	—	0	—	1	—
Witch Island	0	—	0	—	10	1–20	0	—

M, median values of number of parasites seen in 10 min in each infected oyster, by site and sampling date; R, range of parasite number.

TABLE 4.

Gravimetric Condition Indices of Oysters, *O. edulis*, Uninfected and Infected With *B. ostreae*, by Site and Sampling Date.

	Little Point				Mears Cove				Witch Island			
	Uninfected		Infected		Uninfected		Infected		Uninfected		Infected	
Date	M	SD	M	SD	M	SD	M	SD	M	SD	M	SD
10 Jun 1994	107.0	19.8	—	—	138.0	38.5	—	—	115.3	35.0	—	—
25 Aug 1994	89.9	28.0	—	—	82.2	23.7	—	—	136.2	106	—	—
30 Nov 1994	120.7	45.4	106.9	45.7	217.6	527	—	—	139.7	41.8	97.4	27.2
18 Apr 1995	113.9	37.4	67.2	—	133.4	33.9	52.1	—	114.7	25.8	—	—

M, mean; SD, standard deviation.

1994 at Little Point and Witch Island tended to have lower average condition index (104.4 ± 40.4) than noninfected oysters (131.2 ± 44.5), the difference was not significant (*t*-test; P = 0.892).

Comparison of Techniques

The quality of blood smears prepared from heart tissue collected in June 1994 was unsuitable for parasite screening because of dense cell aggregations. Subsequent smears prepared from diluted blood were satisfactory. Even though *B. ostreae* cells stained with Hemacolor were easily observed, diagnosis was uncertain in 2% (5 of 217) of oysters. The parasite was observed principally in agranular cells.

Although 13 oysters were determined by histological preparation to be infected, only 6 were detected with stained blood smears. In contrast, three infected oysters, detected with blood smears, were considered uninfected after histological examination. In each of them, however, only one parasite was observed. The McNemars's test, used to compare the prevalence of infection detected by histological preparations and stained blood smears, revealed no significant differences between the two techniques (Z = 0.90). In five of the six infected oysters detected by both techniques, histological preparations detected a 50% greater number of parasites than did stained blood smears. A comparison of the mean differences between the number of parasites detected by histology and blood smears, however, showed that the techniques were not significantly different (P = 0.34). Low-intensity infections characterized by focal hemocytic infiltration were detected only by histological examination.

Histological results were used as references for immunological tests performed on samples collected in November and April. Sections of uninfected animals, used as controls, showed no background or nonspecific fluoresence with both 20B2-1B12 and 15C2-2F2 at both concentrations (50 and 100 μg/ml PBS). *B. ostreae*–positive slides tested with 20B2 did not fluoresce at either 50 or 100 μg/ml PBS. Seventy-three percent of *B. ostreae*–positive slides tested with 15C2-2F2 showed faint fluorescence associated with parasite cells. Results from one slide were indeterminate, and results from another were negative. The fluorescence pattern was similar at both MAB concentrations (50 and 100 μg/ml).

DISCUSSION

B. ostreae was found at all three sampling sites; however, no obvious *B. ostreae* cells were detected before November 1994 at any site. At Mears Cove and Witch Island, prevalence was 8% or less throughout the year of sampling. Prevalence was 32% at Little Point in November 1994. Past prevalences of *B. ostreae* infections at Little Point, detected by the use of histological preparations, were 34% in June 1991, 45% in June 1992, and 20% in August 1993 (Barber and Davis 1994, Friedman and Perkins 1994). Thus, the prevalence of *B. ostreae* in the Damariscotta River at Little Point has been relatively steady since the parasite was first detected in 1991.

The finding of low prevalence and low infection intensity indicated a general lack of disease development in oysters parasitized by *B. ostreae* in Maine. In addition, a high percentage of oysters had focal hemocytic infiltration, rather than general inflammation. Ninety-two percent of infected oysters had a "low" infection intensity; in many, fewer than 10 parasites were observed. Even though infected oysters examined in 1993 had a higher intensity than those in this study, intensity was still within the "low" range of infection (B. Barber unpublished data). The prevailing low prevalence and low infection intensity of *B. ostreae* infection in Maine oysters may be related to low densities of oysters and prevailing climatic conditions in Maine (long, cold winters followed by short, mild summers), both of which restrict the spread of the disease (Elston and Holsinger 1988). Accordingly, the highest prevalence and intensity of *B. ostreae* recorded in this study occurred at Little Point, where oyster density and water temperature were the greatest.

Parasitic infections have been shown to reduce the condition index of bivalves (Montes and Melendez 1987, Barber et al. 1988). The results of this study revealed no significant differences between condition indices of infected and uninfected oysters collected in November at Little Point and Witch Island, when infection intensity was greatest. Rogan et al. (1991) suggested that condition indices were not appreciably affected in oysters with low infection intensity. All but one infected oyster found in this study had low intensities of infection; this may have contributed to the lack of detection of a significant difference in condition index between infected and uninfected oysters. This lack of effect on condition index is another indication that levels of *B. ostreae* in the Damariscotta River in 1994–1995 were generally not great enough to cause disease.

The detection of *B. ostreae* is difficult in animals with a low infection intensity. The absence of infected oysters in samples collected between June and August 1994 could be the result of the misdiagnosis of lightly infected oysters, a small sample size, or a combination of both factors. A larger sample size would be necessary to detect *B. ostreae* in areas with both low prevalence and low infection intensity (Ossiander and Wedemeyer 1973). Unfortunately, a scarcity of oysters limited sample size throughout this study.

Histological preparations detected more infected oysters than

did stained blood smears, although the difference was not significant. Failure to detect the parasite with stained smears at low infection intensity has been previously reported (Bucke 1988, Bucke and Feist 1985, McArdle et al. 1991). Histological preparations are a time-consuming and expensive diagnostic technique. Without significant differences in detection between these techniques, stained blood smears are thus adequate for preliminary studies or in systematic screening to help determine infection status in a particular area. By the use of blood smears, a larger sample can be screened rapidly, without sacrificing oysters. Histological preparations should be reserved for more exhaustive research, or for situations where the detection of light (early) cases is important (e.g., before moving oysters to areas free of *B. ostreae*).

The immunofluorescence technique developed by Mialhe et al. (1988b), tested extensively for the first time on oysters from Maine, gave unclear results. MAB 20B2 did not bind *B. ostreae* cells on any of the tested slides. Fluorescence of *B. ostreae* cells was observed in 73% of the positive slides tested with 15C2, but the pattern was faint. The differential performance of the MAB solutions may be explained by a different decline in the affinity for an epitope caused by an excess of seawater (V. Boulo pers. comm.). No antigenic differences have been observed between *B. ostreae* from Washington and from Europe (Mialhe et al. 1988a). Potential serological differences between *B. ostreae* and the parasite observed in this study, however, cannot be ignored. *B. ostreae* has been observed in all hemocyte types in *O. edulis* but is found primarily in granulocytes (Balouet et al. 1983, Mourton et al. 1992). The fact that the parasite in this study was observed principally in agranular cells might also indicate a difference between *B. ostreae* from Maine and from Europe. Additional immunofluoresence assays should be developed to improve this technique, which has been shown elsewhere to be as effective as histology and stained blood smears in detecting the disease (Boulo and Mialhe 1989). In addition, because the immunofluorescence assay is an important taxonomic tool (Mialhe et al. 1988a), it could be used to ultimately verify the identity of *B. ostreae* in Maine.

B. ostreae is currently resident in several locations in Maine (Barber and Davis 1994, Friedman and Perkins 1994, this study). Even though the prevalence and intensity of the parasite are at present relatively low, the potential for oyster mortality caused by bonamiasis remains. Further experiments growing oysters under commercial conditions (high density) will be necessary to determine the potential effects of *B. ostreae* on the commercial culture of *O. edulis* in Maine.

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FACTORS AFFECTING THE GRAZING RATE OF THE NEW ZEALAND ABALONE *HALIOTIS IRIS* MARTYN

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ABSTRACT Grazing rates of the New Zealand black foot paua *Haliotis iris* Martyn were measured for individuals supplied with four species of shallow subtidal algal food. Measurements were made on 11 length groups of paua, 16–120 mm. Feeding rates depended on body size, with a decrease in the weight-specific grazing rate with increasing shell length up to 66 mm. Grazing rates for rhodophytes, *Gracilaria sp.* and *Hymenocladia sanguinea* exceeded those on phaeophytes, *Macrocystis pyrifera* and *Lessonia variegata*. The assimilation efficiency of paua feeding on *Gracilaria sp.* was the highest of the four species tested (85%). During winter, abalone grazing rates at a temperature of 10°C were significantly reduced compared with summer values at 17.5°C. Consumption rates on the kelp *L. variegata* were similar during summer and winter. Laboratory experiments investigated the effects of density on the feeding and growth rate of juvenile paua, initial length 35–45 mm, over a year. Although feeding rates were relatively constant with increasing density, annual length increment decreased. At densities greater than 40 individuals per cubicle (400 m^{-2}), there was no length increase and individuals lost weight. The highest growth rate was found at the lowest density, where an annual length increment of 9.2 mm was about half that recorded from field populations. The grazing rates of the black foot paua feeding on natural algal food are discussed in relation to their natural diet, and the algae are evaluated for their potential use as a food supply for abalone mariculture.

KEY WORDS: Abalone, grazing rates, growth, gastropod, *Haliotis iris*

INTRODUCTION

Haliotis iris, the common or black foot paua, is the largest of the three species of abalone found in New Zealand. It is discontinuously distributed along the coast of both main islands in New Zealand, Stewart Island and Chatham Islands (Poore 1969). Although there has been worldwide interest in the ecology and growth of abalone in different parts of the world (Shepherd 1973, McShane et al. 1988, Tegner et al. 1989, Day and Fleming 1992), there is relatively little information about the New Zealand species, which are of increasing interest for commercial culture. Ecological studies on *H. iris* have provided details of natural diet, and growth rates have been calculated from field studies (Poore 1972a, Poore 1972b, Sainsbury 1982, Pirker 1992). These studies suggest variable growth rates depending on geographical location, seasonal temperatures, and food supply.

Recent studies on the feeding physiology of gastropods suggest that dietary composition depends on many factors, including food quality, quantity, palatability, and nutritional value (Paine and Vadas 1969, Steneck and Watling 1982, Carefoot 1987, Brendelberger 1995). Food choice may not be predictable, with some species of gastropod herbivores showing food preferences based on the physical structure of the food rather than its nutritional value (Andrew 1986, Chew 1984). Food availability may also determine the dietary composition of some gastropods including abalone (Shepherd and Steinberg 1992). Poore (1969) examined the gut contents of *H. iris* from subtidal areas close to our laboratory and found that they fed mainly on red algae throughout the year. In contrast, paua collected 200 km south in a more protected locality fed mainly on drift weed consisting mainly of the brown kelp *Macrocystis pyrifera*.

This study was prompted by recent interest in the culture of the black foot paua using natural food supplies. Our goals were to estimate assimilation efficiencies and laboratory grazing rates of paua supplied with four different species of macroalgae. Three of these were locally abundant and known to be part of the natural diet of the paua (Poore 1972a, Sainsbury 1977). The fourth species, *Gelidium species*, was included because it has been identified as a potential food source for the commercial culture of abalone elsewhere (Pickering 1986, Tong 1986). Laboratory experiments were designed to investigate the effects of body size and season on the feeding rate of abalone. In addition, because of interest in land-based paua farming or barrel culture, the effects of density on algal consumption and the growth of *H. iris* were investigated by a laboratory experiment extending over a period of a year.

MATERIALS AND METHODS

A preliminary feeding experiment was undertaken to determine an appropriate level of food supply for the paua and a duration for the consumption experiments. This would be the time period at which variance of the mean consumption rates were least. Five individuals from three length groups (36–40, 66–70, and 96–100 mm) were supplied with a known wet weight of *M. pyrifera*, one of the common species of drift algae found at Kaikoura. The paua were maintained in aquarium conditions with running seawater at a temperature of 12°C. The amount of *M. pyrifera* consumed by each individual was recorded every 24 h over a 15-d period. Daily consumption rates for each size group were calculated as dry weight of algal frond consumed per individual. This experiment showed that, for all three size groups, the variance in the daily algal consumption decreased from 1 to 5 d and then remained similar. As expected, small *H. iris* consumed more algae per dry weight of tissue than larger individuals. On the basis of these results, we chose to investigate the feeding rates of paua using a standard 5-d trial period.

The grazing rates of *H. iris* were estimated during winter (August) when the aquarium seawater temperature was 10°C and summer (January) at a temperature of 17.5°C. These temperatures were approximately 1.5–2°C above the coastal surface seawater temperatures. The paua were collected from intertidal and shallow subtidal areas close to the marine laboratory. They were separated

into 11 5-mm-length groups ranging from 16 to 20 mm (first-year juveniles) to the largest size group, 116 to 120 mm in length. Any paua that had been damaged during collection (broken shell or damaged foot) were excluded. Experimental animals were allowed to acclimate to laboratory conditions over a period of 2 wk, during which they were supplied with a mixed selection of the algae that would be presented to them during the experiments. Faeces and any other material that accumulated at the bottom of the flow-through tanks were removed daily. The tanks received a low-level natural light regime.

Four species of macroalgae were used in the experiments—two phaeophytes, *M. pyrifera* and *Lessonia variegata*, and two rhodophytes, *Hymenocladia sanguinea* and *Gracilaria* species. The *Gracilaria sp.* was collected weekly from intertidal areas close to Christchurch and transported to Kaikoura, where it was kept at 4°C. The other three species were freshly harvested from the same site every 2 d.

At the start of each 5-d experiment, the length (in millimeters) and wet weight of five individuals from the 11 size groups were measured. Individuals were held separately and supplied with a known wet weight of one of the test algae. Algal treatments in each holding tank were assigned randomly. After each 24 h, the remaining algae were removed, reweighed, and replaced with a known quantity of fresh algae. Control tanks containing algae but without animals were used to test for any deterioration or decomposition of the algal food source. Algal wet weight loss was converted to dry weight loss by comparing wet/dry weight relationships from subsamples of algae taken at regular intervals and oven dried at 60°C. The dry weight of tissue from individual paua was estimated from a single sample (n = 30) covering the whole length range. Daily algal consumption rates were calculated for each size group, and consumption rates during winter and summer were compared by use of analysis of variance (ANOVA) (Snedecor and Cochran 1976). If the variances were not homogeneous (Bartlett's test), trends were determined by the use of the nonparametric Kruskal-Wallis test (Siegel 1956, Sokal and Rohlf 1981). The Scheffe comparison of means test was used to compare means of the different size groups.

The assimilation efficiencies of *H. iris* feeding on the four freshly collected test algae were determined by standard bomb calorimetry (Phillipson 1964). Eight paua from each of three length groups were used; 36–40, 66–70, and 96–100 mm in shell length. Animals were acclimated for 2 wk, during which they were supplied with a mixture of the test algae. They were starved for 72 h to evacuate their gut contents before being presented with a preweighed sample of a single alga. After 48 h, the remaining alga was removed and reweighed to estimate the feeding rate. Faeces from the group were collected for up to 48 h. These were washed quickly in distilled water to remove salts and oven dried at 60°C. Other groups of paua were held in similar conditions without food as control groups. The energy value of each algal species was calculated for freshly collected specimens and for those held in a circulating seawater system for 5 d. This latter sample was used to simulate drift algae and to evaluate its potential value as a food source for paua. The assimilation efficiency for each size group of paua was calculated by difference in the energy content (Kcal $\cdot$ g^{-1} dry weight) between ingested and egested food material.

The effects of density on the feeding and growth rates of juvenile paua (length, 35–45 mm) were evaluated for groups of 2, 10, 20, 40, 60, and 80 individuals in each cubicle (39 × 27.5 × 8 cm depth; surface area, 2,136 cm^2). Polythene tubes were cut in half and placed in the cubicles to provide shelter. Preweighed algal food was supplied to the groups at 24-h intervals when any remaining alga was removed. There were four replicates for each density. Individual paua were measured initially after the first and second month and then at 2-mo intervals for a year. Abalone grazing rates were measured during autumn (March), as described in the previous section.

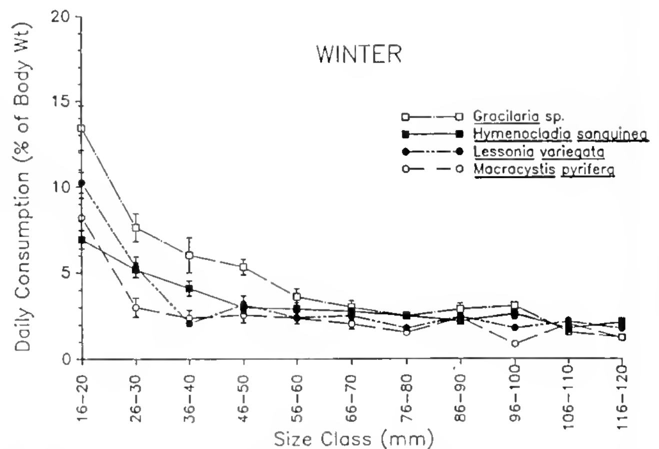

Figure 1. Effect of body length on the algal consumption of *H. iris* during winter. Values are mean values ±1SE; n = 25 for each species.

RESULTS

Effects of Body Size and Season on Algal Consumption

During winter, the mean daily consumption rates for *H. iris* varied with both size and food type (Fig. 1); smaller abalone showed increased consumption compared with larger individuals. The Scheffe comparison of means test showed similar consumption rates for adjacent length groups, and for individuals above 56 mm, the daily consumption rates were similar.

Summer mean consumption rates of abalone were higher than winter values, reaching 18.7% of the body weight for the 16–20 mm-length group (Fig. 2). Mean consumption rates of adjacent length groups were similar (Scheffe comparison of means test), and three size groups, differentiated. These were smaller individ-

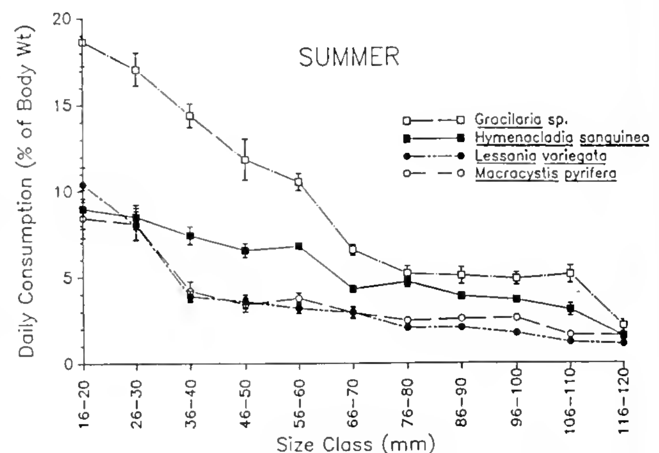

Figure 2. Effect of body length on the algal consumption of *H. iris* during summer.

TABLE 1.

ANOVA Comparing the Effects of Size, Season and Algal Food Type on the Grazing Rate of Paua.

Season	Variable	df	F	P<
Winter	Size class (Kruskal-Wallis)	n = 220	110.8	0.001
	Algal species	3,219	2.2	NS
	Rhodophyte/phaeophyte	1,219	12.4	0.001
Summer	Size class	10,2	30.9	0.001
	Algal species	3,219	25.2	0.001
	Rhodophyte/phaeophyte	1,219	57.2	0.001

The data were normalised by $\log^e$ transformation. NS indicates a value that is not significant.

TABLE 3.

Caloric Values (Kcal g^{-1} Dry Weight) for Fresh and Experimentally Produced Drift Algae.

Algae	Fresh	Drift	% Change	Assimilation Efficiency	Energy Value
Gracilaria sp.	4.4	4.25	−3.4	85.4	3.76
H. sanguinea	3.6	3.25	−9.7	65.3	2.35
L. variegata	3.8	3.75	−1.3	64.3	2.44
M. pyrifera	3.9	3.80	−2.6	75.0	2.93

Also shown are the % assimilation efficiencies of *H. iris* feeding on particular algal species. Energy value is the potential Kcal · g^{-1} algae available to *H. iris*.

uals (16–30 mm in length), intermediate individuals (36–60 mm), and larger individuals (66–120 mm). By the use of $\log^e$ transformed results, it was found that winter feeding rates differed significantly from summer values (Kruskal-Wallis $F = 40.3$; $P = 0.001$; $n = 440$). Analyses of these results grouped into size groups suggest that for the largest individuals there was little variation in the algal consumption rates with season.

Effect of Algal Species on Paua Feeding Rates

In all experiments, individuals less than 70 mm in body length consumed more *Gracilaria* than *Hymenocladia*, with *L. variegata* and *Macrocystis* being eaten in similarly lesser quantities. In summer, abalone consumption of all four algae differed, whereas during winter, when overall consumption rates were reduced, the paua consumed similar amounts of algal species grouped into their taxonomic groups. The winter grazing rates of abalone on the red algae were significantly less than that recorded for the brown algae (ANOVA; Table 1). Results from all size groups were combined to compare the seasonal grazing rates on the four algal foods. These showed significantly greater summer consumption by the abalone for three of the four species tested (Table 2). The paua grazing rate on the small kelp *L. variegata* was similar in summer and winter.

Assimilation Efficiencies

The caloric values (Kcal · g^{-1} dry weight) of fresh and experimentally produced drift seaweeds are shown in Table 3. *Gracilaria* species had the highest value of the four species tested, and values remained high in all species except *Hymenocladia* after 5 d of immersion. The assimilation efficiency of *H. iris* feeding on *Gracilaria* exceeded 85%. The three other algal species had a similar caloric value, but the assimilation efficiency of paua feeding on *Macrocystis* exceeded that for the two red algae. When calculated as the potential energy gain per unit dry weight of algal food, *Gracilaria* remained the most efficient food, followed by *Macrocystis*, *Lessonia*, and *Hymenocladia*.

TABLE 2.

ANOVA Comparing the Effect of Season (Winter/Summer) on the Grazing Rates of Paua Feeding on Four Different Algal Species.

Algal Species	df	F	P<
Gracilaria sp.	1,109	29.2	0.001
H. sanguinea	1,110	23.4	0.001
L. variegata	1,110	0.2	NS
M. pyrifera	1,110	13.4	0.001

Density Experiments

The effect of density on the algal consumption of juvenile paua held long term in aquarium conditions is shown in Figure 3. Feeding rates ranged from 8.2% of mean body weight per day at the lowest density to 5.5% at a density of 60 individuals in each container. One-way ANOVA indicated no significant effect of density on the feeding rate ($F = 2.39$; $P = 0.08$; $df = 5, 23$), but paired tests detected differences between feeding rates of paua held at densities of 2 and those held at 60 or 80 individuals in each container.

During the density experiment, mortality was low. Of the nine individuals that died over the year, six deaths occurred in the first month. These were replaced by individuals of a similar shell length that had been held for a similar period in the laboratory. At the three lowest densities, length and weight increases were seasonal (Fig. 4), with the highest values from November to February and little growth between March and August. The bimonthly mean length increase did not exceed 3 mm, corresponding to a wet weight increase of approximately 2.5 g at the lowest density. Over the experimental period of a year, there was no significant length increase in paua maintained at densities of 40, 60, and 80 individuals per container (Fig. 5). Individuals held at the two highest densities lost weight over the exposure period.

The effects of density on the annual weight and length increase

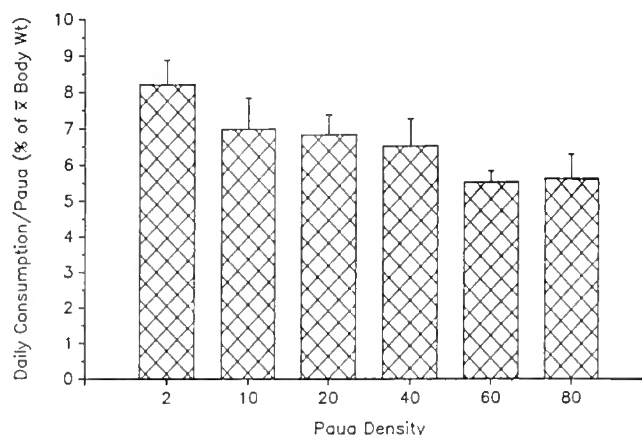

Figure 3. Effect of stock density on the consumption rate (mean ± SE) of *H. iris* feeding on *M. pyrifera* during autumn.

of *H. iris* fed *M. pyrifera* are shown in Figure 5. Growth rate declined with increasing stock density, and paua lost weight at densities of more than 40 individuals per container. The greatest growth rates occurred at the lowest density, where the 9.2-mm increase in body length represented a doubling of the initial wet weight.

DISCUSSION

Laboratory grazing rates of the black foot paua depend on body size, food type, season, and density. Although the feeding rates were maintained at relatively high levels, the slow growth rates achieved during this study might suggest relatively poor potential for mariculture using natural food sources.

Body size is a major factor affecting the feeding rates of gastropods. As in previous studies, the weight-specific grazing rates of smaller paua exceeded those for larger individuals (Paul et al. 1976, Day and Fleming 1992). However, the grazing rate remained constant above 60 mm in length. In *H. iris*, sexual maturity occurs between 60 and 80 mm shell length (Poore 1972b) with gonad maturation over winter. The relative independence of the grazing rate beyond the size at sexual maturity most likely reflects changes in energy allocation. This adaptation would allow comparatively less energy to be used in body maintenance and shell and somatic growth and more energy to develop reproductive tissues. Some support for this can be found from fecundity values in *H. iris*, which increase markedly at shell lengths greater than 80 mm (Poore 1973).

During summer, higher values for the feeding rate of the black foot paua reflect metabolic responses to increased temperature. Like *Haliotis gigantea* and *Haliotis discus hannai* (Ino 1943, Ino 1952), *H. iris* exhibited maximal grazing rates during spring and summer and the lowest values during autumn and early winter. Although this contradicts a prediction made by Poore (1969), his conclusions were based on measurements of gut fullness. Low winter temperatures may affect abalones by reducing feeding activity and increasing gut passage time. This could explain why paua with full guts were found in winter field populations.

For all size groups of *H. iris*, consumption rates on red algae exceeded those for brown algae. Similar findings were reported by Fleming (1995a) working on *Haliotis rubra*. Also, differences in feeding rates between the algal species were more pronounced in summer, when the overall levels were elevated for all size groups. The assimilation efficiencies or digestibility of algae used in our experiments did not correlate with taxonomic divisions. This contrasts with research on *H. discus hannai*, where assimilation efficiency was higher in brown than in red algae (Ino 1952, Sakai 1962).

Steinberg (1985) and Sakata et al. (1988) tried to explain discrepancies in feeding behaviour in terms of the palatability or attractiveness of the alga. Some abalones are thought to avoid algae high in phenolic compounds because they inhibit nitrogen digestion (Fleming 1995b). Preliminary examination of phenol levels from the algae used in our experiments suggested the highest levels for *Lessonia*, intermediate levels for *Gracilaria*, and the lowest values for the other two species (Williams 1990). If low phenol levels make an alga attractive and therefore more palatable, then subsequent choices may be made on the basis of food quality or in response to particular components such as nitrogen levels or trace elements. For example, in the case of *Macrocystis*, its low phenol levels, high energy content, and the ability of *H. iris* to assimilate it makes it potentially a better food source than some red

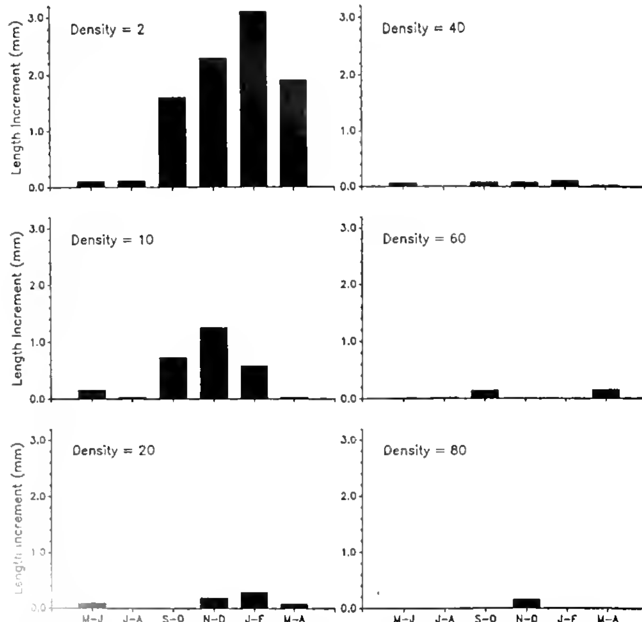

Figure 4. Effect of paua density on individual bimonthly length increase.

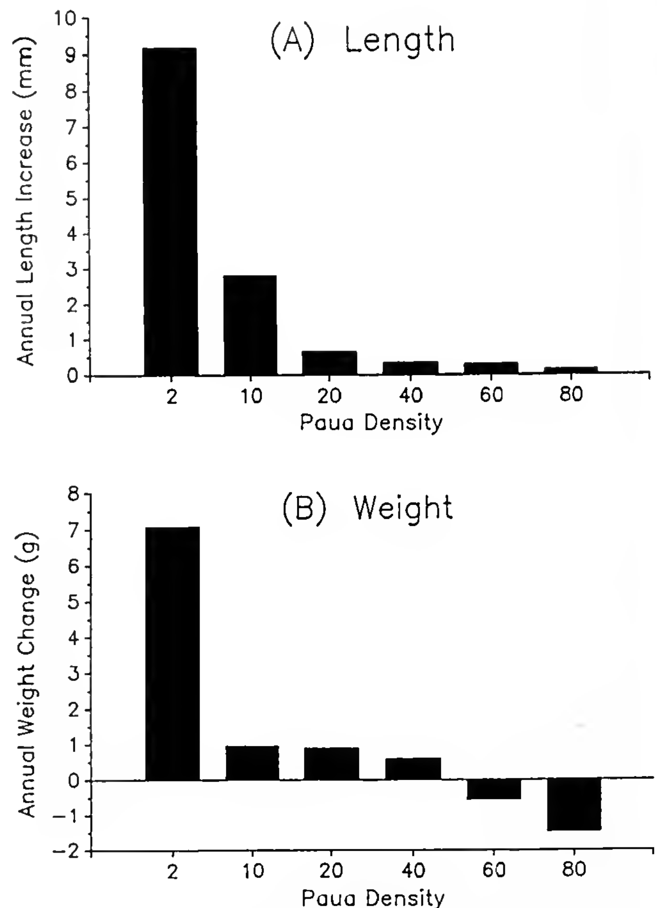

Figure 5. Annual increments for (A) length and (B) weight of *H. iris* held at different densities.

algae. Because it is locally abundant and readily available as drift, it could be used in abalone culture. However, *Gracilaria* was the best food source because it exceeded all of the other species we tested in terms of potential energy gain from algal tissue.

Our grazing experiments did not provide a choice of algae; thus, we cannot comment directly about the relative attractiveness between species. However, in simple two-way choice experiments, Poore (1969) found preferences in the same rank order that we found for the grazing rates. These laboratory preferences, however, may not operate in field populations, and examinations of gut contents have shown that *H. iris* will feed on the relatively unattractive *L. variegata*, in the absence of other food species. This suggests that food availability, rather than food preference, is a likely determinant of the natural diet of *H. iris*.

Several authors have suggested that abalone show consistent food preferences based on food availability in their habitat. This may partially explain some apparent inconsistencies in dietary composition, both within and between localities (Shepherd 1973, Shepherd and Steinberg 1992, McShane et al. 1994). The results of our study suggest that when the most palatable algal food is not available, paua will feed on less favourable food items. Previous knowledge is apparently not required, and paua readily accepted the branching thallus of *Gracilaria*, although this alga is not normally available in the Kaikoura region.

The growth of *H. iris* in this study was low compared with estimates of abalone growth elsewhere (Day and Fleming 1992). The annual length increment in our study was approximately half that recorded from our field data and from tagged paua on the shore (Williams 1990, Pirker 1992). No explanation for this difference can be provided, although the restricted monospecific diet may form a part (Wilson 1987). Recent studies on artificial diets have highlighted the value of variation in the diet, and the inclusion of additives such as trace elements and lipids can affect abalone growth (Hahn 1989, Uki and Watanabe 1992, Mai et al. 1994).

Although a number of experimental field studies have investigated the effects of density on mollusc growth (Underwood 1979, Ortega 1985, Fletcher 1988), there have been few studies on abalone. McShane and Naylor (1995) recently reported similar growth rates for *H. iris* held in field enclosures. At densities of 0.3 and 15 m^{-2}, they concluded that there were no food or space limitations on growth. In our experiments, where the density levels corresponded to between 20 and 800 m^{-2}, we found severely restricted growth rates of *H. iris* with increasing density. Although similar results were found in early studies on *Haliotis gigantea discus* (Ino 1943) and *Haliotis cracherodii* (Leighton and Boolootian 1963), more recent investigations into barrel culture of abalone feeding on *Macrocystis* have yielded high growth rates, at densities similar to those used in our experiments (Aviles and Shepherd in press). Generally, growth rates of abalone held in cages with high water exchange and a high surface-to-volume ratio grow faster than those held in barrels or tubes. Also, Hahn (1989) emphasises the importance of good water quality for abalone growth, which can be inhibited by reduced oxygen levels and bacterial growth.

In our experiments, although we cannot exclude water quality as a limiting factor, the detrimental effects of high density may also be due to interference (Douros 1987) or space limitations. At high density, abalone may be exposed to increased stress, resulting in higher metabolic rates (Gaty and Wilson 1986), and/or increased biochemical or other activity. Paua held at the two highest densities continued to feed throughout the experiment but lost weight and did not increase in overall length. However, despite these trends, a few individuals grew, as evidenced by a different shell colour. These observations suggest high individual variability in response to density stress and a remarkable ability for black foot paua to survive lengthy periods in less than favourable conditions.

In conclusion, this study has confirmed that *H. iris*, provided with a natural food supply, can be maintained in laboratory culture on a small scale, but we have highlighted some problems in maintaining growth rates similar to field values. Further studies are now required, using larger numbers of individuals and investigating the effects of artificial diets (Uki and Watanabe 1992) and dietary supplements (Mai et al. 1994) on feeding, energy conversion, and growth of this potentially important aquaculture species.

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LARVAL SUPPLY TO QUEEN CONCH NURSERIES: RELATIONSHIPS WITH RECRUITMENT PROCESS AND POPULATION SIZE IN FLORIDA AND THE BAHAMAS

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ABSTRACT Surveys were made for larvae of the commercially important gastropod *Strombus gigas* Linne (queen conch) over two spawning seasons in two island chains of the northwestern subtropical Atlantic, the Florida Keys (United States) and the Exuma Cays (Bahamas). Size-frequency distribution of veligers in the Florida Keys was characterized by two distinct modes representing newly hatched larvae (<500 μm in shell length) and larvae near metamorphosis (>900 μm) in approximately equal numbers, with virtually no mid-size larvae. The Florida Keys have a very small spawning stock, yet densities of late-stage larvae were nearly as high as those in the Exuma Cays, where spawning stocks are large. Late-stage larvae in the Keys appear to be derived from distant spawning stocks, probably in Cuba or the western Caribbean Sea. In contrast, only 1% of the larvae in the Exuma Cays were late stages and the juvenile populations appear to depend on local spawning and recruitment. The sizes of juvenile populations were positively correlated with the mean density of late-stage larvae in both the Florida Keys ($r = 0.881$) and the Exuma Cays ($r = 0.759$), indicating the significance of larval supply in determining benthic recruitment on the local scale. The slope of the linear relationship between larval supply and juvenile population size, however, was much higher in the Exuma Cays nurseries than in Florida, suggesting important regional differences in settlement and postsettlement processes. Recruitment of benthic fauna with pelagic larvae must be considered in terms of metapopulation dynamics and both presettlement and postsettlement processes.

KEY WORDS: Bahamas, distribution, Florida, larval transport, length-frequency, *Strombus gigas*

INTRODUCTION

A large proportion of benthic marine animals have "complex life cycles" (sensu Roughgarden et al. 1988) involving pelagic eggs or larvae with high potential for dispersal as well as loss to stochastic, density-independent processes during larval development. Connell (1985) predicted that population sizes of benthic animals would be positively correlated with recruitment density where recruitment rate is low and that density-dependent mortality would rapidly destroy a direct relationship between larval settlement and subsequent recruitment where recruitment rate is high. Numerous studies with fishes (Sale et al. 1984, Victor 1986, Doherty 1987) and invertebrates (Keough 1984, Connell 1985, Gaines et al. 1985, Sutherland 1987, Roughgarden et al. 1988) have examined the relationship between settlement and subsequent population size. Others have made empirical analyses of the relationship between larval supply and settlement and/or recruitment (Yoshioka 1982, Wethey 1984, Gaines et al. 1985, Lipcius et al. 1990, Minchinton and Scheibling 1991, Milicich et al. 1992, Peterson and Summerson 1992, Doherty and Fowler 1994).

The large gastropod *Strombus gigas* Linne (queen conch) forms the basis for one of the most important fisheries of the Caribbean region, with a total annual value of approximately $40 million US between 1988 and 1991 (Appeldoorn 1994). However, queen conch stocks have declined throughout the region over the past 10–20 y, and various forms of catch and size limits have been imposed in most nations (Appeldoorn et al. 1987, Berg and Olsen 1989, Appeldoorn 1994). International trade in conch is now monitored by the Convention on International Trade of Endangered Species (CITES) with the hope of ensuring the species' survival. Despite complete closure of the fishery in the United States in 1985, queen conch stocks have shown little sign of recovery (Berg and Glazer 1995). This lack of recovery is poorly understood, in part because of limited knowledge of early life history, larval abundance, and recruitment processes. The ecology of the juvenile and adult queen conch is relatively well studied (Randall 1964, Weil and Laughlin 1984, Iversen et al. 1987, Stoner and Waite 1990, Stoner and Sandt 1992, Stoner et al. 1995); however, detailed larval descriptions for identifying the larvae of the different *Strombus* species (Davis et al. 1993) and the first analyses of veliger abundance (Stoner et al. 1992, Posada and Appeldoorn 1994, Stoner et al. 1994) have appeared only recently. The recruitment problem is compounded by the fact that queen conch larvae spend ~3 wk in the water column and may drift hundreds of kilometers from parental stocks before settling to the benthos (Davis et al. 1993). As a result, many local populations are probably replenished from distant sources, and stock management for queen conch is a multinational problem (Berg and Olsen 1989). Biochemical evidence suggests a high degree of gene flow among Caribbean populations (Mitton et al. 1989, Campton et al. 1992).

This study was conducted to compare the abundance and size frequency of queen conch larvae within and between two geographically distinct regions. At both sites, collections were made in nursery areas with the broadest possible range of juvenile population size to examine the relationship between larval supply and spatial variation in recruitment. Analyses were made in the Florida

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Keys (United States), where populations have been heavily overfished (Glazer and Berg 1994), and in the Exuma Cays (Bahamas), where conch populations were large and relatively stable (Stoner and Sandt 1992, Stoner and Schwarte 1994, Stoner et al. 1996). Differences in the benthic populations both within and between regions are discussed in terms of potential sources of larval recruitment, long-term juvenile population size, stock recovery, and management strategy.

METHODS

Stations in Florida

Stations in the Florida Keys were chosen on the basis of long-term data for historically important conch nursery grounds in the middle and lower Florida Keys (Glazer and Berg 1994, our unpubl. data). Two stations were in nearshore locations (Tingler's Island [TI] and Big Pine Key [BP]; Fig. 1) that had small, ephemeral populations of juveniles (Table 1). These sites were ~1.5 m deep at mean low water (MLW), and the bottom was a mixture of macroalgae, sponges, sand, and patches of seagrass (primarily *Thalassia testudinum* Konig). Two more stations were located on nursery grounds associated with shoal areas along the Florida Keys coral-reef tract, which lies ~10 km offshore and to the south of the islands. These areas, Delta Shoal and Looe Key National Marine Sanctuary, typically support several hundred to a few thousand conch (Table 1). Delta Shoal is a shallow, coral-rubble area covered with macroalgae, small corals, and patches of sand. Delta Shoal station 1 (DS1) was located in the backreef area along the northern edge of the shoal over a 1.5-m-deep platform of mixed seagrass, rock, algae and sponges. Station 2 (DS2) was ~0.5 km offshore from the shoal, where depth increased rapidly from 20 to 30 m. Looe Key is composed of a very shallow coral-reef tract running east to west, with shallow rubble shoals reaching north from the ends of the reef. Station 1 (LK1) was behind the reef and between the shoals, where there is a shallow sand- and seagrass-covered flat. Station 2 (LK2) was ~0.5 km offshore in depths of 20–30 m. At both Delta Shoal and Looe Key, juvenile conch are found consistently on algae-covered rubble and seagrass in shallow water. Adults are found on the reef flat at Looe Key and in the deep areas surrounding the reefs and shoals at both sites. Spawning occurs principally in these deeper habitats and has not been observed north of Hawk Channel.

Stations in the Bahamas

Five stations were chosen on the basis of long-term data from the Lee Stocking Island area in the southern Exuma Cays (Stoner et al. 1994, Stoner et al. 1995, our unpubl. data) (Fig. 1). Stations at Children's Bay Cay (CBC), Shark Rock (SR), Tug Boat Rock (TBR), and Neighbor Cay (NBC) were all on the shallow Great Bahama Bank to the west of the Exuma Cays (leeward in prevailing summer winds). The fifth station was on the windward island shelf of Lee Stocking Island in a cove off of Charlie's Beach (CHB). As is typical of large nursery grounds in the Exuma Cays, CBC and SR are characterized by moderate-density seagrass (*T. testudinum*), shallow depth (3.0 m at MLW), and strong tidal currents. Both have large aggregations of 70,000 or more juvenile conch in most years (Table 1). TBR, NBC, and CHB have smaller, more ephemeral populations, with TBR being the largest and most consistent (7,000–50,000 individuals), and CHB and NBC rarely having more than 200–2,000 conch (Table 1). TBR is

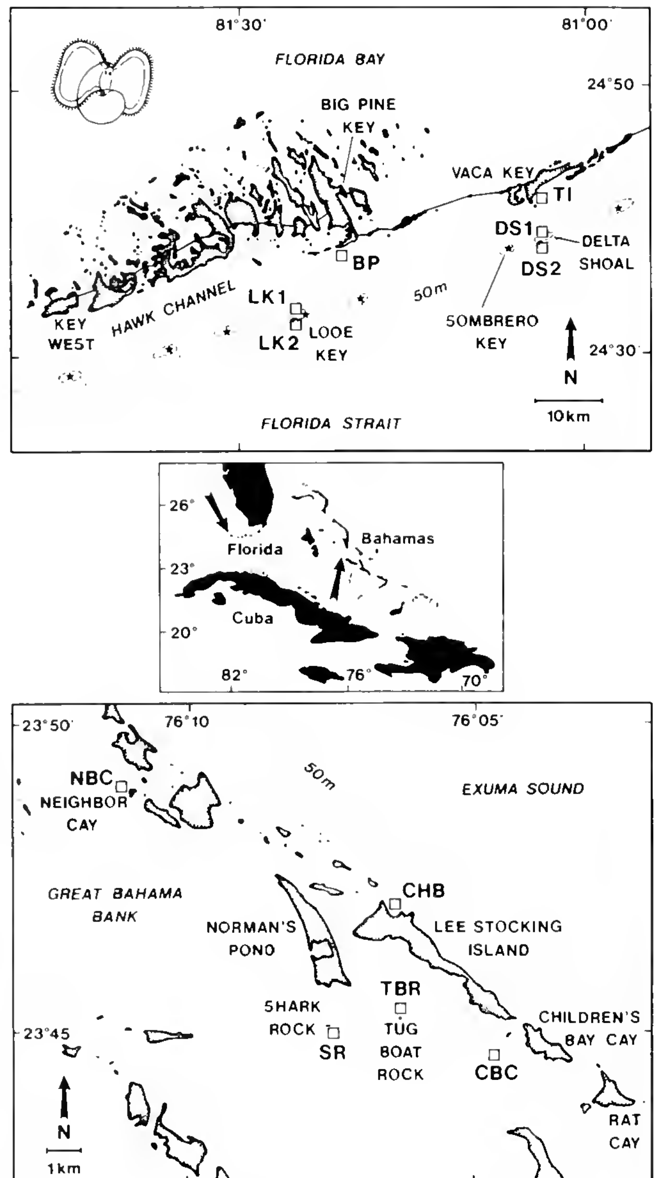

Figure 1. Location of veliger-sampling sites in the Florida Keys (top) and Exuma Cays, Bahamas (bottom). Arrows in the center map show the general locations of the two study sites. Note the scale differences for the Florida and Exuma maps.

2.0 m deep and has sparse to medium-density seagrass and strong tidal currents. Where plankton tows were made in the cove at CHB, depth is 1.5–2.5 m and the bottom is a mixture of bare sand and patches of sparse to dense *T. testudinum*, detritus, and drift algae. Conch at NBC inhabit a depth range from immediately subtidal to approximately 2.0 m in depth in an area grading from a bare sand beach to a sparsely vegetated seagrass bed (Sandt and Stoner 1993). Adult density and spawning frequency are highest in depths of 10–18 m on the island shelf to the east of the Exuma Cays (Stoner and Sandt 1992, Stoner and Schwarte 1994). Spawning is relatively infrequent in bank habitats to the west of the Exuma Cays.

Plankton Collections

Plankton samples were collected with simple conical nets (0.5 m in diameter, 2.5 m in length, 202-μm-pore-size mesh). Repli-

TABLE 1.

Estimates of Juvenile Population Size of Queen Conch at Nine Nurseries in the Florida Keys and Exuma Cays, Bahamas, 1988–1994.

	Florida Keys				Exuma Cays				
Year	Tingler's Island (TI)	Delta Shoal (DS1)	Big Pine Key (BP)	Looe Key (LK1)	Children's Bay Cay (CBC)	Tugboat Rock (TBR)	Shark Rock (SR)	Charlie's Beach (CHB)	Neighbor Cay (NBC)
1988	600	—	13,084	—	—	—	—	—	2,500
1989	1,012	—	1,716	—	—	—	—	—	6,000
1990	170	14,400	78	810	110,000	50,000	220,000	—	—
1991	0	13,800	0	6,890	10,000	500	162,000	281	—
1992	536	845	3,920	722	90,000	7,000	70,000	229	—
1993	228	1,830	368	2,116	145,900	47,600	84,900	50	100
1994	70	1,282	0	2,504	165,600	100,700	85,700	0	0
Mean	374	6,431	2,738	2,608	104,300	41,160	124,520	140	2,150

Population size at stations TI and BP were determined by standard tag and recapture methods (Glazer and Berg 1992), whereas the larger, more dense populations in the Florida Keys (DS1 and LK1) were surveyed by the use of standardized belt-transect methods (Glazer and Berg 1994). The numbers of juveniles in the largest populations (CBC, SR, and TBR) were estimated by measuring the density of conch in highly replicated quadrats within multiple sectors of the aggregation, which were mapped with the aid of the global positioning system (Stoner and Ray 1993, Stoner et al. 1994).

cate tows (~1.0 m · sec^{-1}) were made at each station and sample date near the water surface. Surface sampling was appropriate because queen conch veligers are photopositive (Barile et al. 1994) and most abundant near the surface under relatively smooth surface conditions (Stoner and Davis 1997b). In the Florida Keys, sampling was restricted to mid-day periods when wave height was <0.5 m. In the Exuma Cays, where the nurseries are subject to strong tidal currents, sampling was restricted as described above and confined to time periods 2 h before and 1 h after the high tide. Stoner and Davis (1997a) have shown that the highest concentrations of veligers pass through the inlets at mid-flood tide. We assumed, therefore, that maximum densities at the nurseries would occur close to the high tide. Tow times varied according to the concentration of plankton on the sampling date but averaged 15 min each. Tow volume was determined with a calibrated General Oceanics flow meter suspended in the mouth of the net. Nets were deployed by hand from small boats (<8 m). Plankton samples were preserved in a buffered 5% formalin-seawater mixture.

At both sites, sampling was conducted between late May and late September in 1992 and 1993, seasonal periods that spanned the primary reproductive season at Lee Stocking Island (Stoner et al. 1992) and in the Florida Keys (Glazer, pers. observ.). In 1992, samples were collected every 2 wk at the Florida stations for a total of eight sets of plankton. At the Exuma stations, samples were collected every 9 d, yielding 13 sets. In 1993, the sampling effort was increased to 12 collections in the Florida Keys and 14 collections near Lee Stocking Island. On each sampling date, observations were made on wave height and direction, wind speed and direction, and surface-water temperature.

In the laboratory, plankton samples were rinsed on a 180-μm-pore-size mesh screen and sorted for veligers of *Strombus* spp. with the aid of a dissecting microscope. Even the smallest veligers of *Strombus gigas*, *Strombus costatus*, and *Strombus raninus* can be distinguished by use of the descriptions of Davis et al. (1993). These were identified to species, counted, and measured for maximum shell length (SL). Patterns of abundance for *S. gigas* were analyzed in terms of numbers of veligers per unit volume of water sampled (veligers · 100 m^{-3}) for the total number of veligers and by size class. Classes used were: early-stage (<500 μm SL), mid-size (500–900 μm), and late-stage veligers (>900 μm), most of which were metamorphically competent. In an open system, larvae of different sizes may have different sources and size-specific density data are useful in interpreting larval production and transport processes. For example, early-stage conch veligers are only a few days old (Davis et al. 1993) and reflect local larval production, whereas late-stage veligers may have a source in more distant reproductive populations.

Juvenile Population Size

To test for a relationship between the supply of larvae to nurseries and the subsequent benthic population, regression analysis was performed using the mean seasonal density of late-stage veligers as the independent variable for each station and the estimated total number of juveniles in the population the following year as the dependent variable. That is, 1992 veliger data were paired with 1993 data for juveniles, and 1993 veliger data were paired with 1994 data for juveniles. Juveniles were predominantly 1-y-old conch, 80–120 mm SL. The methods used to estimate juvenile population size (see Table 1) have been described in detail for both the Florida Keys (Glazer and Berg 1992, Glazer and Berg 1994) and the Exuma Cays (Stoner and Ray 1993; Stoner et al. 1994).

RESULTS

Spatial Variation in Veliger and Size Frequency

In 1992, only 209 queen conch veligers were collected in all 96 tows in Florida, whereas over 3,900 were collected in 130 tows in the Exuma Cays (Table 2). Total numbers collected in the two geographic areas were relatively similar in 1993 (2,300–2,700); however, most of the 2,600 veligers at LK1 were newly hatched individuals collected on just one date. In Florida, the highest mean veliger concentration occurred at LK1 during both 1992 (9.1 veligers · 100 m^{-3}) and 1993 (140 veligers · 100 m^{-3}) (Table 2). Over the 2-y survey period, only one queen conch veliger was collected at TI, and only two were collected at BP. Densities of conch veligers were generally higher in the Exuma Cays than in the Florida Keys. Densities near the largest populations at SR, CBC, and TBC were 15–33 veligers · 100 m^{-3} in 1992 and 12–

TABLE 2.

Counts and Density of Queen Conch Veligers (All Stages) Collected in the Florida Keys and Exuma Cays, Bahamas, May Through September 1992 and 1993.

Site and Station	1992 No. of Veligers Collected	1992 Veliger Density (no. · 100 m^{-3})	1993 No. of Veligers Collected	1993 Veliger Density (no. · 100 m^{-3})
Florida Keys	16 tows		24 tows	
Tingler's Island (TI)	0	0 ± 0	1	0.06 ± 0.19
Delta Shoal 1 (DS1)	11	0.86 ± 1.77	71	2.40 ± 6.80
Delta Shoal 2 (DS2)	29	2.60 ± 5.50	ND	ND
Big Pine Key (BP)	1	0.07 ± 0.27	1	0.07 ± 0.22
Looe Key 1 (LK1)	144	9.10 ± 19.8	2,637	140 ± 443
Looe Key 2 (LK2)	24	1.10 ± 1.90	ND	ND
Total	209		2,710	
Exuma Cays	26 tows		28 tows	
Children's Bay Cay (CBC)	939	17.8 ± 14.7	942	12.5 ± 18.0
Tugboat Rock (TBR)	799	15.6 ± 13.7	278	3.57 ± 6.60
Shark Rock (SR)	1,576	32.5 ± 34.8	1,001	12.5 ± 9.7
Charlie's Beach (CHB)	123	2.30 ± 2.70	ND	ND
Neighbor Cay (NBC)	494	9.80 ± 9.40	136	1.70 ± 3.30
Total	3,914		2,357	

Density values are mean ± standard deviation. The number of tows made at each station is shown for each of the 2 y. ND, not determined.

36 veligers · 100 m^{-3} in 1993 (Table 2). Densities near the smaller, more ephemeral juvenile populations were 1.7–9.7 veligers · 100 m^{-3}.

All of the queen conch veligers collected in the Florida Keys were either very small, newly hatched individuals (most were <400 μm SL) or late-stage larvae (>900 μm SL). There were no intermediate stages. Nevertheless, two types of size-frequency distribution were observed at Looe Key and Delta Shoal (Fig. 2). Directly over the nurseries (LK1 and DS1), most of the larvae collected were early stages, probably just 1–4 d old. Conversely, no larvae <1.0 mm SL were collected at offshore station LK2, and only three individuals <1.0 mm were collected at DS2. The differences were highly significant for both station pairs (Kolmogorov-Smirnov tests, $p < 0.01$).

Size-frequency distributions for veligers collected in the Exuma Cays were dominated by early-stage larvae (Fig. 3). At all five stations, small veligers (<500 μm SL) comprised >93% of the total numbers; however, mid- and late-stage veligers were also collected at all stations.

Occurrence of Precompetent Veligers

Densities of early-stage larvae were relatively high (near 25 veligers · 100 m^{-3}) at Exuma Cays stations SR, CBC, and TBR throughout the spawning season in 1992 (Fig. 4), with strong maxima (>100 veligers · 100 m^{-3}) occurring at SR in June and late August. Although larval densities were obviously lower in 1993 than in 1992, densities of early stages were never zero at these three nurseries with large populations of juvenile conch. Among the Exuma Cays stations with low numbers of juvenile conch, early-stage veligers were constantly present at NBC in 1992, but they were present in only 4 of 13 collections made in 1993. At CHB, concentrations were low, but positive, on all but two dates with no veligers collected. None of the Exuma Cays stations showed an obvious temporal pattern in veliger density during the spawning seasons of 1992 and 1993.

Among the Florida stations, early-stage larvae were sufficiently abundant to warrant plotting only at LK1 (Fig. 4). Densities were erratic, with high concentrations interspersed among zero values throughout both 1992 (50% zeros) and 1993 (33% zeros). Eighty-eight percent of all of the conch veligers collected at this station in 2 y were very recently hatched larvae (<400 μm SL) collected on 22 July 1993. Early-stage larvae were never collected at LK2. At DS1, early stages were collected on 4 of 20 sampling dates, with only one density estimate >1 veliger · 100 m^{-3} (24.8), occurring on 8 July 1993. At DS2, early-stage veligers were collected on only two dates in 1992, with values ≤0.7 veligers · 100 m^{-3}.

Density of early-stage larvae showed no particular association with wind direction or speed except that none were collected on the relatively few dates (three dates at Looe Key, one date at Delta Shoal) when wind velocity exceeded 7.3 m · sec^{-1} (15 knots). The very high concentration of newly hatched larvae estimated for LK1 in July 1993 was associated with calm conditions and the highest recorded temperature for the season (32°C).

Whereas no mid-stage conch veligers were collected in the Florida Keys, they were present in 56% of the samples at CBC and 44% of the samples at SR. Densities were much lower than those for early-stage larvae, typically <1–2 veligers · 100 m^{-3}, with maxima occurring irregularly (Fig. 5). Densities of mid-stage larvae were never >1 veliger · 100 m^{-3} at TBR, NBC, and CHB. At all of the stations, mid-stage larvae were relatively uncommon before mid-June in both 1992 and 1993, probably reflecting the beginning of the reproductive season and the subsequent growth of the larvae. As with early-stage larvae, mid-stage veligers were more abundant at SR in 1992 than in 1993. At CBC, mid-stage larvae were more abundant in 1993 than in 1992, but densities were erratic in all cases.

Occurrence of Competent Veligers

Concentrations of late-stage larvae, all of which were competent or near competent, are particularly relevant to benthic recruitment in the nursery habitats; therefore, these were considered sep-

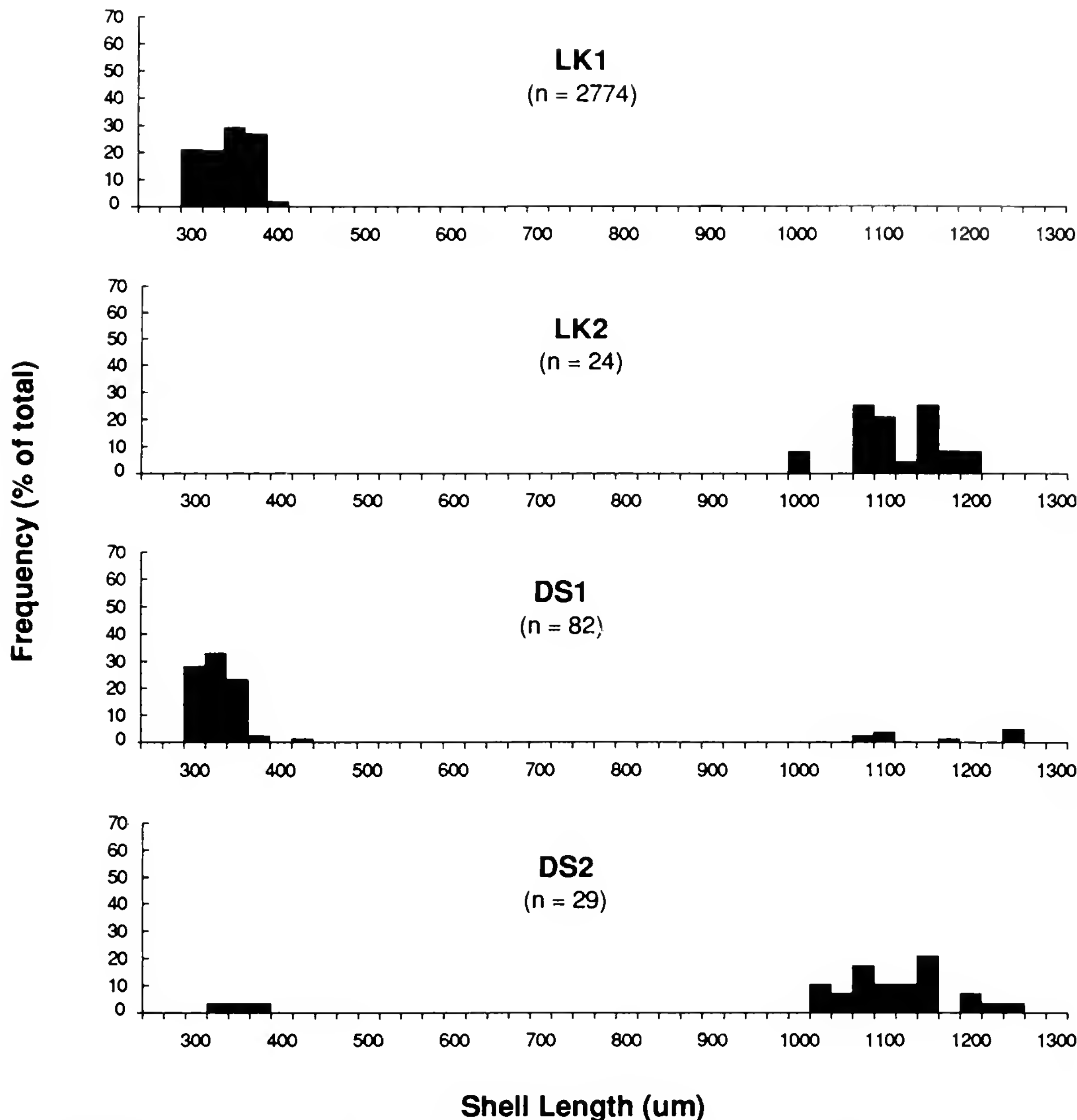

Figure 2. Length-frequency distribution of queen conch veligers at four stations in the Florida Keys in 1992 and 1993. The total number of larvae shown for station LK1 is slightly less than that reported in Table 2 (2,781) because some shells were damaged and not measured.

arately (Table 3; Fig. 6). The highest concentrations of late-stage larvae occurred in 1992, at the offshore Florida stations DS2 (1.34 veligers · 100 m^{-3}) and LK2 (0.85 veligers · 100 m^{-3}), where late stages comprised most of the larvae (Fig. 2). Among the nursery sites, the densities of late-stage larvae ranged from zero at stations characterized by very small populations of juveniles, TI and BP, to 0.34 veligers · 100 m^{-3} at DS1 in 1993 (Table 3). Mean concentrations were relatively consistent between 1992 and 1993 at the Florida nurseries; however, the frequency of occurrence of late-stage veligers was lower in the second year. The highest concentrations of late-stage veligers at nursery sites LK1 and DS1 were associated with onshore winds from the south. Conversely, at all four stations near the reef tract (at Delta Shoal and Looe Key), larval densities were zero whenever the wind was north of east (<90–110° true), suggesting that the larvae were transported on and off the shelf with the surface layer.

In general, the mean densities of late-stage larvae at the Exuma Cays stations were not much higher than those in the Florida Keys (Table 3), despite very large differences in total larval densities (Table 2). The highest density for one date was 5.9 veligers · 100 m^{-3} at CBC in August 1993; however, densities >2 veligers · 100 m^{-3} were uncommon (Fig. 6). Late-stage larvae occurred sporadically, with less than half of the sampling dates yielding late-stage larvae (Table 3), and there was no concordance in the abundance patterns at SR and CBC, which lie in adjacent tidal flow fields and are separated by just 7 km. Consistent with the pattern observed for early- and mid-stage larvae, late-stage larvae were more abundant at SR in 1992 than in 1993.

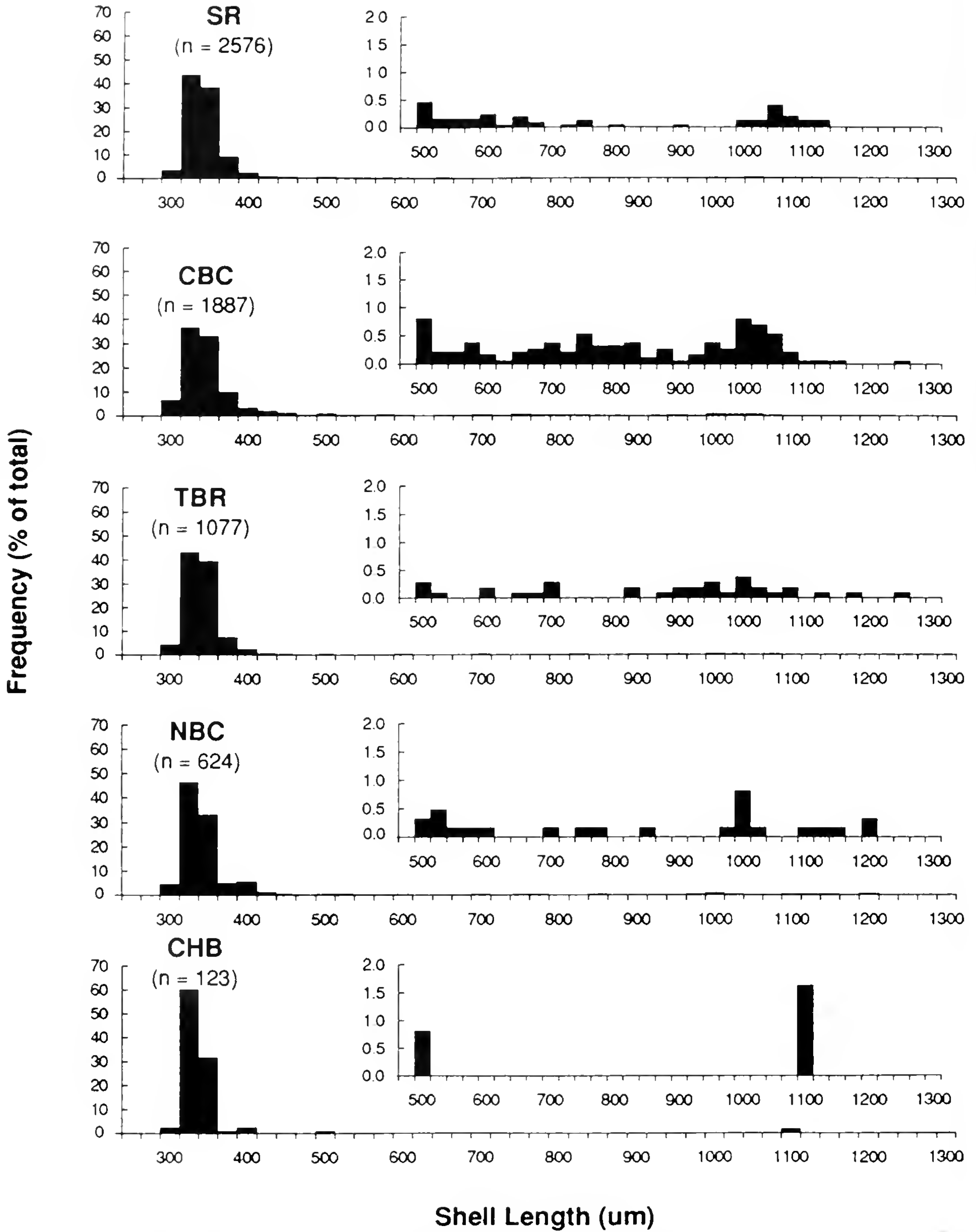

Figure 3. Length-frequency distribution of queen conch veligers at five stations in the Exuma Cays in 1992 and 1993. The frequency distributions of larvae >500 μm SL are shown in the insets. Note the scale difference on the y-axes. Small differences in the total numbers reported here and in Table 1 occur because some shells were damaged and not measured.

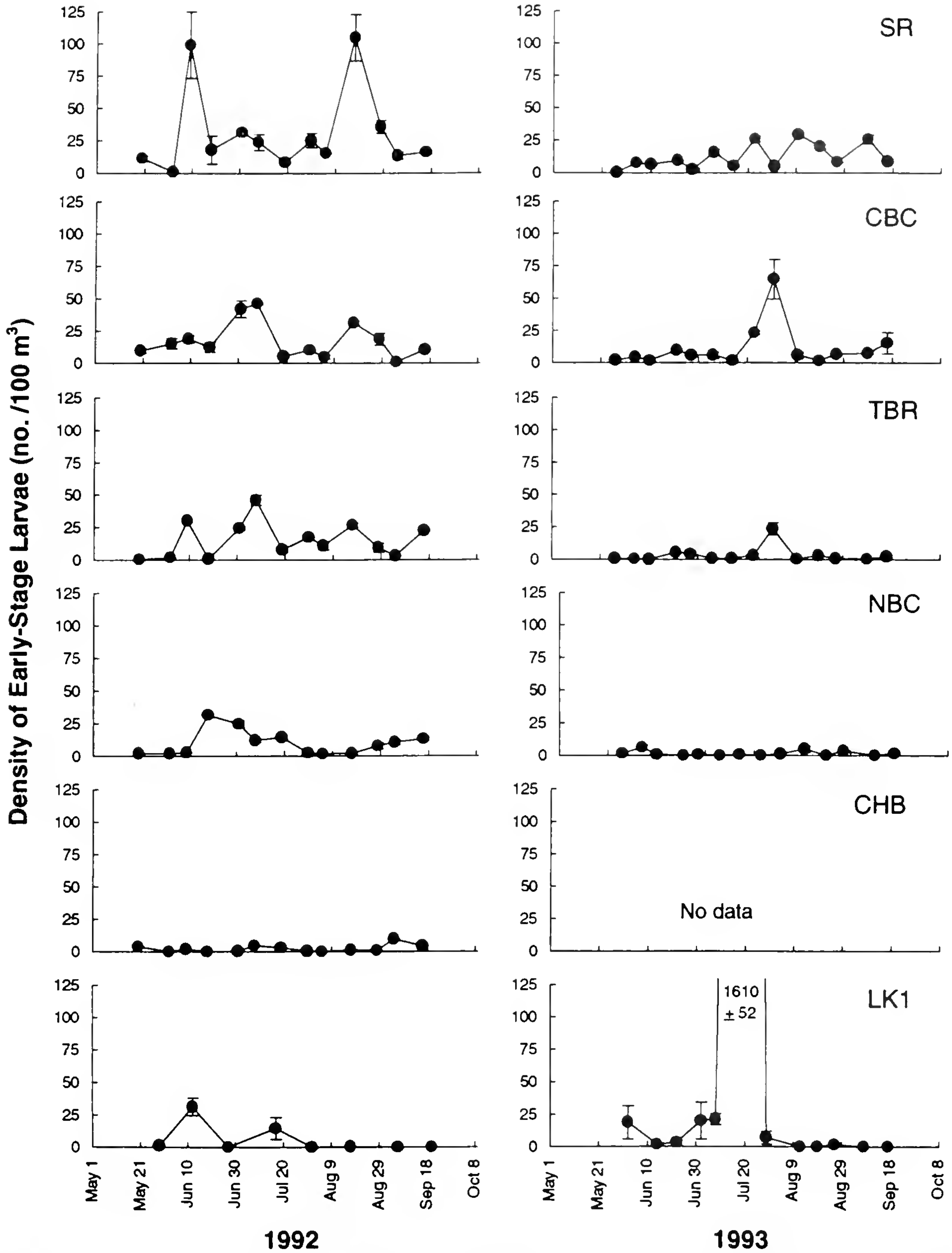

Figure 4. Density of newly hatched queen conch veligers (< 500 μm SL) at five nurseries in the Exuma Cays, Bahamas, and at the Looe Key nursery (Florida Keys) in 1992 and 1993. Numbers of larvae collected at the other study sites were too low to plot. Values shown are mean ± standard error (n = 2).

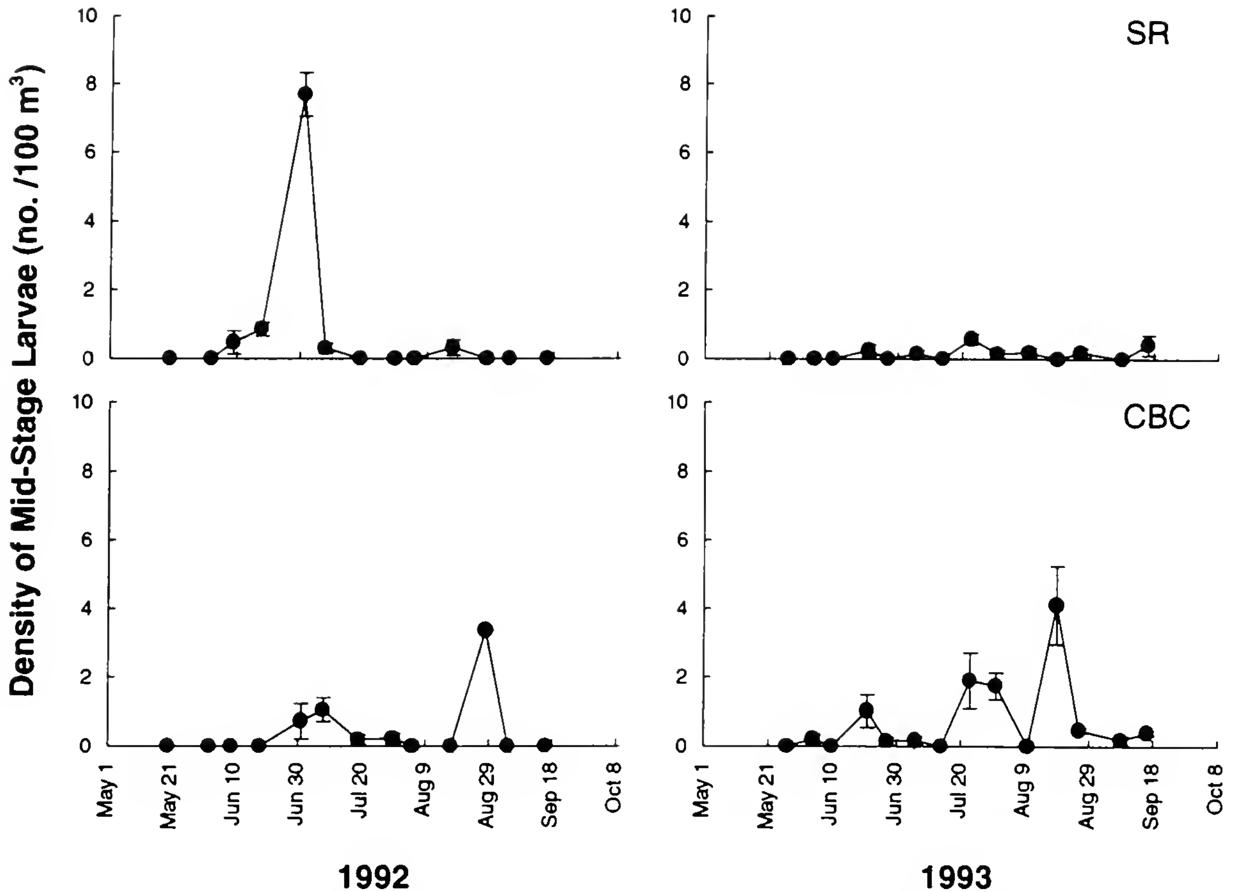

Figure 5. Density of mid-stage queen conch veligers (500–900 μm SL) at two nurseries in the Exuma Cays, Bahamas, in 1992 and 1993. Numbers of larvae collected at the other study sites were too low to plot. Values shown are mean ± standard error (n = 2).

TABLE 3.

Counts and Density of Late-Stage Queen Conch Veligers Collected in the Florida Keys and Exuma Cays, Bahamas, May Through September 1992 and 1993.

Site and Station	1992 No. of Veligers Collected	1992 % of Collections With Veligers	1992 Veliger Density (no. · 100 m^{-3})	1993 No. of Veligers Collected	1993 % of Collections With Veligers	1993 Veliger Density (no. · 100 m^{-3})
Florida Keys	16 tows			24 tows		
Tingler's Island (TI)	0	0	0 ± 0	0	0	0 ± 0
Delta Shoal 1 (DS1)	6	50	0.28 ± 0.34	5	8	0.34 ± 1.04
Delta Shoal 2 (DS2)	26	50	1.34 ± 1.82	ND	ND	ND
Big Pine Key (BP)	0	0	0 ± 0	0	0	0 ± 0
Looe Key 1 (LK1)	2	25	0.10 ± 0.21	5	17	0.16 ± 0.56
Looe Key 2 (LK2)	24	38	0.85 ± 1.65	ND	ND	ND
Total	58			10		
Exuma Cays	26 tows			28 tows		
Children's Bay Cay (CBC)	8	15	0.32 ± 0.79	54	50	0.55 ± 1.55
Tugboat Rock (TBR)	6	15	0.13 ± 0.38	14	36	0.21 ± 0.34
Shark Rock (SR)	23	39	0.52 ± 1.45	6	29	0.08 ± 0.14
Charlie's Beach (CHB)	2	8	0.06 ± 0.23	ND	ND	ND
Neighbor Cay (NBC)	2	15	0.04 ± 0.09	10	43	0.11 ± 0.17
Total	41			84		

Density values are mean ± standard deviation. The number of tows made at each station is shown for each of the 2 y. ND, not determined.

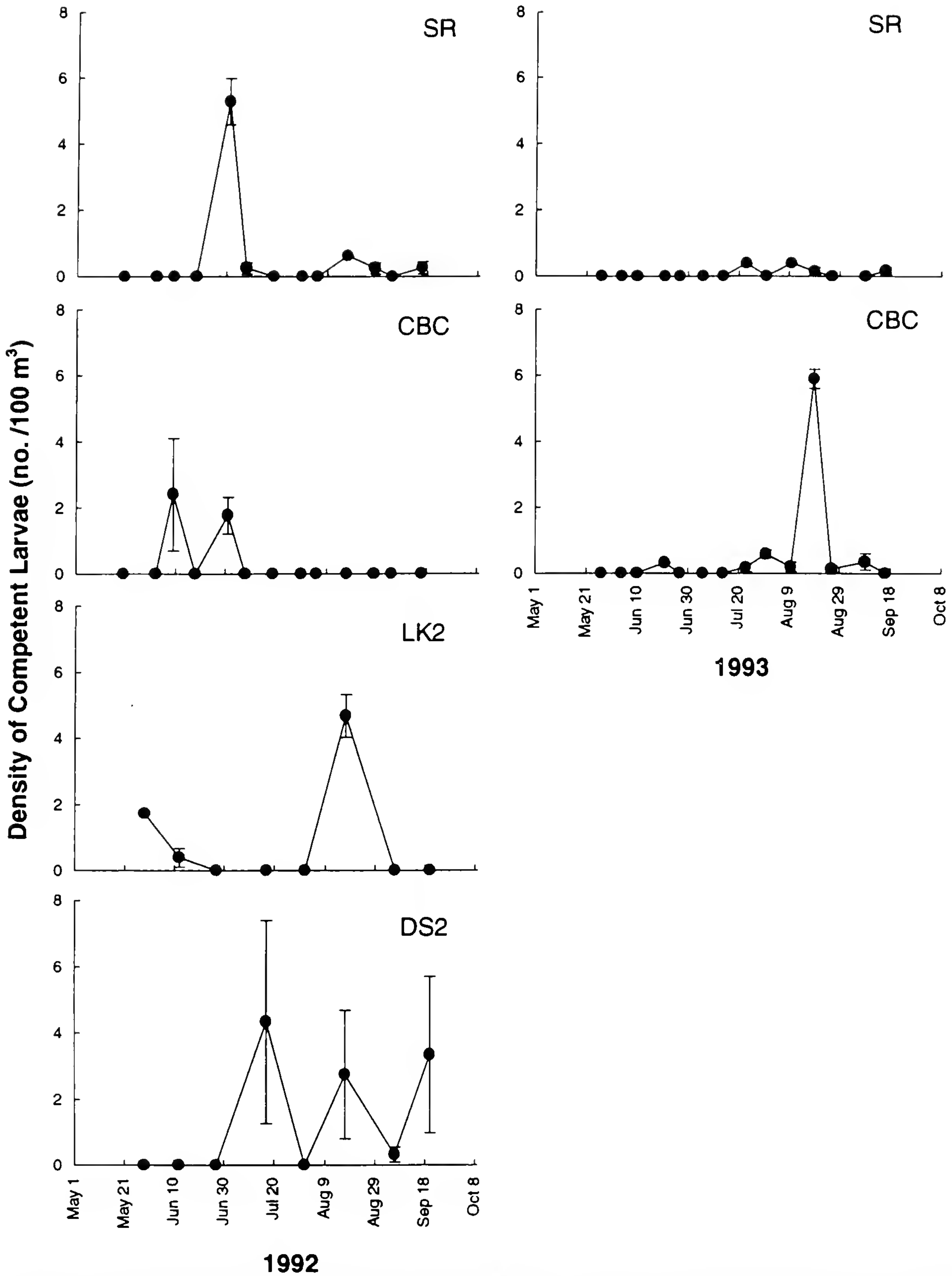

Figure 6. Density of late-stage queen conch veligers (>900 μm SL) at two stations in the Exuma Cays and at two stations in the Florida Keys in 1992 and 1993. Relatively few late-stage larvae were collected at the other sampling stations (see Table 3). Values shown are mean ± standard error (n = 2).

Relationships Between Veligers and Juvenile Population Size

Two general observations can be made about the relationship between the density of veligers and juvenile populations at the study sites. First, veliger densities were consistently low (Table 2) at stations with ephemeral or small juvenile populations, such as TI and BP in the Florida Keys and CHB in the Exuma Cays (Table 1). Second, in the Exuma Cays, veligers were always present, and density maxima were high at nurseries where there were consistently large aggregations of juvenile conch (i.e., CBC and SR). Similarly, the highest larval densities in the Florida Keys occurred at LK1 and DS1, where juveniles were most abundant.

There was a close, positive correlation between larval supply (mean seasonal density of late-stage conch veligers) and the size of the juvenile population 1 y later, both in the Exuma Cays ($r = 0.759$; $p = 0.018$) and in the Florida Keys ($r = 0.881$, $p = 0.004$) (Fig. 7). Analysis of covariance, however, indicated that the slopes of the regression lines for the two regions were different ($F_{(1,13)} = 5.061$; $p = 0.042$). It is clear from the plots (Fig. 7) that the slope for the Exuma Cays stations was much higher than that for the Florida Keys; the difference was >40 times. For example, a density of 0.3 late-stage veliger · 100 m^{-3} in the Florida Keys was associated with ~2,000 juvenile conch, whereas the same concentration of larvae in the Exuma Cays was associated with ~80,000 juveniles.

There was also a significant positive correlation between the abundance of juvenile conch in Florida nursery grounds and the percentage of plankton sampling dates (during the previous year) that yielded late-stage veligers ($r = 0.733$; $p = 0.039$). The correlation was not significant in the Exuma Cays ($r = 0.410$; $p = 0.273$).

DISCUSSION

Veliger Size Frequency and Probable Sources

Detailed length-frequency data for queen conch veligers generated in this study provide important insights into the different mechanisms of recruitment and sources of larvae for the two study areas. At all stations except the offshore non-nursery sites DS2 and LK2 in the Florida Keys, the majority of veligers were <500 μm SL. On the basis of growth curves provided by Davis et al. (1993), these small veligers were no more than 5–6 d old and must have had a local source. With the exception of one collection made in Looe Key National Marine Sanctuary in 1993, the density of newly hatched conch larvae was very low compared with the densities of similar sized veligers in the Exuma Cays, Bahamas. The difference relates to the abundance of spawners in the two areas. Adult conch in the Keys were seriously depleted by overfishing and have not recovered since fishing was ended in 1985 (Glazer unpubl. obs.). In 1992, there were only ~6,000 adult conch in the 200-km-long island chain of the Florida Keys from Carysfort Reef to Western Dry Rocks. Many of these adults were found in Looe Key National Marine Sanctuary, where the highest concentrations of early-stage veligers were collected in both 1992 and 1993.

In contrast with the low numbers of reproductive conch in the Florida Keys, densities of adults near Lee Stocking Island ranged from 2 to 88 individuals/ha in 1991, with an estimate of 89,000 adults in just a 12-km-long section of the island shelf (Stoner and Schwarte 1994). This provides a plausible explanation for the large numbers of early-stage larvae collected in the Exuma Cays compared with the low densities in Florida. An analogous relationship between the abundance of early-stage larvae and adult densities has been described for fished and unfished areas in the Exuma Cays island chain (Stoner and Ray, unpub. obs.).

Temporal variation in the densities of early-stage larvae is influenced by local spawning frequency, egg hatching, and physical factors such as sea surface conditions. The high abundance of newly hatched veligers at LK1 on 22 July 1993, for example, was associated with very warm water temperature (32°C), known to influence spawning (Stoner et al. 1992). Stoner and Davis (1997b) have shown that conch larval abundance in the upper water column is influenced by wave action, and calm conditions probably allowed conch larvae to accumulate both in the surface layer and in backreef areas such as that near Looe Key reef. However, it is impossible thus far to separate the effects of spawning, hatching, and larval behavior and transport on larval abundance patterns.

Densities of mid- and late-stage larvae are more relevant than those of early stages to the recruitment process. The complete lack of mid-size veligers in the Florida Keys and the high abundance of late-stage larvae relative to early stages, particularly in the offshore sites, indicate that the source for these late stages was probably not local. It is possible that the late-stage veligers were spawned in Florida and retained in gyres south of the Keys (Lee et al. 1992, Lee et al. in press). This retention mechanism has been hypothesized for lobsters in the genus *Scyllarus*, which have a 1- to 2-mo larval phase (Yeung and McGowan 1991); however, two lines of evidence indicate that the retention of conch larvae in the Florida Strait is unlikely. First, no intermediate-size larvae have ever been collected in the waters of the Florida Keys or Florida Strait (see below), and second, the densities of late-stage veligers were equal to or higher than those for early-stage larvae. Rates of mortality for queen conch larvae are unknown but assumed to be high.

Given distances, and average current patterns and velocities between the Yucatan Strait and the Florida Strait, coupled with the assumed age of veligers collected in the Florida Keys, it is most likely that late-stage veligers were transported from spawning populations in Cuba, Mexico, or Belize. Such a larval transport mech-

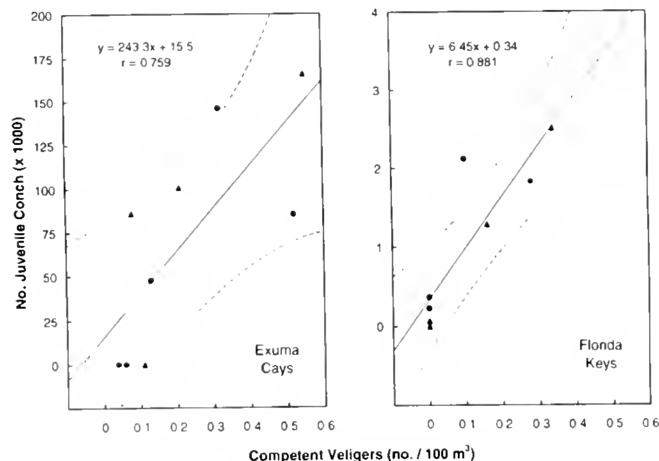

Figure 7. Relationship between the mean density of late-stage queen conch larvae at a nursery ground and the size of the benthic juvenile population in the subsequent year. The relationships are shown for nurseries in the Exuma Cays, Bahamas, and Florida Keys. Circles represent the larval collections for 1992 and juvenile surveys in 1993. Triangles represent larvae in 1993 and juveniles in 1994. Linear regressions and 95% confidence intervals are shown.

anism has been assumed for spiny lobster (*Panulirus* spp.) (Yeung and McGowan 1991) and postulated for queen conch (Berg and Olsen 1989, Mitton et al. 1989, Campton et al. 1991, Davis et al. 1993). Long-distance transport is well documented for a variety of marine molluscs (Scheltema 1971, Scheltema 1986).

Circumstantial evidence supporting the hypothesis that surface currents carry queen conch larvae from the Caribbean Sea to the Straits of Florida was provided in recent collections made in the Florida Current. In June 1993, a mean density of 8.1 veligers · 100 m^{-3} was found 35 km south of Delta Shoal (Stoner unpubl. obs.). All of the veligers collected were >1.0 mm SL and near metamorphic competence. This concentration is an order of magnitude higher than most values found within the Keys, and only one collection, made late in the spawning season at DS2, yielded a higher concentration of late-stage larvae. Given assumed (high) natural mortality rates in conch veligers, it is improbable that high concentrations of late-stage larvae originated in the adult-poor Keys environment, where newly hatched larvae are relatively uncommon. Support for the hypothesis that larvae drift from Cuba to the Florida Keys has been provided recently by the release of surface drifters along the north shore of Cuba (T. Lee pers. commun.). The drogues made direct paths from Cuba to Florida over several days. Under prevailing conditions, the Florida Current front is 10–20 km south of the reef tract, and exchange between the Keys and the current may be uncommon, as shown by the sporadic presence of late-stage larvae at Delta Shoal and Looe Key.

The Exuma Cays island chain is probably a more efficient system than the Florida Keys in maintaining high concentrations of conch larvae close to the island shelf and nursery grounds. During the summer, when conch spawn, winds are nearly always onshore (east to southeast), and the prevailing northwest current (6–12 cm · sec^{-1}) along the Exuma Cays has a significant onshore (cross-shelf) component (N. P. Smith pers. commun.). The larvae are then drawn onto the nursery grounds of the Great Bahama Bank through the island passes by strong tidal currents (Stoner and Davis 1997a). A net flow of water onto the Bank in the pass north of Lee Stocking Island has been observed (Smith and Stoner 1993), and larval concentrations are often higher in nursery areas than offshore near the spawning grounds (Stoner et al. 1992, Stoner and Davis 1997a).

The Relationship Between Larval Supply and Juvenile Populations

Although cause and effect are not established in a descriptive study, differences in larval supply measured in this investigation provide a plausible explanation for the observed differences in juvenile populations of queen conch in both the Florida Keys and the Exuma Cays. This is consistent with Connell's (1985) suggestion that population size will be correlated with recruitment at low recruitment densities. Similar patterns of spatial variation related to larval supply have been observed recently for coral reef fishes (Milicich et al. 1992, Doherty and Fowler 1994) and barnacles (Bertness et al. 1992).

Regardless of the exact source of late-stage larvae for Florida Keys nursery areas, the correlation between larval abundance and juvenile population size in Florida was very high, with just one point lying outside a nearly perfect linear expression. It now appears that ephemeral and small populations of queen conch that exist close inshore along the islands are limited by a general lack of larvae reaching these nursery grounds. Only one veliger (early stage) was collected north of Hawk Channel, where a westward flowing current (N. P. Smith, pers. commun.) may effectively bar the transport of larvae from local spawning grounds, all found along the reef tract.

The correlation between larval abundance and subsequent juvenile population size was also significant in the Exuma Cays, but the relationship was different from that observed in the Florida Keys in two ways. The correlation coefficient was lower in the Exumas, and the slope of the regression was much higher, illustrating that fact that deviation from a linear model of the relationship can vary both within and between sites.

Some of the deviation from the linear relationship among nursery stations in the Exuma Cays can be explained by differences in larval delivery rates. The density of larvae does not measure the actual availability of larvae to a site, and flow past the settlement substratum must be considered (Olmi et al. 1990, Yund et al. 1991). In relative terms, measurements of larval density underestimate larval supply to sites with high flows and overestimate supply at sites with low flows. For example, current velocities at the nearshore stations (CHB and NBC) were relatively low and both had juvenile populations falling below the regression line. Nurseries with the highest tidal current velocities (SR and CBC) had juvenile abundances above the regression line. Attempts to collect queen conch veligers in tube traps, which integrate the abundance of larvae reaching a site over time (Yund et al. 1991), have not been successful, even at station SR, where veligers were most abundant (Stoner unpubl. obs.). Undoubtedly, recruitment to the benthos involves a complex interaction of the density of potential settlers and the regularity and rate of their arrival.

The most remarkable difference in the relationship between mean density of late-stage larvae at a nursery ground and the subsequent juvenile population size in Florida and the Exuma Cays was the difference in slopes. Relatively similar mean densities of late-stage larvae were associated with very much higher juvenile populations in the Exuma Cays; the difference was as high as 40 times. Several explanations for this difference are plausible: (1) *Rate of delivery*—The most productive nurseries in the Exuma Cays are characterized by high current velocities (Stoner et al. 1994, Stoner et al. 1995), with much lower tidal velocities at the Florida Keys nurseries. (2) *Frequency of larval delivery*—Generally, the Exuma sites had higher frequencies of larval delivery to the nurseries than those in Florida, particularly in 1993. (3) *Size of the suitable nursery habitats*—In the Exuma Cays, habitats suitable to queen conch are large and typically have been below their carrying capacity for juvenile conch (Stoner et al. 1994, Ray and Stoner 1994). This provides a large potential settlement area for arriving larvae and may increase the actual numbers of larvae settling. Suitable nurseries in the Florida Keys are associated with relatively specific small backreef and nearshore habitats, more analogous to the small nearshore nurseries in the Exuma Cays (e.g., NBC and CHB) than to the large, open seagrass nurseries of the Great Bahama Bank (e.g., TBR, SR, and CBC). (4) *Settlement cues*—Recent experiments designed to test the response of late-stage queen conch larvae to natural cues from known nursery habitats near Lee Stocking Island (Davis and Stoner 1994) and in the Florida Keys (Stoner et al. unpub. obs.) have shown that settlement and metamorphic responses to sediments and macrophytes in the Exuma Cays are stronger than the responses to analogous substrata in the Florida Keys. Therefore, the frequency of settlement in the Florida Keys may be low. (5) *Postsettlement processes*—Growth and survivorship in the period after settlement

can have a very large influence on the numbers of benthic juveniles animals in a benthic population (Keough and Downes 1982, Luckenbach 1984, Rowley 1989, Keesing and Halford 1992). Mortality rates in young queen conch are highly site specific and inversely density dependent (Ray and Stoner 1994). No comparable experiments have been conducted to compare mortalities between the two sites; however, the small size and low density of juvenile conch densities in Florida may prevent the safety in numbers observed in conch nurseries in the Exuma Cays. It is very likely that all of these mechanisms play at least some role in the low recruitment success of queen conch populations in the Florida Keys after 10 y of fishing moratorium.

Russ (1991) postulated that natural variation in spawning output directly affects local population size in most marine species because of high fecundities and broad dispersal capabilities. However, intense fishing pressure on fishes and invertebrates in the Caribbean has reduced the mean size and abundance of these species in many regions, and some self-recruiting systems with intensive local fisheries may be vulnerable to limitations related to reproductive output and larval supply (Munro et al. 1973, Munro 1983). Overfishing may, in fact, explain the apparent lack of recovery in Florida Keys populations. In the Florida Keys, adult conch populations were probably harvested to the point of recruitment overfishing by the mid-1980s, when a fishing moratorium was established. Today, the populations appear to depend on sporadic influxes of larvae from the Florida Current. If this form of recruitment is not effective in delivering a regular supply of larvae to the Florida Keys, the rehabilitation of queen conch stocks may depend on the success of hatchery rearing and the release of cultured juveniles. However, the limitations of releasing hatchery stocks are well known (Appeldoorn and Ballantine 1983, Jory and Iversen 1983, Stoner 1994, Stoner and Davis 1994).

Future studies will need to determine the relative significance of different larval sources in the Florida Keys and under what conditions larvae in the Florida Current can recruit to the Keys. Most important, species with pelagic larvae and high dispersal potential will need to be managed from the standpoint of metapopulation dynamics rather than on the basis of local populations (Farmer and Berg 1989, Fairweather 1991, Shepherd and Brown 1993, Man et al. 1995). Unfortunately, clear genetic markers have not been found for queen conch (Mitton et al. 1989), and other biochemical markers or new techniques are needed to identify stocks and stock sources. Models integrating oceanographic processes with larval production and behavior may provide another means of answering the important question of larval source. In any case, understanding larval recruitment processes and international cooperation related to spawning-stock maintenance will be crucial to the wise management of queen conch and other fishery resources in the greater Caribbean region.

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THE MANGROVE SNAIL *THAIS KIOSQUIFORMIS* DUCLOS: A CASE OF LIFE HISTORY ADAPTATION TO AN EXTREME ENVIRONMENT

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ABSTRACT This article describes the population ecology of *Thais kiosquiformis* Duclos, the dominant predatory gastropod of the root system of Costa Rican mangroves. *T. kiosquiformis* was shown to cope with the extreme living conditions of its habitat (risk of desiccation and overheating through several hours of daily air and sun exposure, strong salinity, and current changes during the tidal cycle) by using the following strategies: (1) extremely slow growth (~1 mm/y), cessation of growth at the onset of maturity (at ~24 mm in shell length), (2) maintaining high interindividual plasticity in growth and shell thickness as a response to abiotical conditions, food availability, and population density, and (3) migrating ontogenetically for the benefit of lowering desiccation and predation mortality. Because of its high density and biomass (192.2 ± 102.4 g wet weight/m^2), and the predation pressure it exerts mostly on the barnacles of the mangrove roots, *T. kiosquiformis* seems to occupy a central role in maintaining the functioning and productivity of mangroves through "cleaning" their root system from the encrusting fauna.

KEY WORDS: Costa Rica, mangroves, population ecology, gastropods

INTRODUCTION

Thaidid gastropods evolved in the early Miocene (Vermeij 1978) and are distributed worldwide from tropical to boreal areas, indicating the large adaptive potential of this family. They occur in intertidal and subtidal shallow waters, often as a dominant invertebrate predator within their habitats (Menge 1978). Studies on the ecology of thaidid gastropods revealed a complex set of mechanisms to deal with the harsh environmental conditions they encounter in the intertidal habitat. These include prey type selection motivated by energètic considerations (Palmer 1984); prey size choice to minimize the risk of dislodging at wave-exposed sites (Richardson and Brown 1990); optimized foraging behaviour in relation to prey abundance and mortality factors such as predators and desiccation (Menge 1978, Spight 1983, Fairweather 1988); switching to anaerobic metabolism by aperture closing to avoid desiccation (Cantera et al. 1980); size-dependent zonation related to factors like food, predators, shelter, and desiccation (Butler 1979); and modification of shell thickness as a response to different predation pressures by crabs (Kitching 1977, Palmer 1985, Geller 1990). Menge (1978) concluded from his study on predation exerted by *Thais lapillus* on a rocky shore that each snail has to be considered an individual, where life history traits and phenotype are as important as extrinsic factors (e.g., actual habitat conditions). West (1988) also reported high interindividual variation in prey preferences and growth of *Thais melones* on the Pacific Coast of Panama. Great individual plasticity in behavioural, physiological, and morphological responses to a harsh environment seems thus to explain the success of this family.

Thais kiosquiformis Duclos is among the most abundant snail species and probably the most important invertebrate predator in the mangrove swamps of the upper Gulf of Nicoya on the Pacific Coast of Costa Rica (10°N, 85°W). It is distributed along the Pacific and Atlantic Coasts from Baja California to Peru in the intertidal at salinities of 5–30‰, living mainly on mangrove roots, rocks, and rotting trunks (Keen 1971, Cantera et al. 1980). It is strictly carnivorous, preying on balanids, bivalves, and other gastropods by drilling a hole in the prey's shell or by introducing its foot. Cannibalism does not seem to occur. Specimens can survive desiccation up to 9 d by closing the aperture tightly and switching to anaerobic metabolism. The species grows to approximately 50–55 mm in shell length; the strong shell is muddy brownish with well-developed spines. Maturity is reached at 24–32 mm shell length (Cantera et al. 1980).

The objectives of this study were (1) to describe the population structure (density, biomass, horizontal size distribution) of *T. kiosquiformis* at study sites differing in freshwater influence, sediment characteristics, height above low water level, and total mangrove extension; (2) to estimate growth and mortality rates at the different study sites; and (3) to estimate food consumption and identify main prey items. The overall goal of the study was to explain the mechanisms that not only promote survival under the extreme conditions of the mangrove habitat but also allow this species to maintain high abundance and biomass in the mangrove root community.

MATERIALS AND METHODS

Study Area and Sampling Sites

The Gulf of Nicoya is a tectonic estuarine embayment located on the Pacific Coast of Costa Rica, Central America (Fig. 1). The gulf is divided into a shallow upper part (~20 m) and a lower part (>200 m in depth), which is bordered by the Puntarenas Peninsula in the east and San Lucas Island in the west. Because of seasonal upwelling events, this region is the most productive fishery ground in Costa Rica.

Seasonality on the Central Pacific Coast of Costa Rica is very pronounced, with 89% of the annual precipitation occurring from May to October. Average total precipitation for Puntarenas is approximately 1,550 mm/y (Thomas 1988). The annual mean air temperature for Puntarenas is 27.35°C (Janzen 1991); water temperatures in the upper gulf range from 28 to 30°C. The water body of the upper gulf is heavily influenced by freshwater and sediment input of the Rio Tempisque, located at the northern end, and by numerous smaller rivers, especially along the eastern shores (Peterson 1960). The tides are semidiurnal (12.4 h); amplitudes vary between 1.8 and 2.8 m with a mean of 2.3 m (Peterson 1960, Voorhis et al. 1983). During the rainy season, surface salinity in the upper gulf may drop down to 5‰ near river mouths during low

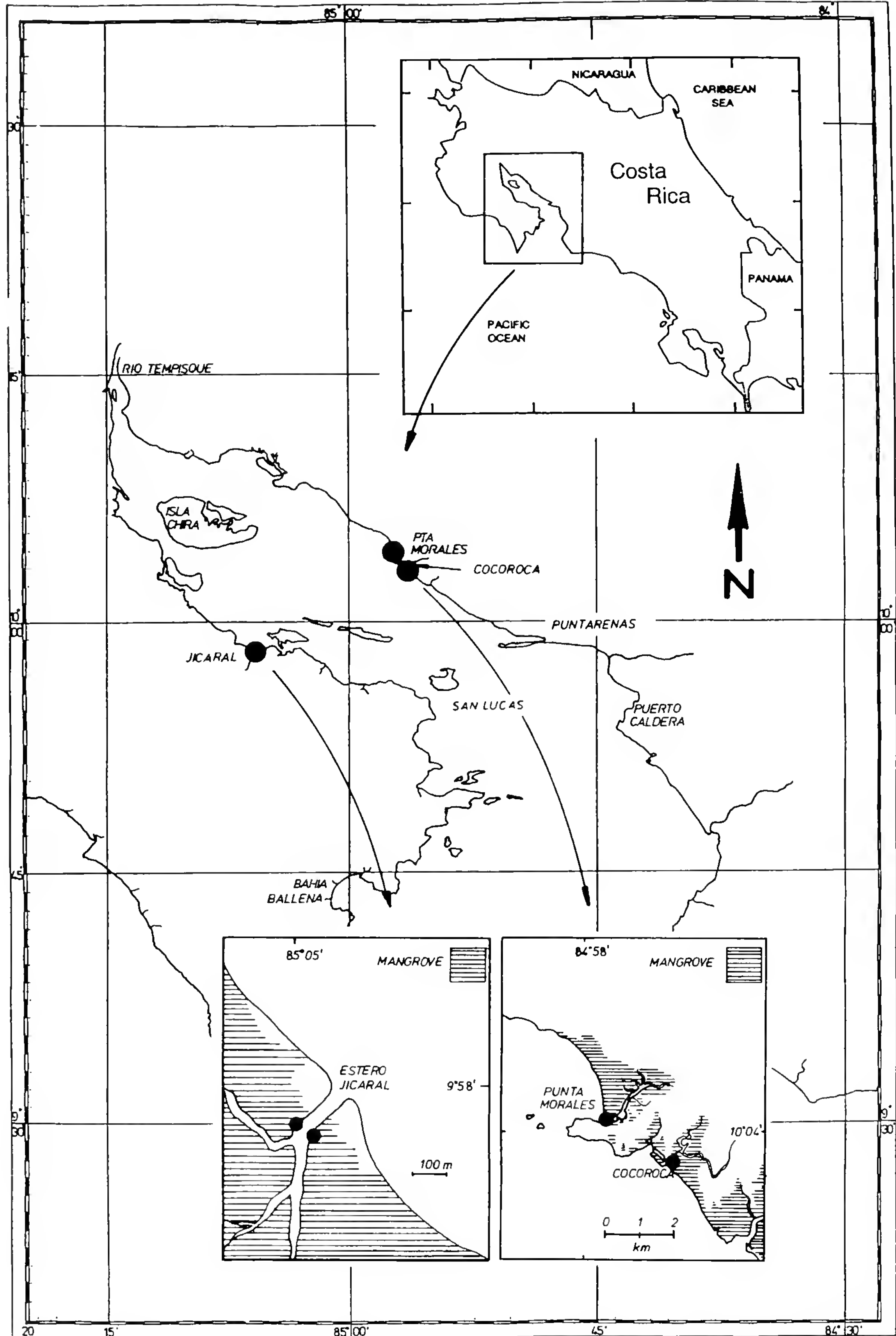

Figure 1. Gulf of Nicoya with the three study sites (solid circles)—Punta (PTA) Morales (P), Cocoroca (C), and Jicaral (J).

tide (this study). The outer coastline of the upper gulf is covered with extensive mangrove stands, dominated by red mangroves (*Rhizophora mangle* and *Rhizophora harrisoni*).

Two sampling sites were chosen in each of the three estuaries of Punta Morales (P1, P2), Cocoroca (C4, C5), and Jicaral (J6, J7) for a representative cross-section of the upper gulf (Fig. 1). The vegetation consisted exclusively of the two *Rhizophora* species mentioned above. Large mudflats extended seaward at each location. Freshwater input was highest at Cocoroca (Rio Lagarto), followed by the Rio Jicaral and the Rio Quebrada Grande in Punta Morales (Fig. 2). The latter only carries freshwater during the rainy season, and even then, the input is very low (Gocke et al. 1981, this study). The increase in salinity in November marks the end of the rainy season. At high tide, the water level at the sampling sites of C and J was approximately 1–1.5 m; in P, it was 1.5–2 m above the sediment, coinciding with the upper limit where barnacles occurred. The sediment in Punta Morales was coarser than that at the other sampling sites, with stones and rocks contrary to the uniform sandy/muddy Cocoroca and Jicaral sites. Accordingly the "sink-in depth" varied considerably: J > C > P.

Sampling Strategy

Twelve length frequency samples were taken at about monthly intervals along transects at P1, P2, and C4 from September 1993 to July 1994. In C5, J6, and J7, only 10 samples were taken from October 1993 to July 1994. An additional sample of tagged snails was taken in February 1995 in P1. Surface water salinity and water temperature were measured at the adjacent water line (refractometer, ±0.5‰; mercury thermometer, ±0.2°C). Each transect consisted of 10 squares of 60 · 60 cm each (0.36 m^2), placed 1 m apart from centre to centre, with a 10-m line tagged at 1-m intervals. This line was positioned vertical to the mangrove edge (Fig. 3). Because a solid frame could not be used because of the mangrove roots, an inch rule was taken instead. All *T. kiosquiformis* found within the square were taken to the laboratory. Total shell length was measured to the nearest 0.5 mm with a caliper. Snails from each square were measured separately to determine length frequency patterns along the transect. After measuring, specimens were returned to the transect to avoid introducing artificial mortality into the population. This was not done in Jicaral because of the large distance to the laboratory (1 h by boat).

On the first sampling date, a different strategy was used to determine the appropriate square size: in addition to the basic design, each square was divided into four subquadrats of 30 · 30 cm each (0.09 m^2). Mean length, standard deviation, and variance of the subsamples were calculated to determine whether the variance changed as a function of sample area. The 60 · 60 cm quadrat size gave homogeneous results even for densities <35 snails/m^2, whereas the smaller quadrat size (30 · 30 cm) did not perform well under low densities, where number of snails, mean length, and standard deviation differed significantly among the subsamples. Therefore, the larger quadrat size was selected for subsequent sampling during the study (length frequency sampling and biomass determination).

Growth and Mortality

The growth of *T. kiosquiformis* was determined from the length frequencies by use of the seasonalized von Bertalanffy growth equation (Pauly and Gaschütz 1979) as implemented in the FiSAT program (Gayanilo et al. 1994):

$$L_t = L_\infty(1 - e^{-(K(t-t_0)+CK/2\pi(t-t_s)})$$

where L_∞ is the asymptotic length, L_t is the length at time t, K is the growth constant, t_0 is the age at length zero, C lies between 0 and 1 and describes the growth amplitude (of a sine function), and t_s is the starting point of the growth oscillation.

The program restructures the data by calculating a running average over five length classes, dividing each length class by this value and subtracting 1 from the result, which creates peaks and troughs. The program traces growth curves with different sets of parameters through the length frequencies and selects the curve that hits as many peaks and avoids as many troughs as possible.

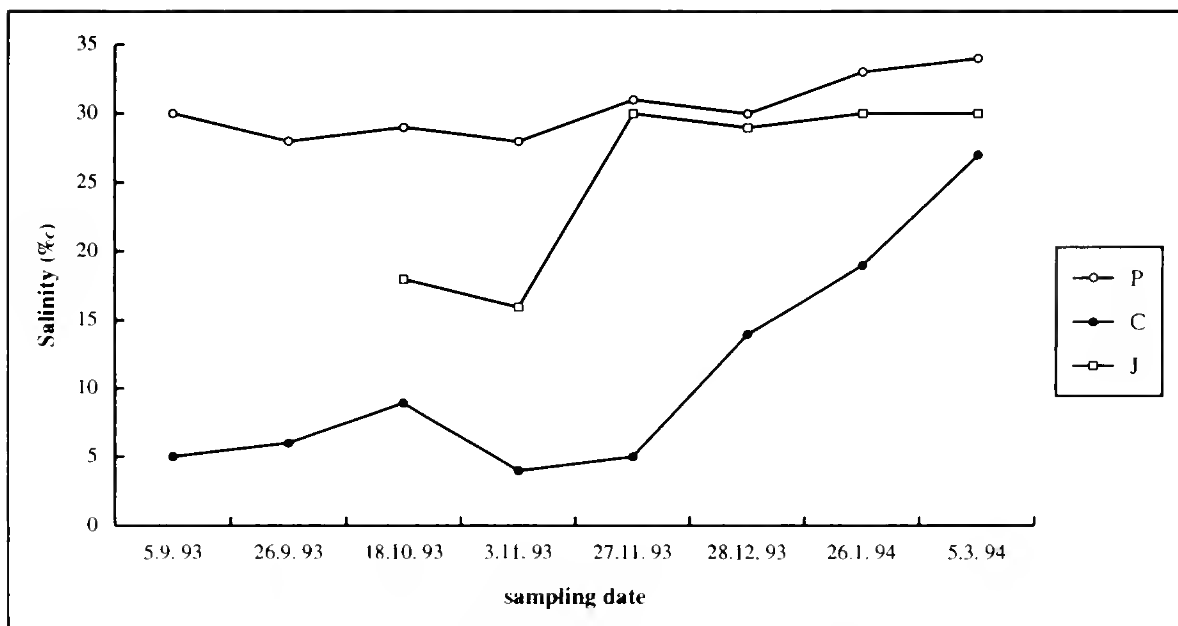

Figure 2. Surface salinity at the study sites P, C, and J during low tide.

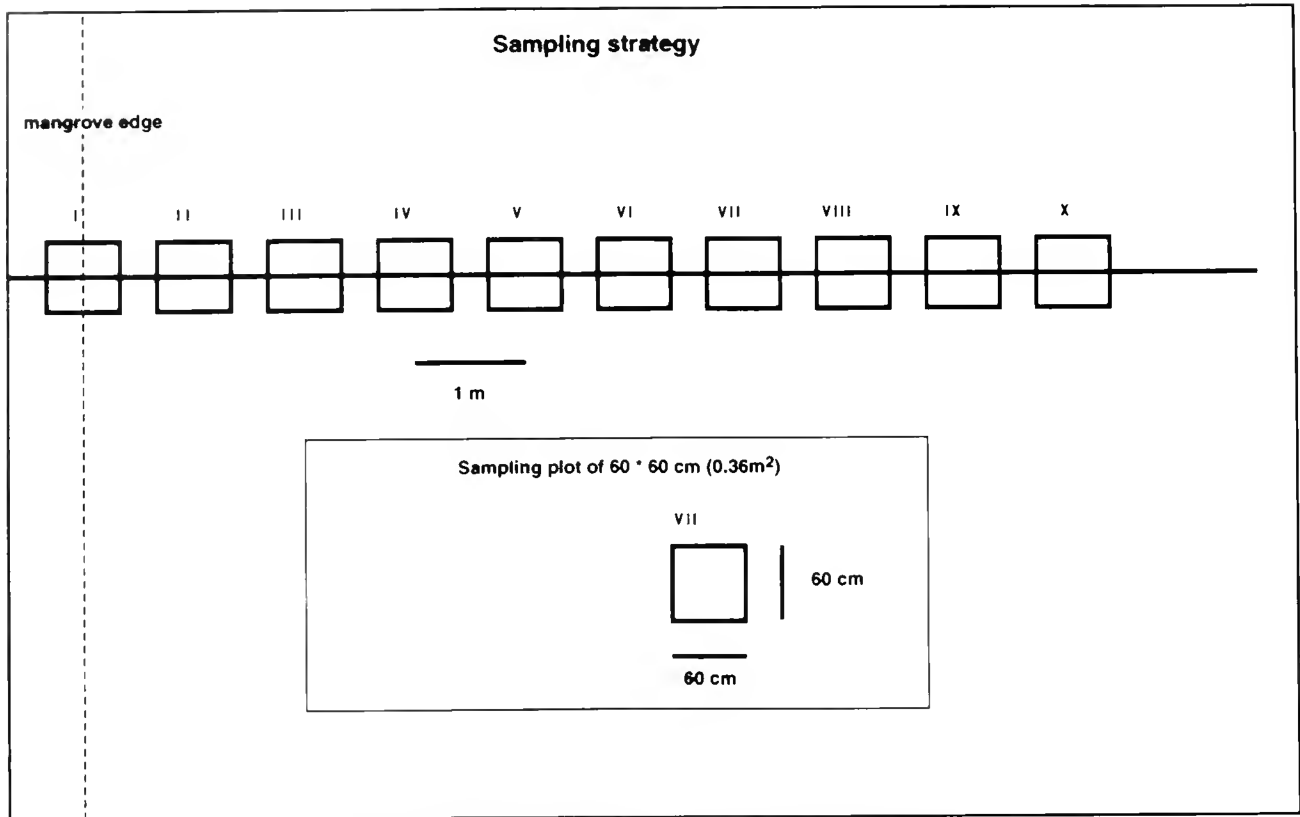

Figure 3. Sampling strategy. Letters (I-X) mark quadrat numbers.

The length measurements of each transect were grouped into 1-mm intervals. Because of the striking differences in biomass and length distributions between the transects, the von Bertalanffy parameters were calculated separately for each transect. Because the analysis of the whole range of length frequencies did not give satisfactory results (goodness of fit, $Rn < 0.2$ in all cases), the youngest visible cohort was determined by eye and reanalysed separately (P1 and P2, ≤23 mm; C4, ≤22 mm; C5 and J6, ≤25 mm), as described in Wolff (1985). In J7, growth was not determined because of the low number of snails found during each sampling date (<30).

The growth performance index (ϕ'), which allows for the comparison of growth between different species (Pauly and Munro 1984), was then calculated with the growth parameters K and L_∞:

$$\phi' = \log_{10} K + 2 \cdot \log_{10} L_\infty$$

As a second method to estimate growth parameters and to compare with the results of the length frequency analysis, 643 snails were marked and monitored over the sampling period. A paint marker was used to mark 230 snails; 186 were marked with quick-drying oil paint. The numbers were written on the shell with waterproof markers. Because both methods failed, a sandwich technique was used, applying two layers of nail paint on the cleaned shell near the aperture. The numbers were written with india ink, and the mark was sealed with "crazy glue." This method was applied to 413 snails, of which 186 specimens had old marks (re-marked). The animals were released near the sampling site P1. On each sampling occasion, a search of about 1 h was conducted for marked snails, which after collection, were measured with a caliper to the nearest 0.1 mm and subsequently released at the same spot.

Estimates of von Bertalanffy growth parameters were derived by the use of the Munro plot (Munro 1982).

$$K_{(t_2-t_1)} = \ln(L_\infty - L_1) - \ln(L_\infty - L_2)$$

where L_1, L_2, t_1, and t_2 are the lengths and times of marking and recapture, respectively, L_∞ is as described above, and $K_{(t_2-t_1)}$ is the growth constant over the time interval. The model allows for calculating the growth constant K for each individual. The sample from February 1995 was treated separately because the transect had been left undisturbed for nearly 1 y, avoiding recapture stress and habitat disturbance through frequent sampling. A linear regression of maximum shell length (L_{max}) versus average density at the transects was calculated, expecting a negative correlation of L_{max} with density. L_{max} was defined as the mean of the largest 3% of the snails at the respective transect (Pauly 1984). Total mortality (Z) was estimated with the mark-recapture data by use of a formula proposed by Gulland (1969):

$$\ln N_r = a + b \cdot r'$$

where N_r is the number of recoveries per time interval r', a is the y-intercept, and b is the slope of the regression, which provides the estimate of Z (with sign changed). This method can only be applied to data where the marking procedure is performed at one time (e.g., a few days), the sampling effort is roughly similar, and mark shedding does not occur (Pauly 1984). Therefore, the October 1993 census was taken as the starting point. Only snails marked or recaptured (and re-marked) at this date were included in the analysis. This ensured that only specimens with sealed marks (no mark shedding) were used for mortality estimation. The February 1995 recapture data had to be corrected for sampling effort because the

area was searched for 4 h, exceeding normal effort by a factor of four.

Population Structure, Biomass, and Density

For each quadrat, the median length was calculated (this was preferred to the mean length because of the lower sensitivity to "outliers") with the pooled data of all samples taken from September to March to prove whether different length groups prefer distinct zones along the 10-m transect from the mangrove border inwards. In addition, the proportion of juveniles (≤20 mm) of the total was calculated for each quadrat to see if they prefer a distinct zone within each transect and to compare the dominance of juveniles between the transects.

Total shell length was converted to dry weight with 87 specimens of *T. kiosquiformis*. To do so, the shell was broken, and the cleaned tissue was placed in aluminum dishes and dried for 100 h to constant dry weight at 65°C. Dry weight was determined with a precision of 0.1 mg. A potential regression of the form $y = a \cdot x^b$ was used (Table 1). The last four samples were excluded from the size distribution and from the biomass calculations because of possible sampling bias because they were not taken by the authors.

Dry weights of all individuals of a length class were calculated from the pooled length frequency data with the regression given above. Biomass values of the length classes were summed and divided by the total area (in square meters) sampled, yielding an average biomass of *T. kiosquiformis* for the upper gulf. The biomass of each sampling quadrat was calculated by pooling the length frequencies for the respective quadrat.

Food Intake

The *in situ* food intake of three size groups of *T. kiosquiformis* (18–22 mm; 23–27 mm; 28–32 mm) was estimated over a period of 14 d in the field (Punta Morales). Ten snails of each group were placed in a mesh wire cage (height, 1 m; Ø, 40 cm), situated approx. 0.5 m above mean low water level at the mangrove edge. The 10 snails were weighed collectively before and after the experiment to the nearest 0.1 g. Wet weight again was converted to flesh weight gain by use of a linear regression of wet weight versus flesh weight (Table 1). Wet weights of each prey species were also registered before the experiment (for balanids that were offered on pieces of aerial roots, percent area covered was estimated). The following eight species (corresponding to the most abundant potential prey in the area) were placed in each of the three cages: *Balanus* sp. (Crustacea), *Littoraria varia*, *Littoraria fasciata*, *Anachis*, *rugosa*, *Cerithium stercusmuscarum* (Gastropoda), *Brachidontis*, *puntarensis*, *Pinctada mazatlantica* (juv.), and *Cardita affinis* (Bivalvia). *P. mazantlantica* is not common in mangrove forests, but a large spatfall had occurred on the mangrove roots shortly before the experiment started, so this bivalve was included. Two months later, this species had disappeared from the area. After the experiment, the surviving prey organisms were counted and weighed together with the empty shells. Because all prey had shells, weight differences measured after the experiment consisted only of flesh weight consumed by the predator. Daily food intake was expressed as % BWD (flesh weight consumed daily/wet (flesh) weight of predator). The weight increment of *T. kiosquiformis* was expressed as % wet weight gain/14 d. Prey preference was not determined; the relative abundance of prey species and their availability in the experiment were not comparable to normal habitat conditions. Some prey specimens (8 of 156) were not found after the experiment and had to be excluded as not eaten.

TABLE 1.

***T. kiosquiformis:* Regression Parameters of Dry Weight Versus Shell Length and Flesh Weight Versus Wet Weight Used for Biomass Estimation and Calculation of Daily Ration.**

Total Length/Dry Weight	Wet Weight/Flesh Weight
$a = 0.00010$	$a = -0.00700$
$b = 3.187$	$b = 0.129$
$r^2 = 0.976$	$r^2 = 0.937$
$n = 87$	$n = 87$

RESULTS

Growth and Mortality

The length frequency analysis yielded similar parameters for the different sampling sites (Fig. 4), where the ranges are: K, 0.18–0.2; L_∞, 48–50 mm; C, 0.575–0.65; W_p, 0.775–1.0. The goodness of fit (R_n) of the growth curves ranged between 0.171 (C5) and 0.341 (J6). The average growth performance (ø') was 2.66, with values for K and L_∞ of 0.19 and 49 mm, respectively.

The tagging experiment conducted in Punta Morales yielded results that differed from those of the length frequency analysis. Recapture data from September 1993 to March 1994 and from March 1994 to February 1995 are presented separately, because the latter consisted of individuals that were left undisturbed for 1 y (Fig. 5). The points can be divided roughly into three groups: specimens with positive K values, >0.02 (I); others with K values around zero, $0.02 > K > -0.02$ (II); and snails with negative K values, < -0.02 (III). Between September and March, only six juvenile and subadult snails (<24 mm) showed positive K values. Most specimens did not grow at all; snails larger than 25 mm tended to show negative K values. For the period from March to February, the smaller animals had the highest K values, decreasing more or less linearly to zero at a shell length of 24 mm. The same division of points was applied here, but because no negative K values occurred (and no specimens >26 mm), only the first two groups are represented. Mean density at the transects was negatively correlated ($R^2 = 0.944$) with maximum shell length (Fig. 6). Highest L_{max} values occurred at J7 and J6, where density (and biomass) were lowest, whereas high densities (P1 and C4) related to low L_{max} values. P2 was excluded from the calculation because the population at this transect consisted almost exclusively of juveniles and subadults.

The estimation of the total mortality rate (Z) from the tagging data (Fig. 7) yielded a value of 0.178/y (c.i. 0.132–0.225). The resulting fit of the regression was good ($R^2 = 0.951$; $p = 0.0002$).

Population Structure, Biomass, and Density

The distribution of length classes along the transects (Fig. 8) shows a clear prevalence of smaller animals in the inner zone. The median length decreases from the mangrove border to the interior part, stabilizing 5–8 m inwards from the mangrove edge. The graph also demonstrates the large differences in median shell length between the transects. The subpopulation in J7 consisted of the largest snails, followed by J6, C5, C4, P1, and P2. In P2, the

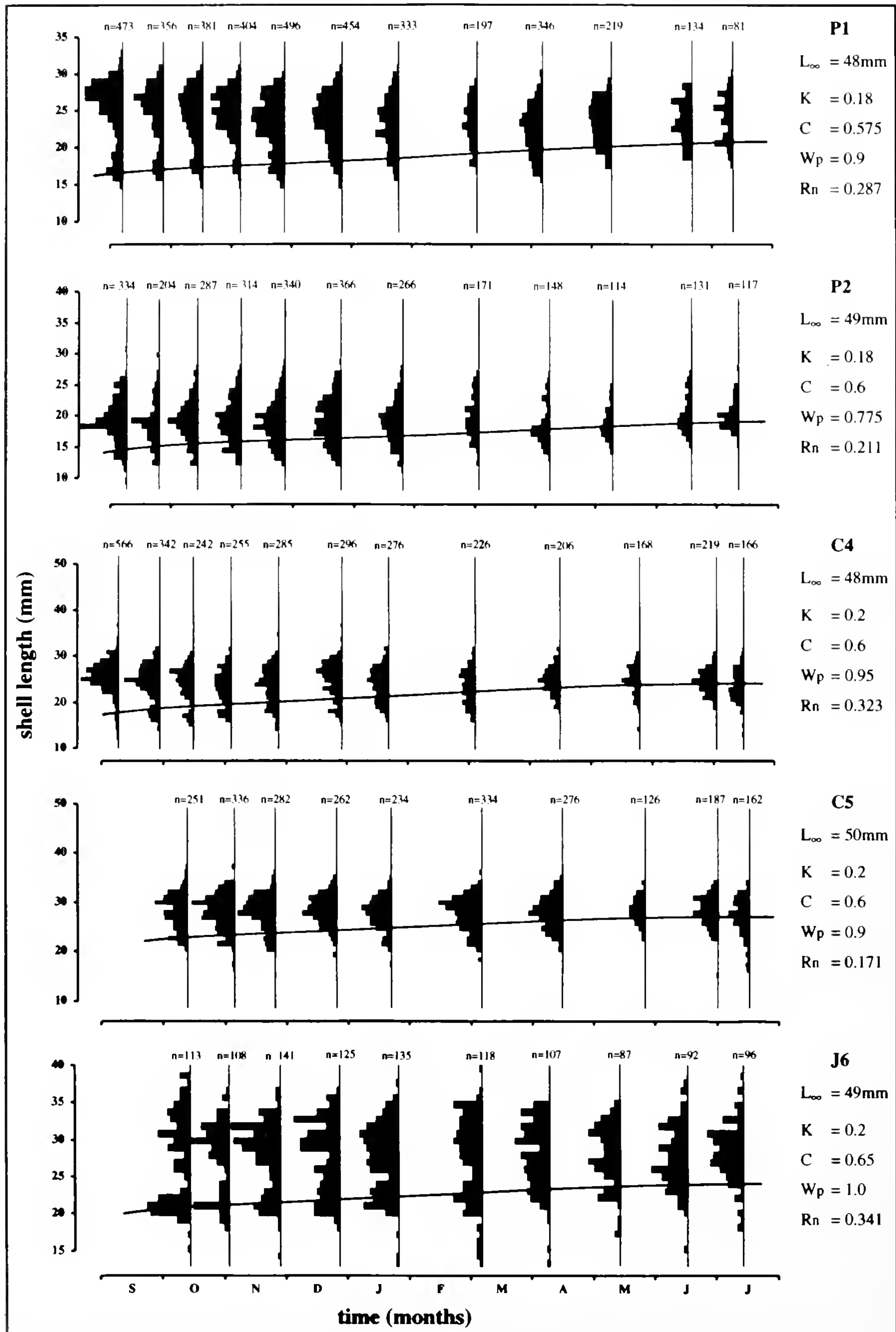

Figure 4. *T. kiosquiformis:* growth at the five transects, calculated from the length frequencies with ELEFAN. L_∞ and K von Bertalanffy growth parameters; C, constant of growth oscillation; Wp, winterpoint; Rn, goodness of fit.

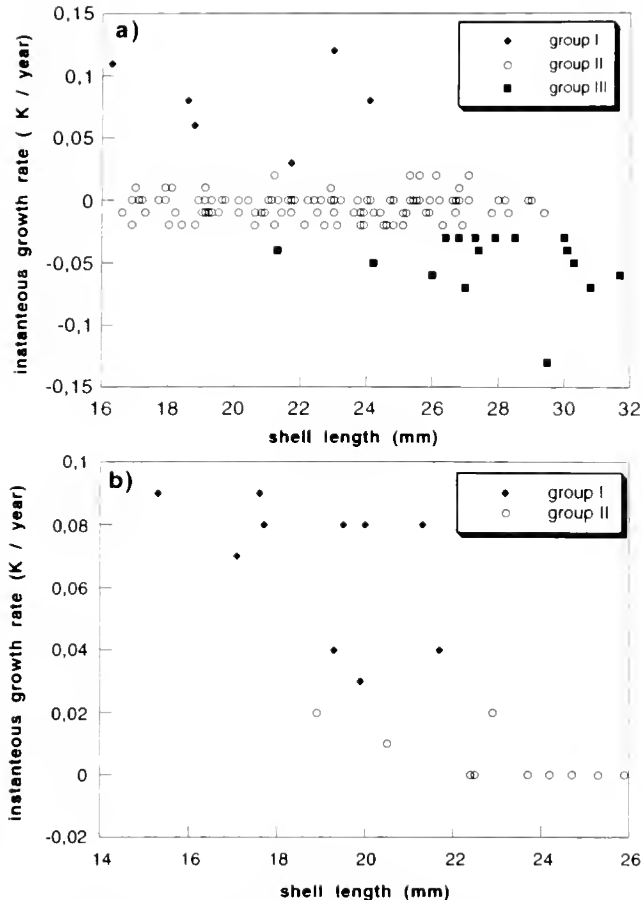

Figure 5. *T. kiosquiformis:* growth constant K as related to shell length for individuals of the marking experiment. (a) Sampling period from September 1993 to March 1994. (b) Sample taken in February 1995. Group I, K > 0.02; group II, 0.02 < K < −0.02; group III, K < − 0.02.

decrease in median length was not very pronounced, but because it was the transect with the smallest snails, large changes in size frequency distribution could not be expected. The dominance of juveniles ≤20 mm is therefore clearly strongest in P2, followed by P1 and C4 (Fig. 9). At the other transects, only 0–10% juveniles were found. At the first three transects, the dominance of juveniles increased inwards from the mangrove edge, indicating a gradient along which snails of different age classes are distributed. Highest density values (ind./m^2), obtained at the transects, were: 222 at P1, 139 at P2, 194 at C4, 111 at C5, 56 at J6, and 19 at J7.

Biomass, averaged over transects and sampling period, was highest in quadrats III–V (2–4 m from the mangrove border) (Fig. 10). The high standard errors are partly explained by the high variation of biomass values between transects, differing by a factor of up to 6. Average biomass and standard deviation of *T. kiosquiformis*, calculated over transects and sampling period, were 6.37 ± 3.41 g dry weight/m^2 (192.2 ± 102.4 g wet weight/m^2).

Food Intake

The average daily ration was lowest in the medium group (1.3% BWD wet weight; 10.7% BWD flesh weight), followed by the smallest group (1.6% BWD wet weight; 13% BWD flesh weight) (Table 2). The largest animals had the highest daily ration (2.0% BWD wet weight; 16% BWD flesh weight). *Littorina* spp. accounted for nearly 70% of prey eaten in the experiments, followed by the pearl oyster *P. mazatlantica* with 19% and the small mytilid *B. puntarensis* with 10%. Under natural conditions, however, *T. kiosquiformis* primarily feeds on balanids (pers. obs.). The results of the field experiment (Table 2) show that only the smallest size group had slight positive growth in wet weight (0.6%/14 d), whereas the larger groups (23–27 and 28–32 mm) lost weight during the experiment (−1.9 and −1.3%/14 d, respectively).

DISCUSSION

Growth and Mortality

The length frequency analysis yielded very low K values (0.18–0.2) for *T. kiosquiformis* for all transects when compared with the reports of Cantera et al. (1980), who gave growth rates of approximately 0.5 mm/mo (K/y ≈ 0,29) for this species on the Colombian Pacific Coast. Although the goodness of fit (Rn) was rather low for our K estimates (0.171–0.341), the calculated parameters are very similar between transects and seem reliable.

The growth performance index (ϕ' = 2.66) is correspondingly low and seems more comparable to that of boreal than of tropical gastropods, which normally exhibit values between 3.2 and 4.7. Only two boreal thaidids and some *Littorina* species are reported to have similar low values (Wolff 1994).

The results of the marking experiment indicate still much lower growth rates than were found with the length frequency analysis. Of nearly 300 recaptured snails, only a few showed positive growth during the sampling period. One might suspect marking and/or recapture stress to be the cause, but this does not seem probable for the following reasons: (1) marking was conducted by three different methods (see Materials and Methods); (2) marking procedure and recapture never lasted longer than 2 h, whereas snails are naturally exposed for up to 5 h during low tide; (3) many marked snails were recaptured the first time alive after 3–5 mo without showing any measurable growth; and (4) the sample taken in February 1995 yielded basically the same results, although the transect had been left undisturbed for nearly 1 y. The decrease in total shell length in some specimens is due to shell erosion at the apex, where the periostracum can be damaged, especially in older animals, leaving the calcareous shell structures in this part uncovered. The interindividual variability in growth—seen in the wide scatter of individual K values—is very high, but several authors report similar variabilities for other molluscs (e.g., Moore 1972, Broom 1982, Sainsbury 1982, Wolff 1987). This variability can probably be explained through small-scale differences in habitat structure but possibly also by genotypical differences between specimens (Wolff 1987).

Unexpectedly, K, generally considered to be constant for a species over its whole life cycle, clearly decreased with size, and it seemed reasonable to divide the individual K values into three groups: positive values for snails ≤24 mm, values around 0 (occurring in the whole size range), and negative K values for snails >24 mm [one exception]. It thus seemed that 24 mm, which marks the lower limit for the onset of maturity (Cantera et al. 1980), can be taken as the turning point for the snails in P1, where growth ceased completely. This suggests ontogenetically based metabolic changes related to the onset of maturity. This pattern can be described for the study site P1, where population densities were highest. However, for the other study sites, L_{max} values were

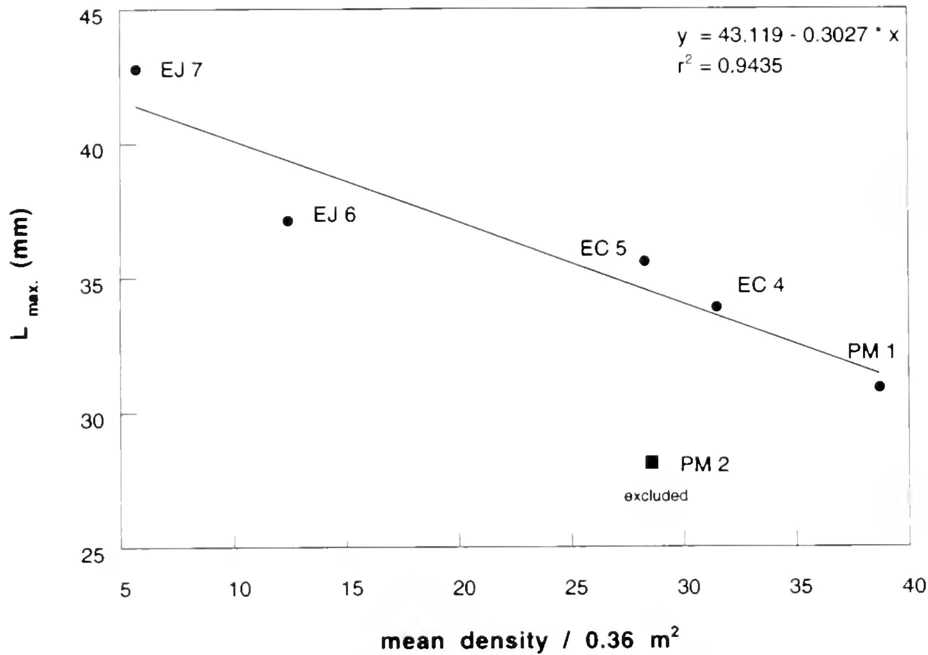

Figure 6. *T. kiosquiformis:* maximum shell length ($L_{max.}$) vs. average density at the six transects. P2 was excluded from the fitted regression (see text).

significantly higher, which suggests that growth in these areas continues in older individuals. Thus, it might be speculated that somatic growth ceases early under high population densities (resulting in smaller maximum length) in response to limiting food supply. Under these conditions, energy may be used exclusively for gonad formation and maintenance metabolism. A negative effect of high densities on growth and maximum length through intraspecific competition for microhabitats and food resources has also been reported for other intertidal gastropods (e.g., Underwood 1978). This phenomenon could naturally not be detected by the length frequency analysis because only juvenile specimens, which were still growing, were used.

The thick shell of *T. kiosquiformis*, being the most robust among the snails occurring at Punta Morales (Borjesson and Szelitowski 1989), is energetically costly (Palmer 1985, Kitching 1986) and might be another factor explaining the very slow growth rate of *T. kiosquiformis*. In related species, thick-shelled morphs were also shown to have a decreased tissue growth (*Thais lamellosa*, Palmer 1981); slower shell growth (*Purpura* species, Wellington and Kuris 1983); higher food intake as the result of increased production, maintenance, and locomotion costs (*T. lapillus* and *T. lamellosa*, Palmer 1992) and less offspring production (*Thais emarginata*, Geller 1990) when compared with thin-shelled morphs. Finally, energy costs for adaptations to environmental stress, which is supposed to be very high in tropical intertidal areas (Moore 1972, Vermeij 1978, Garrity 1984), may contribute a significant part to the total energy budget, further reducing energy available for growth (Russell-Hunter 1985).

We believe that a combination of the above-mentioned factors is responsible for the extremely slow growth of *T. kiosquiformis*. The results of the length frequency analysis possibly reflect the growth potential of this species when conditions are favourable and food supply is not limiting. In J6, this may have been the case, because density was low, animals grew near to their asymptotic size, and goodness of fit (Rn) for the resulting growth curve was very good.

If high population density and biomass are maintained while individual growth is very slow, survival must be maximized. Our mortality estimate ($Z = 0.178$), which is at the low end of the range reported for tropical and subtropical gastropods (0.1–1.66) (Sainsbury 1982, Appeldoorn 1987, Appeldoorn 1988, Prince et al. 1988, Wolff 1989, Debrot 1990), is an indication thereof. This low mortality is probably due to: (1) the strong antipredatory characteristics of the shell (thick shell, narrow aperture, stout spines), which reduce predation pressure by crabs and fish (Vermeij 1978, Palmer 1979, Palmer, 1985, Bertness and Cunningham 1981, Wellington and Kuris 1983); (2) the high tolerance against desiccation (Cantera et al. 1980); and (3) the ability to minimize energy expenditure (extremely slow growth, growth inhibition at the onset of maturity), using a "sit and wait" strategy. These results contradict Alongi's (1989) general assumption that turnover rates as well as predation mortalities in the tropics are higher than in temperate latitudes.

The method used may even have overestimated mortality, be-

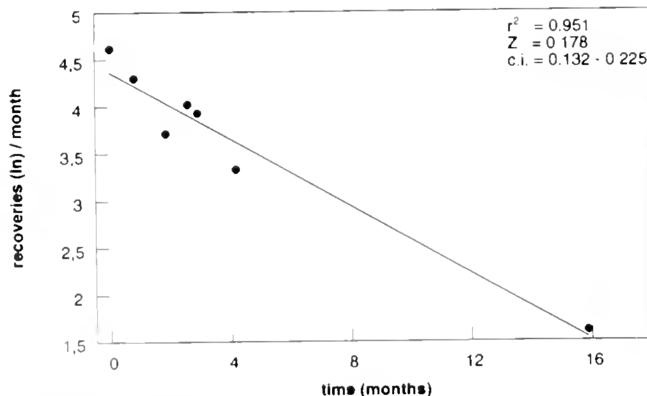

Figure 7. *T. kiosquiformis:* total mortality rate (Z) as derived from Gulland's (1969) method.

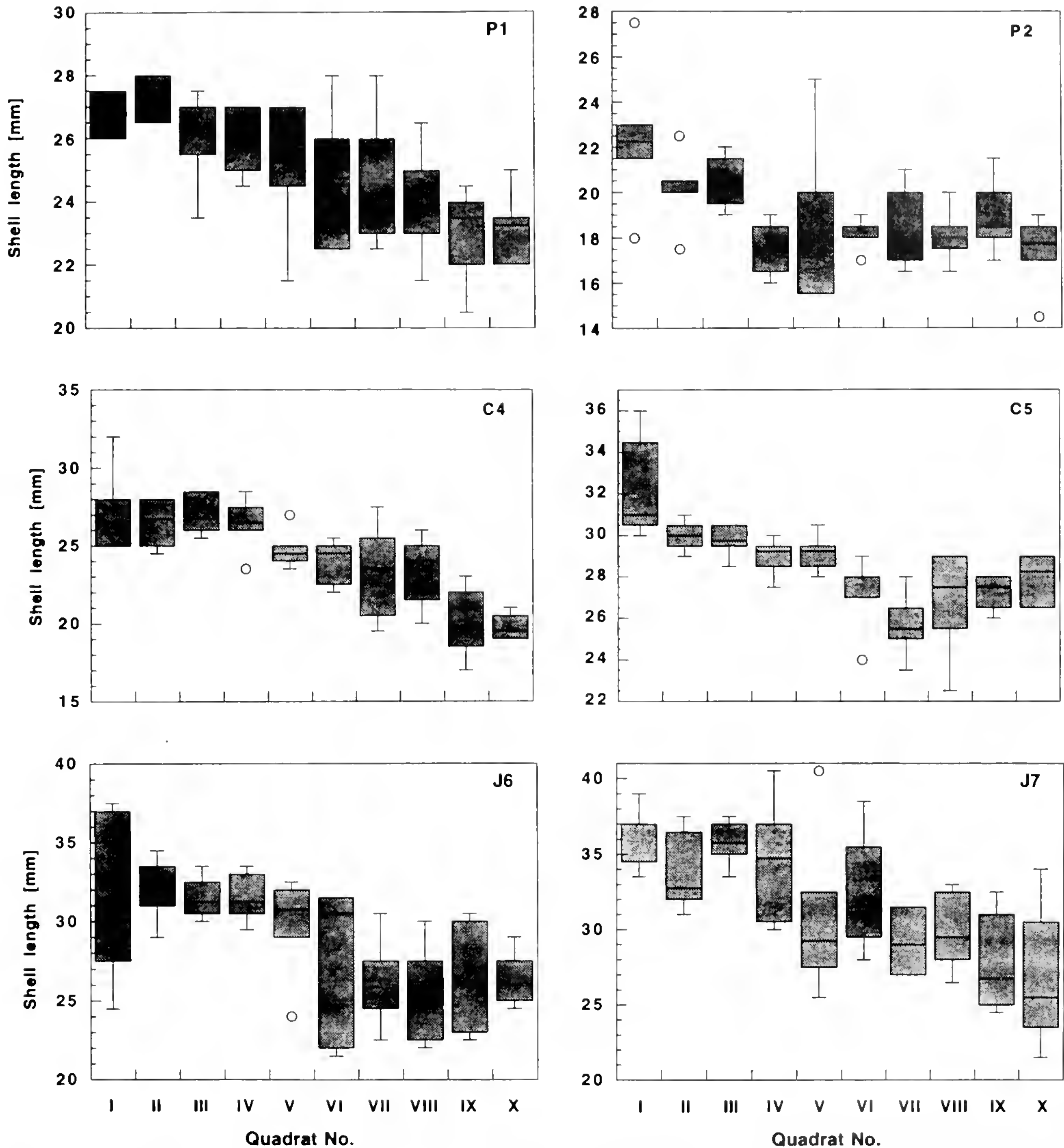

Figure 8. *T. kiosquiformis:* **box plots of the size distributions along the six transects, with the combined data of all sampling dates. Horizontal line within the box indicates the median, first upper and lower quartiles are given by the vertical edges of the box, vertical bars indicate the whole data range, and open circles are extreme outliers, which were excluded from the calculation.**

cause mark shedding may have occurred to a small extent and because snails could have migrated out of the area. In addition, specimens well hidden might not have been found.

Population Structure, Biomass, and Density

The observed distributional pattern—with the juveniles predominating mangrove inwards and the larger specimens towards the mangrove edge—probably results from the active distribution of the respective size groups along a gradient of predation and desiccation, as described for several gastropods (e.g., Vermeij 1972, Butler 1979, Garrity 1984). The most important gastropod predators in the area are puffer fish (*Sphoeroides* species and *Diodon* species) and rays, which were frequently observed foraging in Punta Morales (Jerome 1987, Whitey 1990, pers. obs.). Predation pressure is probably more severe at the mangrove border than inside the forest for the following reasons: (1) the root system is much denser inside, making foraging more difficult here; (2) foraging time is reduced inside the forest because of shorter tidal

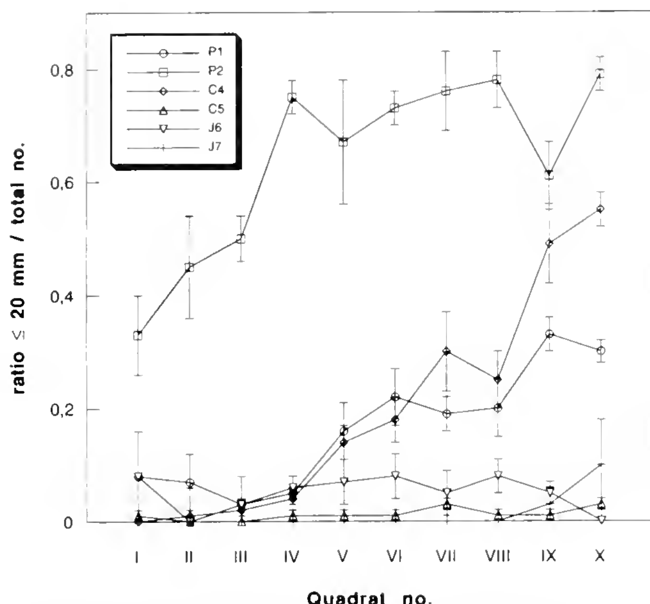

Figure 9. *T. kiosquiformis:* **ratio of juveniles (≤20 mm) to total number of specimens ± standard errors along the six transects.**

inundation (higher elevation of the sediment); and (3) light conditions are better at the mangrove border (less shading through the canopy), making visual recognition of prey specimens easier. Because large *T. kiosquiformis* are less susceptible or even immune to predation, as shown by Palmer (1979), they can live at the mangrove border, where barnacles are more abundant than in the inner zone (pers. obs.), whereas smaller snails are largely restricted to the inner forest, where predation is less intense.

Irradiation is more intense at the mangrove border, where the canopy is less dense than mangrove inwards, resulting in relatively higher temperature and desiccation. Wind is also stronger at the border because air movements are dampened by roots and leaves, reducing desiccation stress in the inner zone. Generally, larger individuals of a given gastropod species can tolerate desiccation much better than juveniles (Moore 1972, Vermeij 1972, Vermeij 1978, Underwood 1978), which allows them to stay further outside than juveniles. Personal observations confirmed the migration pattern of smaller specimens towards the inner zone, whereas large

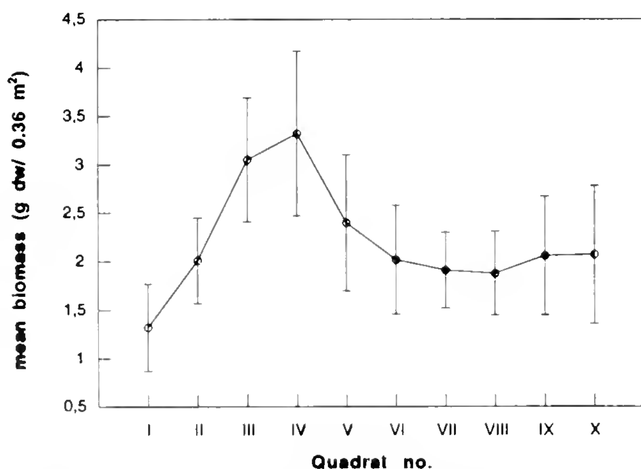

Figure 10. *T. kiosquiformis:* **average biomass distribution ± standard errors for each quadrat along the transect. Data from all transects combined.**

animals dispersed in all directions or stayed at the border (Koch unpubl. obs.).

The biomass distribution along the transect, with its maximum 2–4 m from the mangrove border, can be explained by the above-mentioned mechanisms affecting the distribution of respective size groups. Middle-sized snails, which account for the largest portion of total population biomass, were found in highest densities 2–4 m from the mangrove border, where stress is already reduced but food supply is still high. Further inwards, where food seems less abundant but where stress is minimized, the smallest snails occur and survive the most vulnerable life stages.

The average biomass of *T. kiosquiformis* in the Gulf of Nicoya of 6.37 ± 3.41 g dry weight/m^2 (192.2 ± 102.2 g wet weight/m^2) is very high when compared with values of other mangrove areas. Lalana Rueda and Gosselck (1986) found values between 8 and 17 g dry weight/m^2 for the whole epifauna during the rainy season, with much lower biomasses in the dry season. In Taiwanese mangroves, biomass values of 131–406 g wet weight/m^2 are reported for the whole epifauna (Wu et al. 1992). These values are similar to that of *T. kiosquiformis* and reflect the high secondary productivity of the mangrove community in the study area. In terms of biomass and abundance, *T. kiosquiformis* is the most important species in the mangrove root community of the study area.

Food Intake

The overall estimate for the average food intake of 1% BWD wet weight (9% BWD flesh weight) is similar to that of the related species, *Thais carinifera* (10% BWD flesh weight and to that of

TABLE 2.

T. kiosquiformis: **Food Intake, Percentage Consumed of Each Prey, and Weight Increment of the Three Size Groups During the Field Experiment (14 d).**

Parameter	Size Groups (mm) 18–22 (n = 10)	23–27 (n = 10)	28–32 (n = 10)
Starting weight (wet) of 10 *T. kiosquiformis* (g)	14.71	32.02	44.43
Final weight (wet) of 10 *T. kiosquiformis* (g)	14.8	31.4	43.84
Mean flesh weight of 10 *T. kiosquiformis* (g) during the experiment (14 d)	1.83	4.02	5.62
Total food intake of 10 *T. kiosquiformis* (g)	3.33	6	12.61
Daily ration in % wet weight/day	1.6	1.3	2
Daily ration in % flesh weight/day	13	10.7	16
Weight contribution of each prey species in %			
L. varia	0	2	13
L. fasciata	56	78	54
B. puntarensis	16	3	11
Pinctada sp.	28	17	12
C. affinis	0	0	10
A. rugosa	0	0	0
C. stercusmuscarum	0	0	0
Balanids	0	0	0
Total weight gain of *T. kiosquiformis*/14 d (%)	0.6	−1.9	−1.3

the naticid snail *Natica maculosa* (7.5% BWD flesh weight), which occurs in tropical mudflats (Broom 1982). By the use of average biomass and daily ration, the population of *T. kiosquiformis* consumes approx. 2.5 g flesh weight/m^2 per day. We do not have any reasonable explanation for the differences in daily rations (%BWD) found between the three size groups, with the largest specimens having the highest food intake, followed by the smallest and the middle sized.

The strong preference for littorinid snails in the experiment is most probably not representative of field conditions, where *T. kiosquiformis* was found to feed primarily on barnacles (Cantera et al. 1980, Perry 1988, pers. obs.). Littorinids are not readily available as prey because they occur much higher in the mangroves of Punta Morales (Jerome 1987, Withey 1990) and escape when *T. kiosquiformis* is present (pers. obs.), a behaviour also reported for other littorinid snails (McKillup 1982, Fairweather et al. 1984). Furthermore, thaidid and muricid snails are reported to be capable of choosing the most profitable prey (in terms of energy per unit of effort) and readily switch their preferences when a prey species yielding higher profit is made available (Bayliss 1982, Palmer 1984, Carroll and Wethey 1990).

The weight changes during the experiment seem to confirm the results of the growth analysis, because only the smallest specimens gained weight, whereas the larger snails lost weight during the experimental period. The growth of mangrove trees in the study area is possibly highly dependent on the density and biomass of *T. kiosquiformis*. Perry (1988) reported a 50% growth reduction in the aerial roots of *R. mangle* when covered with barnacles. She found *T. kiosquiformis* and hermit crabs to be the most important predators, but the latter occurred in much lower densities along the transects studied (Koch unpubl. obs.). A similar situation was found in Belize, where mangrove growth also depended on barnacle coverage (Ellison and Farnsworth 1992). The dominant predator in that system was the snail *Melongena melongena*. Our study thus suggests that *T. kiosquiformis* structures the mangrove root community in the study area through the predation pressure it exerts on the community and maintains or enhances the productivity of the mangrove trees by constantly cleaning the root system of its encrusting epifauna.

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EVALUATION OF THREE ANESTHETIC AGENTS FOR CRAYFISH (*ORCONECTES VIRILIS*)

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ABSTRACT Crayfish are important research animals, and their culture in Louisiana and neighboring states is one of the largest aquacultural industries in the United States, however, there are no proven anesthetic agents for use in crayfish. In this study, we compared tricaine methane sulfonate (MS-222), lidocaine-HCl, and ketamine-HCl as anesthetic agents for *Orconectes virilis*. MS-222 was ineffective for crayfish at dosages as high as 1,000 mg/L, applied as a bath treatment. Lidocaine-HCl and ketamine-HCl were administered either intramuscularly (IM) or intrathoracically (IT). IT injections of either agent resulted in inconsistent anesthetization. IM injections of both agents resulted in anesthetization. The minimum effective dose of lidocaine-HCl was greater than 300 μg/g body weight. The duration of anesthetization at the higher doses was between 20 and 25 min. Ketamine was effective at doses of more than 90 μg/g body weight with durations of longer than 1 h. No mortalities occurred during any of the evaluations. On the basis of these data, both lidocaine- and ketamine-HCl are efficacious in crayfish. Lidocaine-HCl can be used for short-term anesthetization, whereas the effects of ketamine-HCl were long term.

KEY WORDS: Crayfish, anesthetic, MS-222, lidocaine, ketamine

INTRODUCTION

Tricaine methane sulfonate (MS-222), applied as a bath treatment, is the only anesthetic agent approved for use in aquatic animals in the United States. It is commonly used to anesthetize fish for research purposes and is routinely used in practical settings to minimize physiological responses to handling. However, the evaluation of anesthetics for use in freshwater crustaceans is rare, although a significant commercial industry exists and freshwater crayfish are routinely used as research animals.

One of the most common methods of immobilizing crustaceans is to place individuals in cold water or freezers before the collection of pertinent measurements or tissues. Recommendations for the anesthetization of marine decapods include chloroform, ethane disulphonate, fresh water, heat (40°C), isobutyl alcohol, methyl pentynol, procaine-HCl, and soda water (Smaldon and Lee 1979). Most of these methods result in slow and inconsistent immobilization, particularly for those applications that may require the rapid acquisition of tissues for physiological measurements. In this study, we evaluated three separate anesthetic agents for their usefulness in freshwater crayfish. The anesthetics evaluated were tricaine methane sulfonate, lidocaine-HCl, and ketamine-HCl.

MATERIALS AND METHODS

Tricaine methane sulfonate was added to individual closed containers so that concentrations were 0, 50, 100, 500, or 1,000 mg/L. Two adult male *Orconectes virilis* were placed in each container. All crayfish were intermolt and Form I. Individual weights of crayfish ranged from 33 to 37 g. Crayfish were acclimated to normal laboratory temperatures (22–26°C), and all evaluations were conducted within that range of temperatures. Crayfish were monitored for 30 min after the onset of anesthetic delivery to evaluate effects. All crayfish were given tactile stimulation approximately every 30 s during this period. If individual crayfish were not anesthetized by 5 min, the dosage was considered insufficient. This entire study was conducted twice.

Lidocaine-HCl and ketamine-HCl were injected into male crayfish of varying weights (27–45 g). Injections were either intramuscularly (IM) into the tail or intrathoracically (IT). IM injections were into the third sternum posterior to the thorax, and IT injections were into the base of the fifth periopod and into the thorax proper. Once the needle was inserted, hemolymph was aspirated for verification of location.

Lidocaine-HCl (20 mg/mL) was injected in the following volumes: 0.01, 0.025, 0.05, 0.10, 0.20, and 0.50 mL. Ketamine-HCl was acquired as a concentration of 100 mg/mL. In the first attempt, the stock solution was used, the administration was IM and the injection volume was 0.025 mL. In further evaluations, the stock solution was diluted to 20 mg/mL with distilled water immediately before the study was started. The diluted solution was then injected in the following volumes: 0.01, 0.025, 0.05, and 0.10 mL. After injection by either route, individual crayfish were monitored as described above. The time to unresponsiveness to tactile stimuli was recorded as the time to anesthetization; then, duration of anesthetization was monitored. Before the plotting of data, all doses were expressed as micrograms of anesthetic per gram of crayfish.

All crayfish exposed to MS-222 and those injected by either route with lidocaine-HCl were placed in a recovery tank. All crayfish administered ketamine-HCl were euthanized by hypothermia after complete recovery.

RESULTS AND DISCUSSION

Tricaine methane sulfonate, regardless of concentration, had no anesthetic effect in either study. Of the crayfish injected IT with lidocaine-HCl, only 6 of 24 became unresponsive to tactile stimuli. Only one test animal (25%) became unresponsive when given volumes of 0.05, 0.2, and 0.5 mL, whereas 75% of those injected with 0.10 mL were anesthetized. The average time to unrespon-

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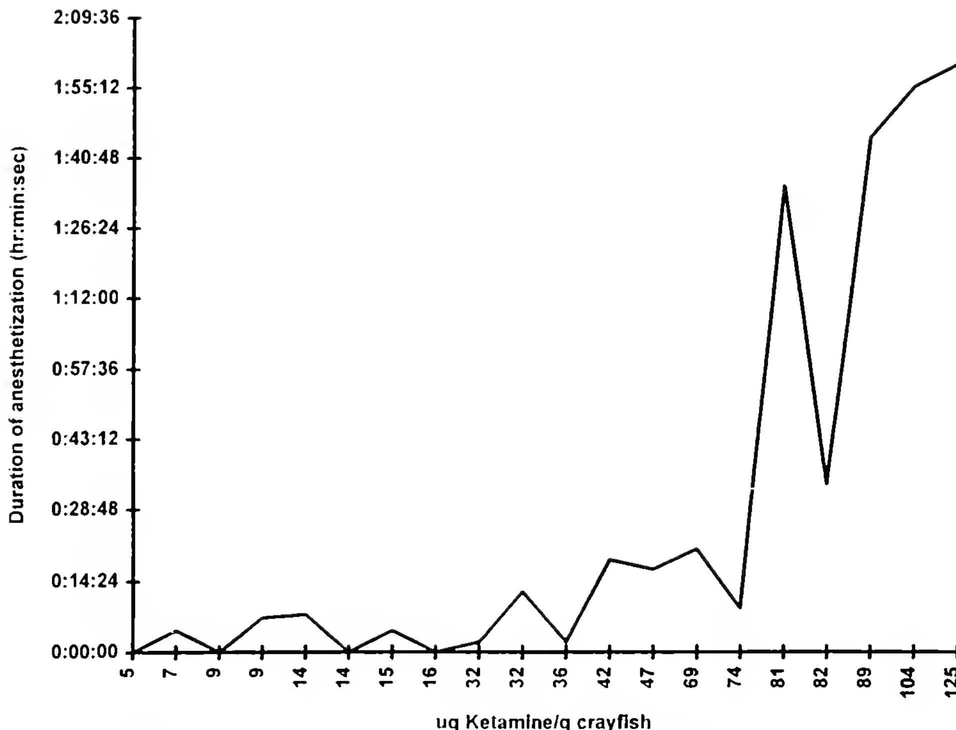

Figure 1. Mean duratioo of anesthetization of crayfish receiving IM iojections of lidocaine-HCl.

siveness was 30 s, and the average duration was 5 min 48 s; the maximum duration of IT lidocaine-HCl anesthetization was 8 min 42 s.

IM injections of lidocaine-HCl had no effect on crayfish until the injection volume was 0.10 mL. Fifty percent of those injected with 0.10 and 0.20 mL became unresponsive, whereas all of those injected with 0.50 mL were anesthetized. Average time to anesthetization was 1.5 min, with a maximum duration of longer than 25 min.

The response of crayfish to IT injections of ketamine was highly variable. Only 5 of 16 crayfish became unresponsive. The duration of anesthetization with IT injection was also highly vari-

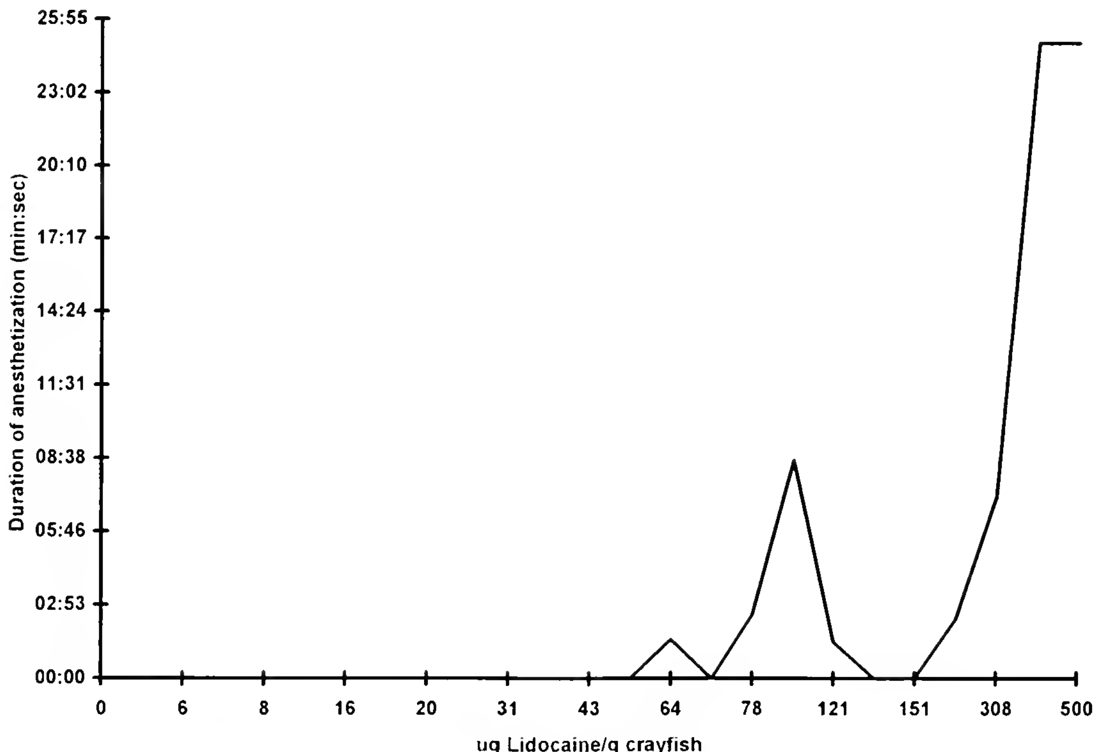

Figure 2. Mean duration of anesthetization of crayfish receiving IM injections of ketamine-HCl.

able. Two of the crayfish remained unresponsive for over 2 h but became responsive and appeared normal after that time, whereas two individuals receiving the same dose did not exhibit any effects of the anesthetic.

Crayfish receiving ketamine IM exhibited the most consistent anesthetic response. All crayfish receiving injection volumes of 0.05 mL and higher of the diluted solution (20 mg/mL) and all of those receiving 0.025 mL of the stock solution were anesthetized. The overall average time to anesthetization was 54 s. The duration of unresponsiveness increased from an average of 8 min 48 s in crayfish injected with 0.05 mL of the diluted solution to 1 h 48 min crayfish injected with 0.025 mL of the stock solution.

The response of crayfish administered IT injections of both lidocaine and ketamine was inconsistent and highly variable. The reasons for this remain unclear. Thus, that route of administration cannot be recommended. The responses of crayfish administered IM injections of both anesthetics were more consistent.

On the basis of the graphical presentation of the duration of anesthetization as a function of dose (micrograms per gram crayfish body weight), the minimum effective dose of lidocaine-HCl for the consistent immobilization of *O. virilis* is more than 300 μg/g (Fig. 1). We recommend the IM injection of doses of 400 μg/g for the light anesthetization of crayfish. Lidocaine-HCl has been used as an anesthetic in fish, but as a bath treatment similar to the administration of MS-222 (Carrasco et al. 1984).

It seems clear from Figure 2 that ketamine-HCl is a more potent anesthetic than lidocaine-HCl. Once doses exceed approximately 40 μg/g, consistent anesthetization of longer than 10 min occurred. Crayfish receiving doses in excess of 85 μg/g were in a plane of deep anesthesia but recovered completely. Thus, dosages of ketamine-HCl of more than 90 μg/kg IM are recommended for deep anesthetization. Ketamine-HCl is approved for felines and subhuman primates and is used in several other species (Alexander 1985, Booth and McDonald 1988). Ketamine-HCl can cause adverse emergence reactions such as hallucinations, confusion, and irrational behavior (Brown 1993). When fish were administered ketamine-HCl at doses above 45 mg/kg, emergence reactions were observed (Williams et al. 1988). Crayfish administered ketamine-HCl in these studies were fully recovered before euthanasia. Recovery was similar to those administered lidocaine-HCl in that no adverse reactions were noted.

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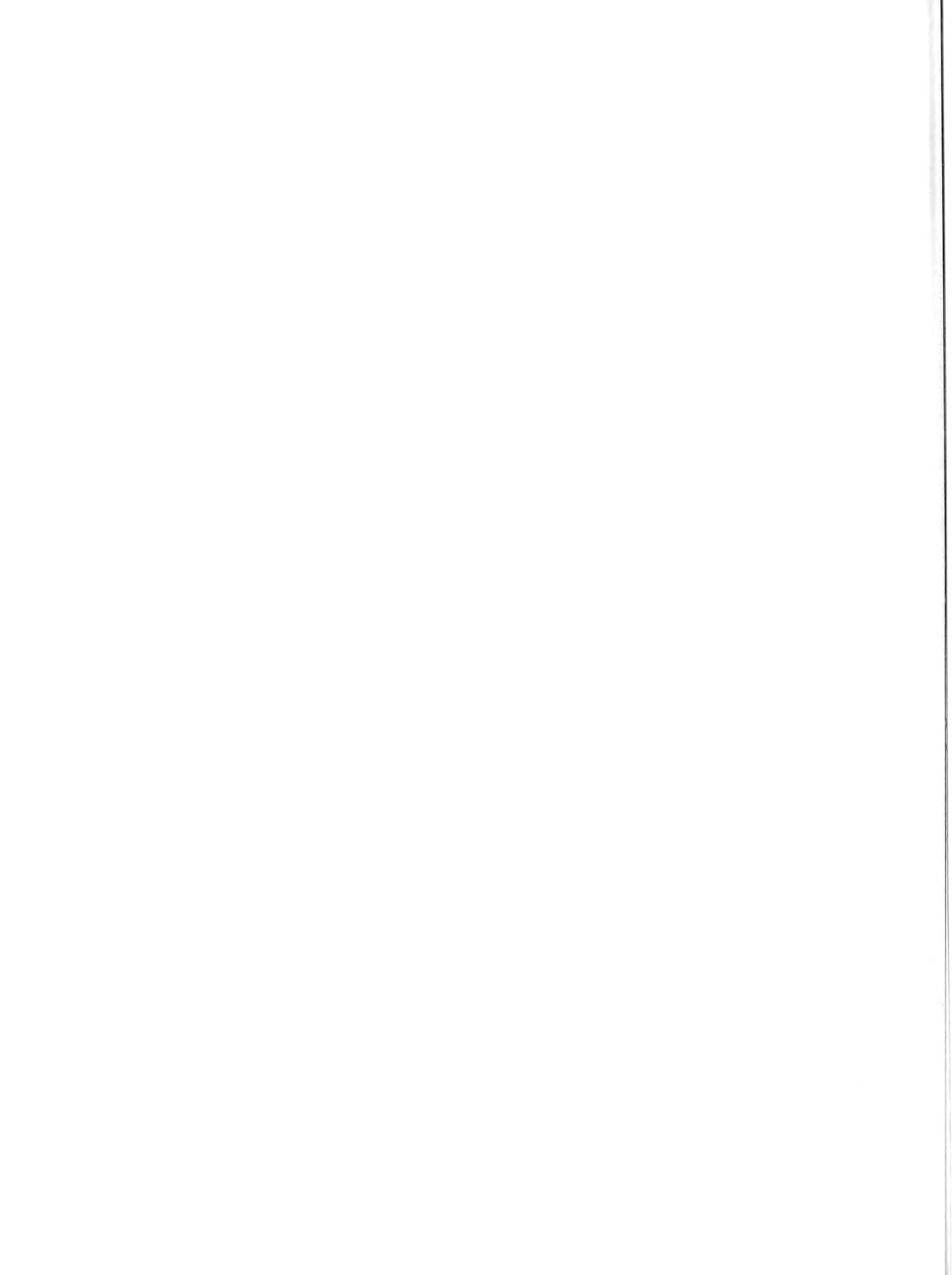

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ELECTROPHORETIC DATA SUPPORT THE LAST-MALE SPERM PRECEDENCE HYPOTHESIS IN THE SNOW CRAB, *CHIONOECETES OPILIO* (BRACHYURA: MAJIDAE)

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ABSTRACT Controlled mating experiments were carried out with the snow crab, *Chionoecetes opilio*, over two female breeding cycles. In this species, electrophoretic patterns of the enzymes glucose-6-phosphate isomerase, phosphoglucomutase, and malate dehydrogenase observed in the parents and the progenies are inherited as simple Mendelian codominant characters. The genetic differences observed between the larvae from the first and second egg clutches of some females mated with males of different genotypes support the hypothesis that the last male to mate with a female before she extrudes an egg clutch fertilizes a large proportion of the extruded eggs.

KEY WORDS: Snow crab, *Chionoecetes*, allozyme, electrophoresis, sperm competition, mating experiments

INTRODUCTION

Few studies have addressed the question of sperm competition in Crustacea. Polyandry and sperm mixing leading to multiple paternity seem to be the rule in two terrestrial isopods, *Porcellio scaber* (Sassaman 1978) and *Venezillo evergladensis* (Johnson 1982). Multiple paternity is relatively rare in wild populations of the amphipod *Gammarus* (Sexton 1935, Yarnold 1935a, Yarnold 1935b, Siegismund 1985), where precedence of the first male mate has been attributed to the fact that sperm is not stored between broods and that fertilization occurs <3 h after copulation (Birkhead and Pringle 1986). However, there appears to be no general trend regarding sperm competition among Decapoda. Although electrophoretic data revealed sperm mixing and multiple paternity in the lobster *Homarus americanus* (Nelson and Hedgecock 1977) and in the crab *Cancer pagurus* (Burfitt 1980), serial mating experiments carried out with irradiated and normal males showed that the last male mate fertilizes a very large proportion of extruded eggs in the crab *Scopimera globosa* (Koga et al. 1993) and in the crayfish *Orconectes rusticus* (Snedden 1990). Last-male sperm precedence has also been observed in the majid *Inachus phalangium* (Diesel 1990).

In the snow crab, *Chionoecetes opilio*, there is a potential for sperm competition to occur within the spermatheca at any stage of the female's reproductive life. Indeed, sperm competition might occur at the primary spawning, because females can copulate with more than one male before extruding their first clutch of eggs (Sainte-Marie et al. unpub. obs.). During their first mating period, females generally receive more than enough sperm to fertilize their first egg clutch, and excess sperm may remain viable in long-term storage within the spermathecae (Sainte-Marie and Lovrich 1994, Sainte-Marie and Carrière 1995). Because females may remate before extruding their second or ulterior egg clutch (Taylor et al. 1985, Conan and Comeau 1986, Hooper 1986), there also exists the possibility of competition between older stored sperm and that acquired more recently in the reproductive life of the female (Elner and Beninger 1992, Elner and Beninger 1995).

Beninger et al. (1991) and Elner and Beninger (1992) proposed for *C. opilio* that the most recently acquired sperm has precedence over older sperm in fertilizing the next egg clutch. However, last-male sperm precedence has yet to be demonstrated in this species by the use of controlled mating experiments (Elner and Beninger 1995). In this article, we provide for *C. opilio* genetic data supporting the hypothesis of last-male sperm precedence based on mating experiments carried out over two female breeding cycles.

MATERIALS AND METHODS

Collection and Mating of Crabs

Males and immature females used in mating experiments were collected in Baie Sainte-Marguerite (ca. 50°06′N, 66°33′W), northwest Gulf of Saint Lawrence, in September–October 1991. All crabs were identified individually with a numbered plastic tag attached to the basipodite of the fourth or fifth pereiopod. Adult males and immature females were kept in separate holding tanks, and the females were checked daily for molting. Less than 12 h after an immature female molted to maturity, which occurred from 8 January to 12 April, 1992, she was introduced to one hard-shelled adult male in a 120-L tank. After mating and hardening, the female was transferred to a female communal holding tank and maintained on a diet of previously frozen shrimp and herring. For those females that extruded fertilized eggs after mating, embryonic development lasted approximately 1 y at a mean temperature of 1.5°C. Berried females were checked periodically, and before the eggs hatched, they were transferred to individual tanks. Hatching periods for individual females lasted from 2 to 24 d. All larvae were collected every 24 h. Several larvae were placed on glass fiber filters on which the hatching date was noted, rinsed in distilled water, and immediately frozen in liquid nitrogen. They were stored at −80°C for subsequent electrophoretic analysis.

Twenty of 54 berried females were given the opportunity to remate at the hatching of their first brood, as one male different from their initial mate was introduced into their hatching tank. The females presumably remated (Sainte-Marie and Carrière 1995) and, after hatching their first egg clutch and extruding a second one, were returned to the female communal tank. For various reasons, only 13 of the remated females were used in this genetic study. Embryos were sampled from different places in the second egg clutch after 3 and 8 mo of incubation. These embryos were

handled and preserved as described above for the larvae. Samples of muscle tissue were collected from the second pereiopod of all male and female parents and stored at −80°C until electrophoretic analysis was carried out. Spermathecae of the females given the opportunity to remate were dissected to determine if they contained fresh ejaculate, confirming that the females had effectively remated. Recent ejaculate appeared as a pale deposit underlying the darker and +1-y-old ejaculates.

Electrophoretic Analysis

Approximately 100 mg of leg muscle tissue from each of the parents was homogenized in a 0.01 M Tris-HCL (pH 8.0) extraction solution containing 30% sucrose, 0.005 M dithiothreitol, and 0.5% polyvinylpolypyrolidone. Homogenates were centrifuged at 15,000 *g* for 60 min at 4°C. First-clutch larvae hatched on different days and second-clutch embryos samples after 3 and 8 mo of incubation were homogenized individually with a pipette tip directly in sample well plates containing 5 to 10 μL of the same extraction buffer. The electrophoretic patterns of the enzymes glucose-6-phosphate isomerase (GPI, EC 5.3.1.9), phosphoglucomutase (PGM, EC 5.4.2.2), and malate dehydrogenase (MDH, EC 1.1.1.37) were studied by use of the cellulose acetate gel electrophoresis techniques of Hebert and Beaton (1989).

RESULTS

Allozyme variation is low in *C. opilio* (Davidson et al. 1985, Sévigny unpub. obs.), and in our experiments, two alleles segregated at the *MDH** and *PGM** loci, whereas three were detected at the *GPI** locus. For any given clutch of eggs, there was no difference in the electrophoretic patterns of larvae with respect to hatching date, so data are pooled in subsequent analyses.

In 17 of the 62 tested crosses, the male and the female genotypes differed at least at one of the three studied loci, and in two crosses (#3 and #11), they differed at both the *MDH** and the *PGM** loci (Table 1). For all crosses, the observed number of genotypes of the progeny was in good agreement with that ex-

TABLE 1.

Inheritance of the electrophoretic patterns for the enzymes MDH, GPI, and PGM in the progenies of monandrous female *C. opilio*.

Cross No.	Female			Male			Larvae		
	MDH	GPI	PGM	MDH	GPI	PGM	MDH	GPI	PGM
1	ala1	ala1	ala1	ala1	ala1	ala2	21 ala1	21 ala1	11 (10.5) ala1 10 (10.5) ala2
2	ala1	ala11	ala2	ala1	ala1	ala1	21 ala1	20 ala1	21 (21.5) ala1 22 (21.5) ala2
3	ala2	ala1	ala1	ala1	ala1	ala2	12 (12.0) ala1 12 (12.0) ala2	22 ala1	10 (10.5) ala1 11 (10.5) ala2
4	ala1	ala1	ala1	ala1	ala1	ala2	21 ala1	10 ala1	10 (10.0) ala1 10 (10.0) ala2
5	ala1	ala1	ala2	ala1	ala1	ala1	10 ala1	10 ala1	12 (11.0) ala1 10 (11.0) ala2
6	ala1	ala1	ala1	ala1	ala3	ala1	11 ala1	6 (5.5) ala1 5 (5.5) ala3	11 ala1
7	ala1	ala1	ala1	ala1	ala1	ala2	11 ala1	11 ala1	12 (11.0) ala1 10 (11.0) ala2
8	ala1	ala1	ala1	ala1	ala1	ala2	11 ala1	11 ala1	11 (11.0) ala1 11 (11.0) ala2
9	ala1	ala1	ala1	ala1	ala1	ala2	10 ala1	10 ala1	109 (106.5) ala1 104 (106.5) ala2
10	ala1	ala1	ala1	ala1	ala1	ala2	11 ala1	11 ala1	23 (22.0) ala1 21 (22.0) ala2
11	ala2	ala1	ala1	ala1	ala1	ala2	10 (11.0) ala1 12 (11.0) ala2	11 ala2	10 (11.0) ala1 12 (11.0) ala2
12	ala1	ala1	ala1	ala1	ala1	ala2	10 ala1	9 ala1	68 (66.0) ala1 64 (66.0) ala2
13	ala1	ala1	ala1	ala1	ala3	ala1	10 ala1	10 (10.5) ala1 11 (10.5) ala3	10 ala1
14	ala1	ala1	ala1	ala1	ala3	ala1	10 ala1	21 (21.5) ala1 22 (21.5) ala3	21 ala1
15	ala1	ala1	ala2	ala1	ala1	ala1	10 ala1	11 ala1	10 (10.5 ala1 11 (10.5) ala2
16	ala1	ala1	ala1	ala2	ala1	ala1	6 (6.0) ala1 6 (6.0) ala2	11 ala1	11 ala1
17	ala1	ala1	ala1	ala1	ala2	ala1	11 ala1	12 (11.0) ala1 10 (11.0) ala2	22 ala1

The numbers for each genotype expected for Mendelian codominant characters are shown in parentheses for the larvae of each cross.

TABLE 2.

Inheritance of the electrophoretic patterns for the enzymes MDH, GPI, and PGM in two successive progenies of biandrous female *C. opilio* with mates of different genotypes.

Cross No.	Genotypes of First Mates									Genotypes of Second Mates				
	Female			Male			Larvae			Male			Larvae	
	MDH	GPI	PGM	MDH	GPI	PGM	MDH	GPI	PGM	MDH	GPI	PGM	GPI	PGM
9	ala1	ala1	ala1	ala1	ala1	ala2	10 ala1	10 ala1	109 (106.5) ala1 104 (106.5) ala2	ala1	ala1	ala1	NT	78 ala1 0 ala2
12	ala1	ala1	ala1	ala1	ala1	ala2	10 ala1	9 ala1	68 (66.0) ala1 64 (66.0) ala2	ala1	ala1	ala1	NT	73 ala1 0 ala2
14	ala1	ala1	ala1	ala1	ala3	ala1	10 ala1	21 (21.5) ala1 22 (21.5) ala3	21 ala1	ala1	ala1	ala1	71 ala1 0 ala3	NT

The numbers of each genotype expected for Mendelian codominant characters are shown in parentheses for the larvae of each cross. Family numbers refer to those in Table 1. NT, not tested

pected for Mendelian codominant characters ($p > 0.67$ in the 19 χ^2 tests of goodness of fit). The offspring of a homozygous individual mated with a heterozygous one are expected to segregate in a 1:1 ratio, as was observed in these mating experiments (Table 1).

Among the 13 females that were presented with a second mate before extrusion of their second egg clutch, 11 had recent ejaculate in their spermathecae, indicating that they had remated with the second male. Of these 11 females, 3 had second mates whose genotype differed from that of the first male parent (Table 2). The three females were homozygous at the three tested loci, whereas their first male mates were heterozygous at the *PGM** (crosses #9 and #12) and *GPI** (cross #14) loci. Their second mates were all homozygous at the three loci and were of the same genotypes as the females. The genotypes of the larvae in the first and second clutches of each female differed. Indeed, the heterozygotes that were observed at the *PGM** locus in crosses #9 and #12 and at the *GPI** locus in cross #14 (Tables 1 and 2) were not detected in larvae from the second clutch, which were all homozygous at the three studied loci (Table 2). Such a result would be expected if the second male mate had sired most or all of the eggs in the second clutch.

DISCUSSION

The first mating experiment shows that MDH, GPI, and PGM electrophoretic patterns are inherited as Mendelian codominant characters and that ontogenetic changes are not important factors in our experiments. The absence of heterozygous individuals in the second egg clutch of all three females of the second mating experiment indicates that, under the laboratory conditions presented here, the last male to mate with a female before she extrudes eggs sires most or all of the progeny. Although a contribution of the sperm from the first male mate to the pool of homozygotes from the second clutch would not be detected, the absence of heterozygotes in the second clutch indicates that such a contribution, if any, is very limited. The fact that 32 of 34 females that were not remated extruded a fertilized second egg clutch (Sainte-Marie and Carrière 1995) strongly suggests that females that were remated still had viable stored sperm remaining over from their first mating.

Our results are in agreement with the hypothesis that sperm from the last male has precedence in fertilizing eggs in *C. opilio* (Beninger et al. 1991, Elner and Beninger 1992, Elner and Beninger 1995). However, our experimental design does not allow us to make any inference regarding the mechanisms that might lead to last-male sperm precedence in *C. opilio*. The observation that the first gonopod of male *C. opilio* is hook shaped led to the hypothesis that males can extract previously stored ejaculate from the spermathecae (Beninger et al. 1991, Elner and Beninger 1992, Elner and Beninger 1995). This mechanism differs from that described in another majid, *I. phalangium*, where last-male sperm precedence is ensured by the displacement of previous sperm deposits deeper into the spermatheca (Diesel 1988, Diesel 1990). Sperm displacement resulting in last-male sperm precedence has also been predicted or demonstrated to occur in other brachyurans with ventral-type spermathecae (Murai et al. 1987, Diesel 1988, Diesel 1990, Koga et al. 1993, Orensanz et al. 1995), including *Chionoecetes bairdi* (Paul et al. 1983). Further laboratory experiments will be necessary to elucidate the mechanisms underlying last-male sperm precedence in *C. opilio*.

Our study does not rule out the possibility that multiple paternity can occur under some circumstances for *C. opilio*. For instance, a female that extrudes a second clutch of eggs without remating might draw on sperm reserves obtained from different males during the first mating cycle. Another case that would most certainly lead to multiple paternity would arise when a female is taken over by another male during oviposition and is then inseminated anew, as may happen (Sainte-Marie unpub. obs.). Further investigations are currently under way to describe the ecological contexts that might favor single or multiple paternity.

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COMPARISON OF EXCHANGE AND NO-EXCHANGE WATER MANAGEMENT STRATEGIES FOR THE INTENSIVE POND CULTURE OF MARINE SHRIMP

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ABSTRACT Most of the potential and realized adverse environmental effects of shrimp farming are associated with routine water exchange. This study compared shrimp production and water quality in triplicate ponds operated with and without water exchange. No statistical differences were detected in growth or survival among treatments, although there was a trend towards slightly smaller mean size at harvest and lower survival in the ponds operated without water exchange. The ponds operated with and without routine water exchange had average production of 5,888 and 5,444 kg/ha per crop, respectively. Differences in harvest size and survival also influenced food conversion efficiency. The ponds operated without water exchange had higher nutrients and biochemical oxygen demand (BOD) at the end of the study and, thus, discharged more nutrients and BOD in the drain harvesting process. However, the continuous discharge from the ponds operated with water exchange probably resulted in a much larger total nutrient and BOD load to the adjacent estuary. Heavy precipitation resulted in higher turbidity and total suspended solids in ponds with water exchange near the end of the study. Energy costs were 31.5% higher for the ponds operated with water exchange than for the no-exchange ponds.

KEY WORDS: Shrimp, ponds, water exchange

INTRODUCTION

Although shrimp farming is a relatively benign activity compared with many other types of agriculture, industry, and residential development, it may adversely affect coastal environments, especially where the regulatory framework is weak or inappropriate and shrimp farm development is intense. In the United States, where shrimp farms are widely dispersed and operate within rigid environmental protection guidelines, it is unlikely that significant environmental effects will occur. However, this is not the case in many other areas, where production collapses have often accompanied intensive, poorly regulated development (e.g., Taiwan [Liao 1992], Thailand [Lin 1995], China [Wang et al. 1995]). The effects are felt by both the shrimp farmer and other users of public water resources.

The potential environmental effects of shrimp farming include: (1) wetland destruction for the construction of shrimp ponds; (2) hypernutrification of estuarine ecosystems by shrimp pond effluent; (3) "biological pollution" of native shrimp stocks through escapement of aquaculture stocks; (4) excessive water use and entrainment of estuarine biota by pumping; (5) release and spread of disease organisms; and (6) discharge of treatment chemicals into estuarine systems (Hopkins et al. 1995a). Of these, all but the first can be addressed directly through improved water management, particularly as it relates to routine water exchange and discharge.

Discharges from shrimp ponds may occur as a result of: (1) runoff from heavy precipitation or additions to maintain salinity during periods of high evaporation; (2) routine, intentional water exchange to dilute pond water column nutrients, solids, and biochemical oxygen demand (BOD) (Lee and Wickins 1992); and (3) drain harvesting (Hopkins et al. 1995b). The magnitude and effect of these categories vary considerably, but routine water exchange generates the largest volume of effluent and is the easiest to avoid.

Water exchange practices are seldom based on nutrient monitoring or other "hard" data or used in response to fluctuating environmental conditions. Instead, exchange is often based on a set schedule (Hopkins and Villalón 1992). In extensive production systems, water exchange is sometimes used to introduce additional forage prey (Allan and Maguire 1993) and/or recruit wild postlarvae/juveniles (Whetstone et al. 1988). However, for the most part, systematic investigations to determine how much water exchange is actually needed are generally lacking (e.g., see Allan and Maguire 1993; Hopkins et al. 1993, Hopkins et al. 1995b, Hopkins et al. 1995c).

Despite the widespread belief that regular water exchange will improve pond water quality, there is also potential for the opposite. For example, a shrimp farmer in South Carolina began exchanging large amounts of water in reaction to slightly increased, but not lethal, water column ammonia concentrations (Waddell Mariculture Center [WMC] unpublished data). The rapid water exchange effectively flushed the phytoplankton population from the pond, but because feeding was not discontinued, it did not affect the production of ammonia through metabolic processes occurring on the pond bottom (e.g., shrimp metabolism, microbial decay processes). Phytoplankton and nitrifying bacteria attached to detrital particles in the water column are probably the primary routes of water column ammonia recycling in pond systems. By removing these components, rapid water exchange subsequently increased the amount of free water column ammonia. Once water exchange was stopped or reduced, ammonia concentrations quickly climbed to very high and potentially toxic levels. Had massive water exchange not disrupted the system's ability to reach equilibrium at the feeding rates being used, the pond ecosystem should have been able to react to the increased ammonia concentration through increased primary production and denitrification. Only by reducing water exchange were phytoplankton and nitrifying bacteria populations allowed to rebound and affect a long-term reduction in ammonia.

Similarly, BOD is dictated by respiratory and decay processes at the pond bottom and in the water column. Sources of oxygen are atmospheric diffusion across the water surface and oxygen generation by photosynthesis. Diffusion rates are a function of the con-

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centration differential across the surface boundary and are enhanced by the surface disturbance and mixing caused by wind or aeration equipment (Boyd 1990). With adequate sunlight, the oxygen released through photosyntheses during the day exceeds combined day and night phytoplankton respiration.

The effect of water exchange on oxygen diffusion rates is minimal, and diffusion can be increased more efficiently through the use of aeration equipment. Even when the inlet water is saturated with oxygen, the pumping head and pump efficiency generally make it less expensive to operate aerators than to pump water. The effect of water exchange on photosynthetic oxygen enrichment depends on the sunlight available to algae. Available sunlight is a function of solar radiation, pond depth, density of the phytoplankton population, and amount of other nonphytoplankton turbidity. The phytoplankton population can become so dense that it causes a self-shading effect where light is absorbed before it penetrates far into the water column. On very cloudy days and/or at times of very dense phytoplankton populations, the available sunlight does not induce enough photosynthetic oxygen production to exceed the 24-h algal respiration or produces an excess of oxygen that is too small to offset other respiratory demands. Thus, when solar radiation is low and algal populations are very dense, phytoplankton dilution through water exchange may increase light penetration and improve the overall oxygen balance. However, as in the case of ammonia dilution noted above, excessive flushing of phytoplankton may increase the oxygen deficit if the diminished phytoplankton population cannot effectively use the solar radiation for oxygen production. Ideally, the phytoplankton population would reach an equilibrium where its density is controlled by available sunlight at a level that stabilizes oxygen production at a level sufficient to meet all pond respiratory requirements.

Discharges from intentional water exchange in shrimp farming can be substantial. The production of a metric ton of shrimp typically uses 55,000–86,000 metric tons of water (Hopkins and Villalón 1992). However, large differences in the reported water consumption between species and systems cannot be explained by interspecific differences in metabolism or physiological tolerances. This suggests that there is room for much improvement in water management (Hopkins and Villalón 1992, Phillips et al. 1991). In the past, the amount of water exchanges has generally been increased with increasing intensity of production (although not in strict linear fashion), reaching ≥30%/day at very high stocking densities (Sandifer et al. 1991).

In response to the need to reduce the potential for environmental effects from shrimp farming, researchers at WMC in South Carolina began examining the importance of water exchange in 1990 (Hopkins et al. 1991, Hopkins et al. 1993, Hopkins et al. 1995c, Hopkins et al. 1995d; Browdy et al. 1993, Sandifer and Hopkins 1996). Those authors found that water exchange could be reduced to low levels (≤4%/day) or eliminated altogether without negatively affecting shrimp production as long as adequate levels of dissolved oxygen (DO) were maintained (see Hopkins et al. 1995a and Hopkins et al. 1995b for review). However, these studies generally lacked replication or were conducted only in tanks. This experiment was undertaken to provide a more systematic comparison of exchange and no-exchange water management strategies for the intensive culture of shrimp in earthen ponds.

MATERIALS AND METHODS

The study was conducted during 1995 at the WMC, a field station of the Marine Resources Division, South Carolina Department of Natural Resources. Three replicate 0.1-ha (1,008-m^2), 1,300-m^3 ponds were used for each of two treatments: 0 and 15% of pond water volume exchanged daily. The treatments were instituted after an initial 49-d period of no water exchange immediately after stocking. These ponds have a 2:1 length:width ratio, 3:1 side slopes, and a sloped bottom where depth ranges from 1.3 to 1.5 m; they are lined with 1-mm-thick high-density polyethylene that is overlaid on the bottom with 26 cm of the sandy native soil. A concrete drain structure incorporates screen tracks, an overflow weir, and a drain valve. The ponds were designed to drain into an effluent ditch that carries water back to an adjacent estuary some 800 m from intake pumps. This estuary, the Colleton River, is a high-salinity, vertically mixed embayment.

The ponds were filled to within 20 cm of the overflow structure 1 wk before stocking. As ponds were filled, water was screened through a mesh sock. Ponds were stocked with shrimp (*Penaeus vannamei*, Boone) postlarvae at a density of 38.2 animals/m^2 on April 11–12, 1995. The postlarvae were raised at a commercial shrimp hatchery in South Carolina from nauplii produced from specific-pathogen-free broodstock at WMC.

Shrimp growth was monitored at 14-d intervals by the shrimp being captured and individually weighed to the nearest 0.1 g, a random sample of 100 shrimp. The ponds were harvested after 153 d, at which time the entire crop was weighed *en masse* and subsamples of shrimp were weighed individually to estimate harvest size and survival. Production characteristics of the two treatments were compared statistically using a t-test.

Ponds were fed once daily from a tractor-drawn pneumatic feed

TABLE 1.

Production characteristics of intensive shrimp ponds operated with and without water exchange.

Treatment		15%/Day Water Exchange			No Water Exchange	
Pond/replicate designation	S-06	S-07	S-08	S-10	S-11	S-12
Replicate mean harvest weight (g)	15.4	17.5	16.7	15.5	15.1	16.3
Treatment mean harvest weight (g)		16.5			15.6	
Replicate survival (%)	93.8	91.3	95.1	87.0	90.6	96.1
Treatment mean survival (%)		93.4			91.2	
Replicate production (kg/ha per crop)	5,510	6,096	6,058	5,134	5,233	5,965
Treatment mean production		5,888			5,444	
Replicate feed conversion efficiency	1.58	1.43	1.44	1.70	1.66	1.46
Treatment mean FCR		1.48			1.60	

TABLE 2.

Average of weekly water quality analyses for each pond at the beginning of the study, the treatment mean, and the number of samples (n) Each Value Represents.

Treatment	Pond	pH	TURB	SAL	BOD	TAN	PHOS	TSS
With exchange	S-06	8.2	6.3	26.0	21.5	0.15	1.18	103.3
	S-07	8.3	4.0	25.8	17.2	0.86	0.89	69.5
	S-08	8.2	3.0	25.5	17.6	0.82	0.99	74.2
	Mean	8.2	4.4	25.8	18.8	0.61	1.02	82.3
Without exchange	S-10	8.2	2.8	25.3	14.8	0.42	1.58	88.7
	S-11	8.3	3.8	25.3	16.8	0.76	1.17	82.8
	S-12	8.2	2.7	25.3	15.1	0.64	1.14	83.8
	Mean	8.2	3.1	25.3	15.6	0.61	1.29	85.1
	n	4	4	4	3	2	1	3

TURB, nephelometer turbidity; SAL, salinity; TAN, total ammonia nitrogen, PHOS, reactive orthophosphate; TSS, total suspended solids.

blower. The feed was a 40% protein, 3-mm-diameter × 12-mm pellet manufactured for shrimp by Rangen Inc. (Buhl ID). In contrast to typical feeding procedures where the amount of feed presented varies with shrimp size and estimated standing crop biomass (e.g., see Clifford 1985), a constant daily feed amount of 5.67 kg/pond per day (57 kg/ha per day) was used in this study. This approach minimizes fluctuations in organic loading due to feeding in an effort to stabilize chemical and microbial processes in the ponds.

Each pond was aerated continuously with a modified 1-hp "Taiwanese-style" paddlewheel aerator. A single paddlewheel provided enough supplemental aeration to maintain dawn dissolved oxygen concentrations within an acceptable range. However, to ensure that an aerator malfunction did not result in dawn dissolved oxygen depletion, a second 1-hp paddlewheel was turned on and off automatically by a time switch between 0100 and 0700 h each day. The total aeration rate was, thus, 12.5 hp-day/ha per day. Aerators were situated in opposite corners of ponds so as to create a gyre that effectively swept most of the pond bottom and deposited sludge particles in the center. Power consumption and electrical costs for pumping and aerating water were monitored throughout the study.

Permit conditions for raising nonindigenous shrimp species in South Carolina require that discharge structures be double screened and that there be no discharge until the shrimp reach a mean size of 1 g. This stipulation is designed to prevent postlarvae and small juveniles from moving around the screen frame and escaping to the estuary via the discharged water. Therefore, water exchange was begun in three of the six ponds on day 49, after the first bi-weekly sample where the shrimp size averaged more than 1 g. The water exchange rate was adjusted to 15% of the pond volume per day with a calibrated weir tube. The flow rate was checked daily and adjusted as necessary. The three no-exchange ponds received no additional water except that contributed by precipitation. During August, heavy rains generated an estimated discharge of 0.6% of pond volume/day or about 0.15%/day over the production cycle.

Temperature and DO were measured daily at dawn by thermistor and polarographic meter. We intended to measure a variety of other water-quality parameters as well, but the Center's water-quality chemist became seriously ill and was unable to conduct those analyses. Near the end of the study, a graduate student determined the pH, salinity, nephelometer turbidity, BOD, total ammonia nitrogen, reactive orthophosphate, and total suspended solids three times at roughly weekly intervals for each pond using standard methods (APHA 1989).

RESULTS AND DISCUSSION

Mean shrimp size at harvest size ranged from 15.1 to 16.7 g, and there was considerable overlap between treatments (Table 1). The overall mean harvest size for all ponds with water exchange was 0.9 g greater than that of ponds without water exchange, but this difference was not statistically significant when compared

TABLE 3.

Average of weekly water quality analyses for each pond near the end of the study, the treatment mean, and the number of samples (n) each value represents.

Treatment	Pond	pH	TURB	SAL	BOD	TAN	PHOS	TSS
With exchange	S-06	7.7	28.7	21.0	26.31	0.89	0.17	57.5
	S-07	7.8	29.3	22.2	13.43	1.35	0.05	53.0
	S-08	8.1	23.0	22.7	14.67	0.51	0.01	97.5
	Mean	7.9	27.0	21.9	18.14	0.92	0.08	69.3
Without exchange	S-10	7.9	14.7	19.5	42.81	3.50	0.38	44.5
	S-11	7.9	18.7	18.8	39.00	2.95	0.57	46.0
	S-12	7.8	14.3	17.0	36.65	3.02	0.33	38.5
	Mean	7.9	15.9	18.4	39.49	3.16	0.42	43.0
	n =	3	3	3	3	3	3	2

See footnote to Table 2 for explanation of abbreviations.

TABLE 4.

Estimated nutrient load discharged from ponds with and without routine water exchange on a continuing daily basis during the final 2 wk of the study and during the drain-harvesting process. All values are expressed as kg/ha.

Discharge	BOD	TAN	PHOS	TSS
Daily				
Exchange ponds	35.4	1.8	0.2	135.2
No-exchange ponds	Trace	Trace	Trace	Trace
One-Time Harvest				
Exchange ponds	235.8	11.9	1.0	901.3
No-exchange ponds	513.3	41.0	5.5	559.0

All values are expressed as kilograms per hectare. See footnote to Table 2 for explanation of abbreviations.

with a t-test (P = 0.2637). The size distributions were quite similar for both treatments, and all pond shrimp populations fell into the same market category (26–30 whole shrimp per pound) with the same value ($3.75/kg whole weight).

Survival in all ponds was excellent, ranging from 87 to 96%. Again, although the average survival in the no-exchange ponds was slightly lower than that for the exchange ponds (Table 1), this difference was not significant when examined by t-test (P = 0.4946).

Production, a function of growth and survival, ranged from 5,134 to 6,096 kg/ha per crop whole weight, with considerable overlap between treatments. Average production for the exchange and no-exchange treatments was 5,888 and 5,444 kg/ha per crop, respectively, and this differences was not significant (P = 0.2416).

Because all ponds received the same feed input, the feed conversion ratio (FCR) (kilograms of dry feed:kilograms of whole shrimp) was also a function of growth and survival and ranged from 1.43:1 (apparently the lowest yet reported for intensive pond culture of shrimp) to 1.69:1. A t-test found no statistically significant difference in FCR between treatments (P = 0.2395).

For all ponds and all days of the 153-d study, DO averaged 5.15 and 5.04 mg/l for the water exchange and no-exchange treatments, respectively. This difference was significant (P = 0.0007). Excluding the first 49 d, when there was no water exchange in either treatment, DO averaged 4.92 and 4.73 mg/l, respectively, for the exchange and no-exchange treatments. The minimum dawn DO for all days and all ponds was 2.5 mg/l.

At the beginning of the study, indicators of water quality were similar in all ponds, although turbidity and BOD tended to be slightly higher in the exchange ponds (Table 2). Near the end of the experiment, when pond water quality was sampled at weekly intervals, there was more variation in pH within than between treatments (Table 3). By the end of the study, salinity was 3–4 ppt lower in the no-exchange ponds because of precipitation. The Colleton River estuary has naturally high turbidity and suspended solids, especially after heavy rains. Therefore, water in the ponds with continuous water exchange had higher nephelometer turbidity and total suspended solids than did water in the no-exchange ponds. As expected, total ammonia nitrogen, reactive orthophosphate, and 5-d BOD were higher in the no-exchange ponds than in the ponds with water exchange (Table 3).

Actual electricity costs at WMC, where industrial rates apply, averaged $0.058/kwh (range, $0.053–$0.070/kwh) over the course of the study. The electrical efficiency of the aerators was 1.3 kwh/h per rated horsepower. With an average aeration rate of 12.5 hp-h/h per hectare, the electrical cost for aeration over the 153-d study was $3,461/ha per crop for all ponds. WMC pumps water from the estuary to the ponds with 30-hp centrifugal pumps. The efficiency of the pumps is 7.24 m^3/kwh. All ponds required $89/ha per crop for electricity to fill them initially. By exchanging water at a rate of 15% of the pond volume per day for 104 d of the study, the ponds receiving water exchange had an additional pumping cost of $1,634/ha per crop. Therefore, the ponds receiving water exchange and the no-exchange ponds had electrical pumping costs of $1,723 and $89/ha per crop, respectively. The total cost for electricity (aeration + pumping) was $5,184/ha for the exchange ponds vs. $3,550/ha for the no-exchange treatment in this study. Thus, for the WMC system, the no-exchange water management strategy resulted in a 31.5% savings in electrical costs.

In the United States, the Environmental Protection Agency (EPA) and/or the state agency authorized to administer EPA regulations typically requires that a National Pollution Discharge Elimination System (NPDES) permit be issued to warmwater aquaculture facilities that produce more than 45.4 metric tons (100,000 pounds) of product per year and discharge water more than 30 d/year. With production goals similar to those used in this study (i.e., 5,000–6,000 kg/ha per crop), a shrimp farm with 8 ha or more of ponds operated with routine water exchange would likely require a NPDES permit. On the basis of our experience with requirements of NPDES permits for aquaculture operations in South Carolina (which may *not* be representative of other states or regions), we estimate compliance costs at approximately $400/ha per year for an 8-ha intensive shrimp farm. In addition, farms designed for no-exchange technology could reduce the scope and costs of water pumping and distribution systems.

The more important cost associated with water exchange is the environmental cost. In regions with poorly regulated shrimp-farming activity, the environmental cost may include a collapse of the shrimp-farming industry or disruption of other public uses of coastal waters. In countries like the United States, where there is a more comprehensive regulatory structure, industry expansion may be prohibited if all farms use routine water exchange.

The no-exchange ponds discharged more BOD, ammonia nitrogen, and phosphorus during the drain-harvesting process than did the ponds with water exchange, but the ponds with water exchange released more total suspended solids (Table 4). As noted by Hopkins et al. (1993), the total load of nutrients discharged through the production process (water exchange plus drain harvest) is much higher when routine water exchange is used. As can be seen from Table 4, the differences in drain harvest discharge load between ponds operated with and without water exchange would be overshadowed by the daily discharge associated with routine water exchange. Without routine water exchange, *in situ* assimilation and digestion processes mineralize and deposit much of the nutrient mass in pond bottom sediments or volatilize them to the atmosphere.

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ABSTRACTS OF TECHNICAL PAPERS

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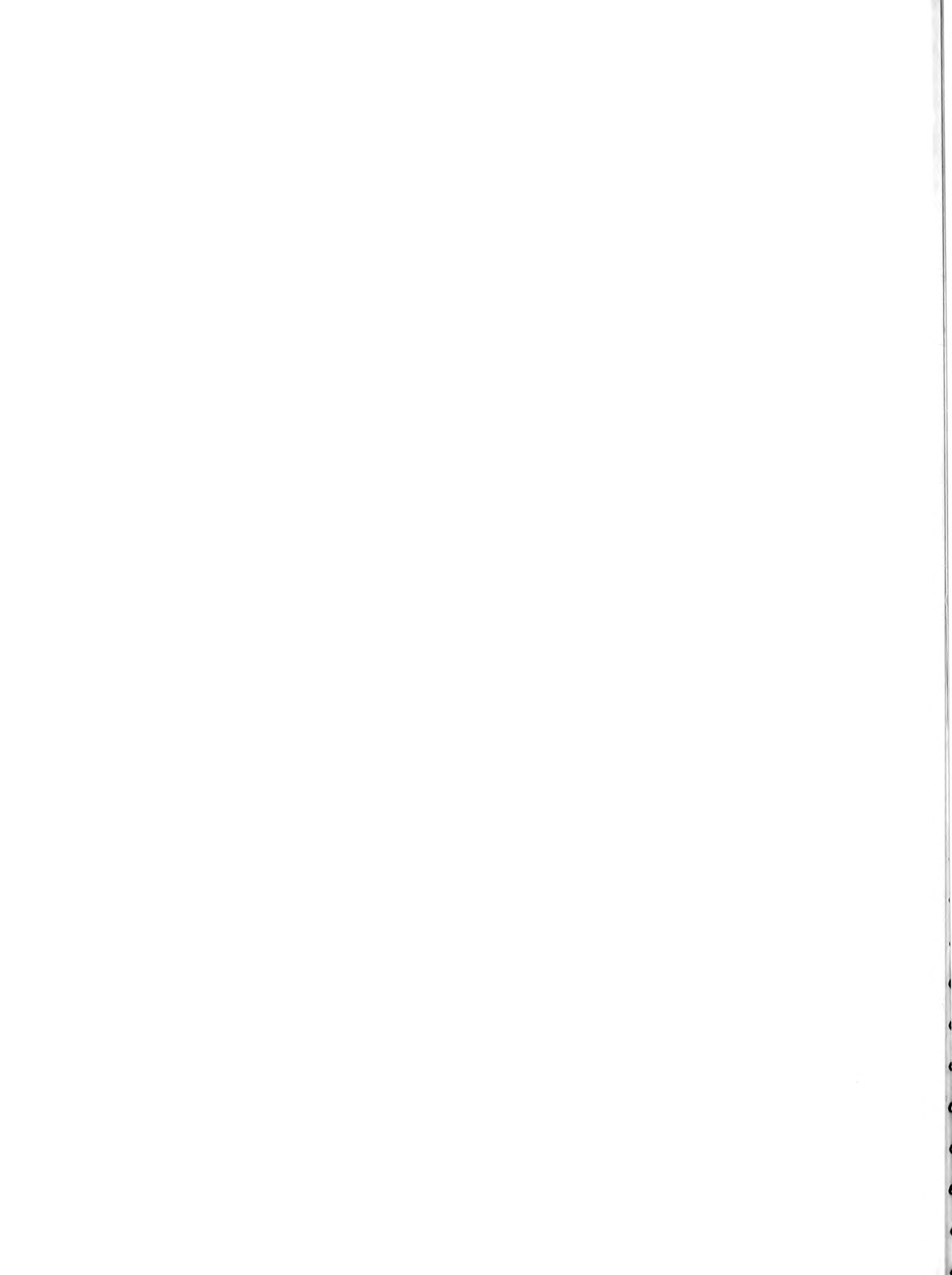

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OVERVIEW, 16TH MILFORD AQUACULTURE SEMINAR. Walter Blogoslawski, U.S. Department of Commerce, National Oceanic and Atmospheric Administration, National Marine Fisheries Service, Northeast Fisheries Science Center, 212 Rogers Avenue, Milford, CT 06460.

The 16th Annual Milford Aquaculture Seminar attracted 37 eminently qualified speakers, whose topics ranged from shellfish and finfish disease and propagation through sponsorship of specific programs designed to assist displaced fisherman in the development of aquaculture ventures. The 160 attendees from the United States, Canada, the United Kingdom and the People's Republic of China met in formal and informal sessions to discuss the latest developments in technology and government extension activities. It was noted with interest by attendees that many of the concerns of the industry, such as overharvesting in the late 19th century, were the same as those influencing shellfish farmers today.

Speakers from 12 states, Canada, and the United Kingdom discussed sea and bay scallop propagation and the control of shellfish diseases as well as aquaculture training and culture of finfish. Twenty-six marine laboratories and hatcheries and twenty universities, were represented during this exchange. Genetics, nutrition, and disease, as related to the culture and survival of shellfish, were prominent topics of discussion.

Sponsorship of this seminar included the National Marine Fisheries Services, Milford Laboratory, Milford, Connecticut and the United States Department of Agriculture, Northeastern Regional Aquaculture Center in North Dartmouth, Massachusetts. Their support is most gratefully acknowledged.

SCALLOP LARVAL FEEDING EXPERIMENTS: SOME SURPRISES AND UNANSWERED QUESTIONS. Jennifer H. Alix,[1] Mark S. Dixnn,[1,2] Barry C. Smith,[1] and Gary H. Wikfors,[1] [1]USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford, CT 06460; [2]Marine Sciences and Technology Center, University of Connecticut, Groton, CT 06340.

Controlled feeding studies with larval bay scallops (*Argopecten irradians*) are being pursued with an ultimate goal of reducing larval rearing to seven days or fewer as a convenience for hatchery work schedules. Initial experiments compared larval scallop survival, growth, and time to metamorphosis when fed unialgal diets from a wide variety of microalgal taxa. Cells larger than about 7–8 μm were too large for first-feeding larvae, and a number of small chlorophytes and eustigmatophytes with cellulosic cell walls were indigestible. Two diatoms used widely as larval diets for other bivalves, *Chaetoceros calcitrans* and *Thalassiosira pseudonana*, were poor relative to a number of chrysophytes and prymnesiophytes. Two rarely-cultured strains of the flagellate, *Pavlova*, CCMP459 and CCMP609, supported particularly good survival and growth; these strains contain remarkably high levels of essential fatty acid.

Subsequent experiments investigated mixed algal diets, either from first feeding or with sequential replacement during larval life. Mixed diets including CCMP459 supported larval growth equivalent to several other mixed diets, but consistently resulted in early metamorphosis in eight days at a smaller size of 140–160 μm than mixed diets not including this alga, which resulted in metamorphosis in 10–14 days at sizes generally >190 μm. Further experiments will be required to determine if CCMP459 induces metamorphosis through some possible chemical mechanism.

We also investigated the possibility of replacing a portion of a CCMP459 diet with high-lipid *Tetraselmis* strains on days 3, 5, or 7 of larval rearing. Two *Tetraselmis* strains with a size range of 12–15 μm were not ingested prior to day 5 or 6; however, a 10 μm *Tetraselmis* strain (PLAT-P) was consumed between days 3 and 4. Scallop larvae fed mixed CCMP459 + PLAT-P grew as well as larvae fed CCMP459 alone, but metamorphosed two days later at a larger size. Further work will be required to determine if early metamorphosis on diets including CCMP459 is an advantage or disadvantage with regard to subsequent postset performance.

This project was funded partially by a University of Connecticut, Marine Sciences and Technology Center Grant.

COMPARISON OF THREE LONG ISLAND SOUND SITES FOR THE GROW-OUT OF BAY SCALLOPS, *ARGOPECTEN IRRADIANS*. Joseph Choromanski,[1] Sheila Stiles,[1] Daniel Schweitzer,[1,2] Matthew Mroczka,[3] and Paul Dinwoodie,[3] [1]USDOC, NOAA, National Marine Fisheries Service, Milford Laboratory, Milford CT 06460; [2]Marine Sciences and Technology Center, University of Connecticut, Groton, CT 06340; [3]Cedar Island Marina, P.O. Box 181, Clinton, CT 06413.

A preliminary study of growth and survival for selected strains of bay scallops was conducted at three sites in Long Island Sound. Float-supported lantern nets held off the bottom were utilized at off-shore sites in Groton and Milford. In Clinton, a suspension-culture rack system in a protected marina was used and evaluated for the first time. Various genetic lines of selectively-bred scallops were cultured from native Connecticut stock in our laboratory hatchery and held in raceways prior to deployment. Sampling was performed on a monthly basis, during which times we were able to assess conditions and equipment at the sites.

This project was funded partially by a University of Connecticut, Marine Sciences and Technology Center Grant.

A REPORT ON THE PROJECT FOR SUSPENSION CULTURE OF BAY SCALLOPS IN LONG ISLAND SOUND. John Curtis, Bridgeport Regional Vocational Aquaculture School, 60 St. Stephens Road, Bridgeport, CT 06605.

In December of 1994, the Bridgeport Regional Vocational Aquaculture School, in cooperation with the People's Republic of China, initiated a scallop restoration project using funds from the State of Connecticut's Long Island Sound License Plate Program

to develop a small demonstration long-line farm in the waters off Fairfield, Connecticut. With the combined efforts of Dr. Luning Sun from Academia Sinica, and the students and staff of the Aquaculture school, a complete scallop hatchery, as well as a grow-out farm, were constructed and maintained throughtout this past year. Students from the school grew algal cultures, spawned native scallops and made qualitative and quantitative growth measurements. Using proven scallop culturing techniques from China, the project participants successfully harvested more than 10,000 scallops this last December. The project has been continued in order to study various culture techniques and experimental factors and to investigate its potential commercial feasibility in the area.

This restoration project has proved to be successful both theoretically and practically. It has involved regional high school students and provided the opportunity for them to work closely with the students and professors from several local universities, as well as the staff from various federal laboratories.

DIVISION RATES OF FIVE HIGH-LIPID *TETRASELMIS* STRAINS AT TEMPERATURES FROM 10°C TO 35°C. **Mark S. Dixon,**[1,2] **Jennifer H. Alix,**[1] **Barry C. Smith,**[1] and **Gary H. Wikfors,**[1] [1]USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford, CT 06460; [2]Marine Sciences and Technology Center, University of Connecticut, Groton, CT 06340.

Temperate climates present challenges to molluscan aquaculture; in spring, water and air temperatures are below optimal and in summer, above. Regardless of temperature conditions a continuous supply of quality microalgal food is an essential component of shellfish culture. The cost of temperature control can be lessened by selecting algal strains that grow well at low or high temperatures to match the season. Five *Tetraselmis* strains were grown in test tubes under identical light conditions but at various temperatures (10°, 15°, 19°, 25°, 30°, and 35°C) in temperature-controlled incubators. Previous studies have shown that these five high-lipid *Tetraselmis* strains have excellent potential for maximizing shellfish growth. Microalgal growth rates were recorded as divisions per day calculated from both direct cell counts and optical density measures.

MC:2 (*Tetraselmis sp.*) and PLY429 (*Tetraselmis chui*) had greatest division rates when grown above 20°C, and showed reduced growth rates below 20°C. PLY429 had reduced growth above 25°C, but MC:2 continued to divide rapidly up to 30°C. PLAT-P (*Tetraselmis striata*) and UW445 (*Tetraselmis chui*) both exhibited maximal growth rates below 20°C with a drop of division rate above 20°C. PLY429S (*Tetraselmis chui*) showed peak growth below 15°C and reduced growth above 15°C. Temperatures above 35°C were lethal to all strains within 10 days and resulted in negative growth in the short term. Typical peak division rates were in the 0.5 div/day range, and reduced growth rates were less than 0.1 div/day in cultures that were sluggish but still growing. These division rates are consistent with algae grown on a larger scale in carboys.

The results of this experiment, coupled with previous work showing that MC:2, PLAT-P, and PLY429 support fast growth in scallops, can be used to design an effective feeding strategy. By growing each strain during the season of its most suitable temperature, heating and cooling costs associated with year-round, large-scale microalgal culture can be reduced. These results indicate that MC:2 and PLY429 would be good warm weather choices, and that PLAT-P would be a suitable cool weather choice.

This project was funded partially by a University of Connecticut, Marine Sciences and Technology Center Grant.

TRURO SEA SCALLOP AQUACULTURE PROJECT. **Judy Dutra,** Truro Aquaculture Project, 43 Shore Road, North Truro, MA 02652.

The purpose of the Truro Aquaculture Project is to demonstrate the feasibility of cultured growth and development of the giant sea scallop (*Placopecten magellanicus*) in Cape Cod Bay, Massachusetts. The 10 acre site is located 2 miles off Truro, MA in water 60–70 feet deep and, as a designated critical habitat for the Northern Right Whale, requires certain adaptations to minimize hazards and risk encounters for the whales. Working with National Marine Fisheries Service, the U.S. Army Corps of Engineers, and Cape Cod Resources, the facility design has eliminated all vertical lines, all hanging mid-water gear and 'spar buoys' have replaced traditional buoys. Plans for monitoring, emergencies and entanglements have been developed.

Spat have been produced by the Martha's Vineyard Shellfish Group for grow-out at the Truro site. Spat collecting in the wild, using traditional methods, is also being investigated. Grow-out will consist of bottom enhancement, bottom caged culture and a bottom technique in which scallop spat is glued to ribbons of plastic mesh.

The project director is a commercial fishermen and has experience and expertise in handling heavy gear in deep water. His understanding of structures in seawater and repair of marine structures are important assets for a project of this type. Underwater video and still photography are being used to document and monitor the facility as well as the habitat and animals.

The restrictions placed upon the Massachusetts fisherman and the impact of the industry on stocks have lead to the development of alternative methods of shellfish and pelagic harvesting. Sea scallop aquaculture has the potential to prove an economically sound business venture thereby attracting interest from the local fishing community. Sea scallop ranching and farming is one option for an industry in need of change.

A NOVEL TECHNIQUE FOR FIELD DEPLOYMENT OF POST-SET *ARGOPECTEN IRRADIANS:* AVOIDING THE JIFFY-POP SYNDROME. **Frank A. Dutra, Scott C. Feindel,** and **Robert Garrison,** Nantucket Research and Education Foundation, 0 Easton Street, Nantucket, MA 02554.

Rapid growth rate makes the bay scallop, *Argopecten irradians*, a prime candidate species for aquaculture. During the nursery phase, however, this factor can become a constraint for all but the largest facilities. An abrupt weekly doubling and occasional tripling of volume can become problematic as optimal stocking sizes are attained and juvenile scallops overflow existing nursery systems. To avoid overcrowding, labor forces are severely taxed in an attempt to thin and deploy large numbers of scallops in a relatively brief time span. Deployment techniques that rely on fine mesh for post settlement retention are susceptible to reduced flow as a result of site-dependent particulates and fouling organisms. Increased mortality, stress, and reduced growth rates can result. Remote sites lacking direct access to hatchery facilities may find it difficult or impossible to employ these techniques. In addition, savings gained through an abbreviated hatchery and nursery period can be over-shadowed in the subsequent painstaking and labor intensive grading and washing processes. The inability of many growers to employ current post-set technologies has resulted in a wide array of field and short-based nursery systems, including up-wellers, raceways, and floating nursery trays.

In an attempt to lower overall production cost and minimize constraints of these existing nursery systems, experimental post-set stocking techniques were developed as part of Nantucket's NOAA-sponsored aquaculture program. Equal volumes of 3-week-old (400–800 μ) post-set scallops were provided with a variety of substrata in hatchery down-welling trays and left overnight for byssal attachment. Substrate sections with attached scallops were then deployed directly to the field in 2.5-mm mesh pearl nets. Densities were determined from randomly selected sections under a dissecting microscope. Anticipated losses due to the inadvertent release of 400–800-μ spat through 2.5-mm openings were minimized by an apparent gregarious settlement preference, as reported for other bivalve species. Errant scallops were observed attached to the outsides of nets in randomly-distributed, oval shaped clusters. Predation and further detachment appeared to be negligible as scallops matured and were removed subsequently from the outsides of the nets after 3 weeks (5–10 mm). Overall retention was estimated to be greater than 70%. Growth rates of post-set scallops substantially exceeded those of nursery-reared cohorts, presumably due to an increase in available food supplies and a minimization of stress associated with repeated handling and overcrowding. Mortality and deformity rates were negligible throughout the trials. Market-sized scallops (35–40 mm) were evident (10% of total) within 90 days of spawning. This technique has proved feasible at any stage of development from eyed larvae up to a 2-mm size range, thus effectively extending the stocking period of any particular batch of scallops, and thereby minimizing the peak labor loads associated with stocking on demand. It should be noted that further study and refinements of this post-set technology are needed, including the feasibility of shipment from hatcheries to remote sites. The initial results may well be site-dependent and of a temporal nature.

RESISTANCE STUDIES FOR JUVENILE OYSTER DISEASE (JOD); 1995 CONTINUATION. **C. Austin Farley,[1] Earl J. Lewis,[1] David Relyea,[2] Joseph Zahtila,[2]** and **Gregg Rivara,[3]** [1]USDOC, NOAA, National Marine Fisheries Service, Oxford, MD 21654-9724; [2]Frank M. Flower Co., Oyster Bay, NY 11771; [3]Cornell University, Cooperative Extension, Southold, NY 11971.

The F_1 progeny from 1991 brood stocks selected on the basis of survival of exposure to juvenile oyster disease (JOD) were exposed to the disease at two infective sites in 1994 along with susceptible seed (FCT) from naive Connecticut brood stocks. In this study, the presumed resistant F_1 population demonstrated up to 7 times better survival.

In the 2nd year of this study, susceptible progeny, F_1 resistant progeny, and F_2 progeny from 1993 F_1 generation brood stocks were produced from June 1995 spawnings at the Frank M. Flower hatchery in Bayville, NY. Seed were placed in nursery raft trays on August 4, 1995 and deployed at 7 sites in the Long Island area on August 28: (1) Oyster Bay, LI Sound; (2) Mattituck Creek, LI Sound; (3) Cedar Beach (SCMELC), Peconic Bay (PB); (4) Parrino Pond, PB; (5) Parrino Lease, PB; (6) Grothe Lease, PB; and (7) Pickerell Lease, PB. Evaluations for JOD (size, shell checks, conchiolin prevalence in live and dead oysters, and mortality) were made from weekly or biweekly samples between August 28 and November 6, 1995.

No mortality was seen on August 28, 1995, at the Flower site. Size-culled FCT susceptible runts (16–20 mm) had mortalities of 75% by October 30, 1995. Size-culled resistant F_1 and F_2 runts had mortalities of 0 to 3% during this time. Unculled FCT seed oysters had a maximum mortality of 15% by October 30, while the F_1 and F_2 seed had mortalities of $<2\%$ over the same time period. At site 2, unculled FCT seed had mortalities of 72% by October 30, 1995. The F_1 and F_2 seed had mortalities of $<15\%$ on the same dates.

At the 5 Peconic Bay sites, all unculled, mortalities of 43–72% were seen by October 30, 1995, in the FCT seed, while the F_1 and F_2 seed mortality was between 1% and 15%. Conchiolin prevalences for all samples averaged 40% in FCT live seed, and 5% in the F_1 and F_2 live seed.

The results of this study demonstrate that seed from JOD-surviving brood stocks are 7 to 25 times better able to survive exposure to this disease than susceptible control populations. Use of these brood stocks on a commercial scale has brought production at the Frank M. Flower Co. back to pre-JOD levels for oysters.

RESISTANCE STUDIES OF CHESAPEAKE BAY, MARYLAND, OYSTERS: EPIZOOTIOLOGY OF TWO POPULATIONS EXPOSED TO *HAPLOSPORIDIUM NELSONI* AND *PERKINSUS MARINUS*. **C. Austin Farley,**[1] **Roy Scott,**[2] and **Leon Williams,**[2] [1]USDOC, NOAA, National Marine Fisheries Service, Oxford, MD 21654-9724; [2]Maryland Department of Natural Resources, Deal Island, MD 21821.

A brood stock from Wilson Shoals Bar, Nanticoke River, was selected from 120-mm survivors of *Haplosporidium nelsoni* (MSX) and *Perkinsus marinus* (Perki) epizootics dating back to 1987. F_1 progeny (DIRS) were produced in May of 1994 from this brood stock at the Maryland Deal Island Hatchery along with an F_1 progeny (DISS) from a naive brood stock from the upper Potomac River (Beacon Bar). Seed oysters were held at the Deal Island site from May 1994 until the present (November 1995). Mortality evaluations, thioglycolate cultures, and histologic samples were taken periodically (quarterly to monthly) from October 1994 through November 1995.

Mortalities were first observed on August 21, 1995: 48% in DISS and 19% in DIRS. Cumulative mortalities were 75% and 40% on September 7, 85% and 60% on September 25, 94% and 76% on October 31, and 95% and 78% on December 4, 1995, respectively.

Haplosporidium nelsoni prevalences and weighted prevalences in DISS and DIRS, respectively, were: 0 (0) and 10% (0.4) on May 31, 37% (1.1) and 40% (1.8) on June 19, 40% (0.8) and 60% (1.8) on August 1, and 0 (0) and 3% (0.03) on August 29, 1995.

Perkinsus marinus prevalences and weighted prevalences in DISS and DIRS, respectively, were: 0 (0) and 0 (0) on May 31, 7% (1.8) and 7% (1.7) on June 19, 50% (1.6) and 30% (0.8) on August 1, 100% (5.8) and 97% (4.7) on August 29, 100% (6.3) and 100% (4.7) on September 26, and 100% (6.6) and 100% (5.9) on October 31, 1995.

Results from the first year of study suggest that some resistance to both diseases is present based on mortality and disease intensity patterns. However, the intensity of disease precludes optimism at least in disease-intense areas. Continuation into F_2 trials, and deployment in locations with less disease pressure, is warranted for future studies.

POLYCULTURE OF SEA SCALLOPS SUSPENDED FROM SALMON CAGES. **Alexander Gryska,**[1] **G. Jay Parsons,**[2] **Sandra E. Shumway,**[3] **Kristin P. Geib,**[4] **Ian Emergy,**[5] and **Sue Kuenstner,**[6] [1]New England Fisheries Development Association, 451 D Street, Boston, MA 02210; [2]Aquatic Industries Ltd., P.O. Box 294, St. Andrews, NB E0G 2X0; [3]Natural Science Division, Southampton College, L.I.U., Southampton, NY 11968; [4]P.O. Box 103, West Boothbay Harbor, ME 04575; [5]Snug Harbor Scallop Farm, P.O. Box 17200, Pembroke, ME 04666; [6]New England Fisheries Development Association, 15 Grigg Street, Greenwich, CT 06830.

Commercial culture of the sea scallop, *Placopecten magellanicus*, is an expanding industry in Atlantic Canada and New England. In an experiment designed to examine the commercial feasibility of polyculturing scallops with Atlantic salmon, we measured the growth and survival of sea scallops grown in suspension on two salmon aquaculture sites in northeastern Maine. One site was in Johnson Cove, Passamaquoddy Bay, and the other was located off Treats Island, near Lubec. Sea scallop spat (11 months of age and 10.2 mm shell height) were grown in standard pearl nets and were deployed on drop lines containing ten nets in August 1994. One drop line of ten nets was sampled about every four months and scallops were counted, measured for shell height, and tissue weights determined. Water samples for chlorophyll and scallop tissue samples for PSP phycotoxin content were also obtained. Scallop growth at the two sites was 35.2- and 38.5-mm shell height after four months and survival was >90%. After one year, shell heights were about 49 and 57 mm, wet adductor muscle weights were 2.8 and 4.5 g, and growth rates were 0.11 and 0.13 mm per day. These growth rates were comparable to sea scallops cultured in Atlantic Canada. Reduced rates of survival were found during the latter part of the experiment and were attributable, in part, to heavy fouling by blue mussels. The potential for supplemental income, diversification of the salmon aquaculture industry, and logistics of culturing scallops in conjunction with salmon will be discussed.

ALGAL FOULING AND PREDATION OF ARTIFICIAL SPAT COLLECTORS IN THE WESTPORT RIVER ESTUARY, MASSACHUSETTS. **Bart Harrison,**[1] **Karin A. Tammi,**[2] and **Wayne H. Turner,**[1] [1]Water Works Group, P.O. Box 197, Westport Point, MA 02791; [2]Department of Fisheries, Animal and Veterinary Science, University of Rhode Island, Kingston, RI 02881.

In 1993, the Bay Scallop Restoration Project (BSRP) was established with a focus on returning the once profitable and rich scallop fishery to the Westport River with the use of artificial spat collectors and spawner sanctuaries. Researchers have observed that spat collectors (2- to 4-mm plastic mesh onion bags containing monofilament) frequently become fouled by various organisms, especially algae and sediment. Typically, both brown (*Sphacelaria sp*) and green (*Enteromorpha sp* and *Cladophora sp.*) algal species foul the exterior of the bag, reducing the effectiveness of the spat collector. Beginning in June 1995, multiple longlines containing 20 spat collectors were deployed weekly to each of 5 study areas. After soaking for approximately 3 weeks, 20 spat collectors were harvested to assess predation and fouling. Spat-collector fouling was recorded on a scale from 1 to 5, with 5 being heavily fouled. Researchers continued to visit each study area throughout the summer and fall at 3-week intervals to record increasing fouling levels. Additionally, at Corey's Island, fouling and predation also was compared to assess the performance between the original onion bag collector with that of a new commercial fine-mesh collector. After soaking for 3 weeks, all study areas had low fouling ratings (1). With the exception of Corey's

Island, fouling indices for all study areas remained low (between 1 to 2) even after six weeks of soaking. At Corey's Island, fouling of onion-bag collectors remained low (1 to 2 rating), but the fine-mesh collector displayed a greater degree of fouling and sedimentation (3 rating), most likely due to the smaller mesh size of the collector. In general, fouling indices gradually increased between the fourth and sixth study week, probably resulting from increased water temperature and influx of nutrients into the estuary. Longlines harvested in the fall were heavily fouled by *Enteromorpha, Sphacelaria* and *Cladophora*. In addition, these collectors were fully colonized by sea squirts, *Mogula sp.*, and mud crabs, *Panopeus sp.*, with the latter being the primary predator. Scallop recruitment over the summer was unaffected by the algal fouling to the collectors. However, as fouling increases, it may make the collector an inhospitable environment by restricting flow through the bag and depleting nutrients, thereby hindering the growth of scallops within the collector. In addition, if there is a second spawning event in the fall, a heavily fouled collector may not function efficiently. This study indicates that fouling and predation to artificial spat collectors is minimal from June to August. Scallop recruitment during this time is not noticeably affected by the fouling of the spat collector.

DESIGNING AN ACCESS SYSTEM FOR OCEAN MARICULTURE. **Porter Hoagland** and **Di Jin**, Marine Policy Center, Woods Hole Oceanographic Institute, Woods Hole, MA 02543.

Ocean mariculture operations have been proposed as alternatives to traditional commercial wild harvests in the U.S. exclusive economic zone (EEZ). Unlike marine fisheries, ocean mariculture operations are designed to constrain the stocks being raised to specific geographic areas using nets, pens, or other technologies. The site-specific nature of ocean mariculture operations requires "security of tenure" (limited property rights) to designated areas of ocean space, possibly including the underlying seabed and neritic and surface waters. Although the allocation of exclusive or proprietary rights to ocean space will be a contentious issue, without security of tenure, the potential exists for other uses of the ocean to impinge upon mariculture operations. Further, the availability of investment capital for ocean mariculture operations is likely to be extremely limited in the absence of security of tenure.

The United States has sovereign rights in its EEZ over the exploitation of commercial living resources. Historically, the United States has exercised those rights through policies designed to manage wild, open-access fish stocks. At present, there is no coordinated policy in the United States governing the use of the EEZ for ocean mariculture operations. U.S. policy is not fully developed with respect to the siting of such operations, and permitting is likely to proceed on an inefficient, ad hoc basis.

A systematic approach to the design of an access system for ocean mariculture operations should involve the following steps: (1) drawing lessons from the design of access systems for other public resources; (2) examining historical practice and current operation of access systems in other jurisdictions; (3) developing a description of the resource to be allocated (ocean space), its relevant attributes, and any potential economic side-effects (positive or negative) that are likely to occur; (4) identifying relevant social objectives; (5) developing an analytic framework within which to analyze tradeoffs among the relevant social objectives; (6) positing a relevant set of policy attributes that would enable the specified social objectives to be met, including property right transfers (partial or complete, permanent or temporary); revenue generation; performance requirements (time limits, fees); information management; environmental protection; and fairness or equity considerations; among others.

DEVELOPMENT OF A GROUNDFISH AQUACULTURE INDUSTRY IN NEW ENGLAND: REVIEW OF EFFORTS TO DATE. **Hunt Howell,[1] Linda Kling,[2] Terry Bradley,[3]** and **Larry Buckley,[3]** [1]University of New Hampshire, Department of Zoology, Durham, NH 03824; [2]University of Maine, Orono, ME 04469; [3]University of Rhode Island, Kingston, RI 02881.

The decline of groundfish populations has caused a renewed interest in growing cod, haddock and flounder species for food and/or stock enhancement. A number of research projects, aimed at raising groundfish species on a commercial scale, are currently underway. This presentation will review the research being done for cod and haddock. Summaries of studies done over the last year, as well as work planned for the future, will be included.

HATCHERY CULTURE TECHNIQUES FOR THE GIANT SEA SCALLOP *PLACOPECTEN MAGELLANICUS*. **Richard C. Karney,** Martha's Vineyard Shellfish Group, Inc., Oak Bluffs, MA 02557.

Under funding from the National Marine Fisheries Service Fishing Industry Grants Program, the Martha's Vineyard Shellfish Group adapted hatchery culture methods for bay scallops to the successful culture of the giant sea scallop (*Placopecten magellanicus*). Field-collected broodstock were sufficiently ripe in early March (sea water temperature, 4–5°C) to spawn just over seven million eggs. The fertilized eggs were transferred to a 400 liter larval conical, with 1-μ filtered, aerated, seawater at 12°C. After 48 hrs, the conical was drained and about three million scallops (ciliated blastulae and trochophores) were recovered and resuspended. Straight-hinge larvae were not observed until the second drain down on day 4. Larval culture protocol throughout the lengthy 40 day larval period included a daily feeding of *Isochrysis sp* (T-ISO) and/or *Chaetoceros neogracile*, with a drain down and sizing every other day. The larvae were cultured in three 400-liter conicals of 5 μ bag-filtered seawater, heated to about 15° C (range 13–17°C). Between days 28 and 38, 1,350,000 pediveliger larvae (about 250 μ) were moved to downweller sieves. The first fully set juvenile was observed on day 32. Set scallops were cultured on

downweller sieves (130–300 μ) with a flow of bag-filtered water (10–50 μ) at seawater temperatures of 8–16°C. By June 8, (day 90) the largest seed measured 2-mm and were moved to 1-mm mesh Korean spat bags in a cage anchored off the dock of the shellfish hatchery. By July 3, a total of 519,000 2-mm seed were successfully transferred to the inshore field culture systems.

All of several potential off-shore growers were frustrated in their attempts to secure proper permits from the regulatory agencies. Over 80% of the 519,000 seed scallops were lost during the month of July when water temperatures reached 22°C and regulatory delays prevented transfer of the seed to cold water grow-out sites. At the end of July, the remaining 90,000 seed were finally permitted to be moved to the deep water (65′) sites of the Truro Aquaculture Project in Cape Cod Bay.

OVERVIEW OF SEED HARD CLAM WINTER MORTALITY STUDIES IN NEW JERSEY, NEW YORK AND MASSACHUSETTS. **John Kraeuter,[1] John Aldred,[2] Paul Bagnall,[3] Richard Crema,[4] Gef Flimlin,[9] Susan Ford,[1] Dale Leavitt,[5] George Mathis,[6] Gregg Rivara,[7] Roxanna Smolowitz,[8]** and **Margy Wintermyer,[1]** [1]Haskin Shellfish Research Laboratory, Rutgers University, Port Norris, NJ 08349; [2]Town of East Hampton, NY 11937; [3]Town of Edgartown, Edgartown, MA 02539; [4]R. F. Crema and Family, Oceanville, NJ 08231; [5]Woods Hole Oceanographic Institution, Woods Hole, MA 02543; [6]Mathis and Mathis Enterprises, Egg Harbor, NJ 08251; [7]Cornell University Cooperative Extension, Riverhead, NY 11901; [8]University of Pennsylvania and Marine Biological Laboratory, Woods Hole, MA 02543; [9]New Jersey Cooperative Extension, Toms River, NJ 08753.

Hard clam (*Mercenaria mercenaria*) aquaculture is one of the most widespread forms of marine aquaculture on the U.S. east coast. The industry is based on hatchery seed production and planting of these seed in protected beds for growout. In the Northeast and mid-Atlantic, seed planted late in the season is subject to variable (up to 50% in some beds) and unpredictable mortalities. Similar observations have been made for manila clams on the Pacific coast. The reasons for the variable success of late plantings of 8–15 mm seed have not been systematically investigated, but are believed to be related to seed "condition" (stored energy reserves) and its interaction with environmental variables or pathogens. One solution to late planting mortality is to hold the clams in nursery systems during winter. Unfortunately, a second, but apparently related, mortality occurs when seed clams are overwintered in nursery systems. Once seed begin to die in these systems, losses can be as high as 5% per day.

The current work is developing and testing methods that could be used by culturists to evaluate the "condition" of seed clams prior to planting or overwintering. The goal is to provide a quantifiable means of evaluating alternatives to mitigate losses and to integrate the methodology into an economic/biological decision matrix. The work underway will evaluate whether disease (bacterial or other infections), lack of energy reserves, or their interaction is the primary cause of winter mortalities. The overall experimental protocol is: 1. Evaluate methods for assessing condition on specific size classes of seed. 2. Test seed and experimentally manipulate condition of specific seed sizes. 3. Re-analyze these manipulated seed and plant at ½ commercial scale. 4. Place additional seed, at specific temperatures, during the fall in field nursery systems. 5. Test growth, survival and condition of all seed in spring.

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THE USE OF VACCINES IN THE CULTURE OF PENAEID PRAWNS. **J. W. Latchford,[1] S. B. Prayitno,[2]** and **A. Alabi,[1]** [1]School of Ocean Sciences, University of Wales Bangor, Menai Bridge, Gwynedd, UK; [2]Department of Fisheries, Diponegoro University, Semarang 50242, Indonesia.

The increased incidence of diseases in cultured shrimp coupled with a growing awareness of the problems of the use of antibiotics in controlling such diseases has led to the development of alternative methods of disease control. Vaccines against several strains of luminous and non-luminous bacterial pathogens were tested for their efficacy in both small scale and commercial scale culture systems of *Penaeus indicus* and *P. monodon* larvae and postlarvae.

Formalin-killed bacteria and vaccines consisting of live attenuated strains of pathogenic bacteria produced by UV light mutagenesis gave a significant degree ($p < 0.05$) of protection against subsequent infection by virulent pathogens when compared to non-vaccinated controls in small scale culture systems.

EXPERIMENTAL EVALUATION OF *VIBRIO* SPP. AS ETIOLOGICAL AGENT OF JOD. **Mijin Lee,[1] Gordon T. Taylor,[1] Monica Bricelj,[1]** and **Susan E. Ford,[2]** [1]Marine Sciences Research Center, State University of New York at Stony Brook, Stony Brook, NY 11794; [2]Haskin Shellfish Research Laboratory, Institute of Marine and Coastal Sciences, Rutgers University, Port Norris, NJ 08349.

The potential role of *Vibrio* spp. in juvenile oyster disease (JOD) was experimentally evaluated during the summers of 1994 and 1995 with isolates collected from juvenile oysters at the Frank M. Flower and Sons Oyster Co. during the JOD episode. To establish which of the isolated *Vibrio* induced JOD symptoms, six challenge experiments were performed at the Flax Pond Marine Laboratory of SUNY Stony Brook. For the first, second, and third challenge experiments, bacterial suspensions were delivered via injection into the mantle cavity of juvenile oysters (size of oysters = 17.5–35.4 mm). For the fourth, fifth, and sixth challenge experiments, oysters were exposed to high concentrations of bacte-

rial suspensions in tank water instead of being injected (size of oysters = 12.5–20.0 mm). All bacterial isolates caused mortality in excess of controls, but one isolate, phenotypically similar to *Vibrio anguillarum,* consistently produced higher mortalities, within 7–14 days of exposure, than all other isolates. Conchiolin deposits similar to early JOD shell deposits were found on oysters in some experiments; however, they were not restricted to a single bacterial isolate. The results of these experiments clearly show that *Vibrio* spp. isolated from JOD-affected oysters, especially a strain similar to a *V. anguillarum,* can be pathogenic to juvenile oysters under certain conditions, and that these isolates can elicit conchiolin layering similar to JOD deposits. No single isolate produced clear or consistent JOD symptoms, however, suggesting that outbreaks of the disease may not be associated with a single bacterial strain but have a multiple-factor etiology.

1995 JUVENILE OYSTER DISEASE (JOD) TRANSMISSION AND BACTERIOLOGICAL STUDIES. **Earl J. Lewis,[1] C. Austin Farley,[1] Ana Baya,[2]** and **Rosangela Navarro,[2]** [1]USDOC, NOAA, National Marine Fisheries Service, Cooperative Oxford Laboratory, 904 S. Morris St., Oxford, MD 21654–9724; [2]Maryland Department of Agriculture, Animal Health Diagnostic Laboratory, 8077 Greenmead Drive, College Park, MD 20740.

Three laboratory transmission experiments for JOD were conducted in 1995. As in previous experiments, material from 3000–3500 gallons of ambient water at a JOD-affected facility was filtered sequentially through bag filters (1–100 μm). Material was backflushed from each filter into 1 gallon of synthetic seawater and added to an aquarium containing susceptible oysters to test the relative infectivity of the material. In addition, 97 water samples and 280 oysters from field and experimental studies were cultured for bacteria.

Samples for the first transmission experiment were collected in May 1995, 10–11 weeks prior to the onset of JOD. Water temperature at the time was 15°C. JOD was minimally transmitted by the 10 μm material at room temperature (24–26°C). Mortalities in these oysters were only 4%, but 75% of the dead oysters had typical JOD conchiolinous shell lesions. Mean vibrio counts in aquaria water at the beginning of the experiment ranged from 10,000 to 1,000,000 colony forming units (CFUs)/ml. Unlike vibrios, the JOD infective agent apparently was not present in the water column in large quantities at this time of the year.

From water sampled in July 1995, JOD was transmitted at room temperature (21–23°C) using material held in 5, 10, 25, 50 and 100 μm bag filters. Oysters held in the 10 and 25 μm filtered material experienced the highest JOD-related mortalities, 33 and 36%, respectively. Mean vibrio counts in aquarium water ranged from 23,000 to 160,000 CFUs/ml at the beginning of the experiment.

An October 1995 experiment also transmitted JOD at room temperature, but the resulting mortalities were influenced by temperature fluctuations. Onset of JOD-related mortalities began in the 5, 10, 25, 50 and 100 μm exposures after 2–3 weeks, but did not progress after the water temperature dropped to 12°C. Mortalities and conchiolinous shell lesions began to reappear 2–3 weeks after heaters were placed in aquaria to maintain temperatures above 20°C. Mean vibrio counts ranged from 4000 to 28,000 CFUs/ml. Disease transmission was not evident in oysters exposed to 1 or <1 μm material from any of the experiments.

Bacteriological cultures of oysters and water samples from laboratory transmission experiments and regional samples from New York, Maine, and Rhode Island yielded 17 known *Vibrio* spp. As in our previous work, there was no consistent association of a particular *Vibrio* sp., or group of vibrios, with JOD. All commonly isolated vibrios (prevalence >9%) were isolated from uninfected control samples and JOD-infected samples. *Vibrio* sp. were isolated with nearly the same prevalence from each group.

DIAGNOSIS AND PREVALENCE OF *PERKINSUS* SP. IN CHESAPEAKE BAY SOFTSHELL CLAMS, *MYA ARENARIA:* AN UPDATE. **Shawn M. McLaughlin,** USDOC, NOAA, National Marine Fisheries Service, Cooperative Oxford Laboratory 904 S. Morris St., Oxford, MD 21654–9724.

Perkinsus sp. was rarely observed in Chesapeake Bay softshell clams (*Mya arenaria*) prior to 1990 based on histological analyses. An unusual occurrence of the parasite was observed in Chesapeake Bay softshell clams from seven sites at prevalences ranging from 3–53% during 1991–1993 based on rectal assays in fluid thioglycolate medium (FTM). Prevalences decreased in 1994 and remained low at sites examined in February 1995. In August 1995, softshell clams from three Chesapeake Bay sites were diagnosed by rectal FTM assays to have significant prevalences of *Perkinsus* sp. Prevalences of the parasite were 13% at Swan Point, 37% at Piney Point, and 64% at Cedar Point. Analyses of tissue sections indicate the initial infection site of softshell clam *Perkinsus* sp. is often the gills. A comparison of softshell clam tissues incubated in FTM was conducted. Subsamples of hemolymph, gill demibranchs, and rectal tissue collected from each of 30 Swan Point softshell clams were incubated separately in FTM for 5 days, stained with Lugol's iodine, and examined. Results showed *Perkinsus* sp. prevalences of 0% for hemolymph, 13% for rectal tissue, and 90% for gill tissue in the Swan Point sample. The experiment was repeated using hemolymph, gill, rectum, and labial palps collected from 30 additional Chesapeake Bay softshell clams. Prevalences of *Perkinsus* sp. were 0% for hemolymph, 43% for rectal tissue, 90% for gill tissue, and 100% for labial palps. Softshell clam labial palps are now routinely incubated in FTM during histologic processing at this facility. The change from rectal to palps thioglycolate tests increases the sensitivity of the softshell clam *Perkinsus* sp. assay and eliminates the time consuming task of locating and excising softshell clam rectal tissue. This study also indicates under-reporting of *Perkinsus* sp. in Chesapeake Bay softshell clams has occurred.

FIG'S AND AQUACULTURE—A LOOK BACK AND A LOOK AHEAD.

Harold C. Mears, USDOC, NOAA, National Marine Fisheries Service, One Blackburn Dr. Gloucester, MA 01915.

In March 1994, the Secretary of Commerce announced the availability of $30 million under provisions of the Northeast Fisheries Assistance Program to address the needs of those directly affected by the decline of the traditional fisheries in the Northeast. The initiative included $9 million for Fishing Industry Grants (FIGs) administered by the National Marine Fisheries Service. Aquaculture-related investigations comprise $4.1 million, or about 46%, of the total funding under the FIG Program. The project objectives for these 22 studies are to help restore overfished groundfish and shellfish stocks in the Northeast Region, as well as to provide new business opportunities for displaced fishermen.

Study activities are focusing on cod, haddock, summer flounder, Nori (*Porphyra*), scallops, quahogs, oysters, surf clams, and sea urchins. The work funded under this program is providing an impetus for facilitating resolution of issues concerning aquaculture start-up costs, technology transfer, environmental impacts, user group conflicts, fishery enhancement, and potential for industry expansion and employment. The knowledge gained from the FIG Program is forging more effective communications and partnerships between the private, state, federal, and academic sectors, as well as further specifying research and management priorities for future projects concerning aquaculture development.

CULTURE OF THE BAY SCALLOP, *ARGOPECTEN IRRADIANS*, WITHIN A SMALL-BOAT MARINA ON LONG ISLAND SOUND (CONNECTICUT).

Matthew Mroczka,[1] **Paul Dinwoodie,**[1] **Ronald Goldberg,**[2] **Jose Pereira,**[2] **Paul Clark,**[2] **Sheila Stiles,**[2] **Joseph Choromanski,**[2] **Daniel Schweitzer,**[2,3] and **Nancy Balcom,**[4] [1]Cedar Island Marina, P.O. Box 181, Clinton CT 06413; [2]USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford CT 06460; [3]Marine Sciences and Technology Center, University of Connecticut, Groton, CT 06340; [4]University of Connecticut, Sea Grant, Marine Advisory Office, University of Connecticut, Groton, CT 06340.

An innovative suspension-culture rack system was designed to evaluate the potential of intermediate grow-out of shellfish seed within a marina in Clinton, Connecticut. Conventional dock space in the marina was modified by cutting out sections of the decking to gain access to the water below. The cut-out sections were replaceable, allowing normal use of the dock. Wire-mesh cages (1 × 0.5 × 0.5 m) containing four shelves were suspended below the modified docks. Shellfish seed were contained on the shelves of the cages within flexible plastic-mesh bags with temporary closures of slit PVC pipe at the ends. To evaluate the growth potential of hatchery-reared bay scallop seed in the marina environment, about 6,000 animals with an initial shell height of 15.5 mm were reared at different densities and in different mesh size cages and bags from June through November of 1995. Survival of scallops in all treatments was very high, averaging about 90 percent. The fastest growing group of scallops reached an average shell height of 54.1 mm by the end of November, with many individuals larger than 60 mm. Stocking density and mesh size were inversely proportional ($P < 0.05$) to growth of scallops over a wide range of sizes. Seawater current flow, temperature and oxygen regimes, and ambient phytoplankton densities were adequate at this location to support substantial growth with low mortality. The use of space under the docks of marinas for shellfish culture caused limited interference with marina activity. There is a good potential for intermediate grow-out of scallop seed at marinas as a step in intensive aquacultural production or in seed transplant efforts to restore scallop fisheries to natural habitats.

This work was partially funded through grants from the University of Connecticut's Marine Technology Center and Sea Grant, Marine Advisory Program, Groton, CT.

CULTURE OF SUMMER FLOUNDER, *PARALICHTHYS DENTATUS*, AT GREATBAY AQUAFARMS.

George Nardi, GreatBay Aquafarms, 153 Gosling Road, Portsmouth, NH 03824.

GreatBay Aquafarms, Inc. (GBA) was established in 1995 for the culture of marine fish, principally summer flounder. The initial objective of GBA is the production of 5–10 gram juveniles from its Portsmouth, NH hatchery. The hatchery is a 10,000 square foot facility which had been a warehouse for the Public Service Company of New Hampshire, an electric utility. The hatchery began production in January of 1996. The facility includes a lab, phytoplankton and zooplankton culture rooms, and three recirculating systems for the broodstock, weaning and nursery stages. The egg and early larval stages are flow-through water systems. At capacity, the hatchery is expected to produce between 300,000 and 400,000 juveniles per year. Including office staff, seven people are employed at GBA.

Our production techniques are based both on university research undertaken here in New England and on a transfer of technology from the European flatfish culture industry. GBA maintains 5 stocks of broodfish which will be induced to spawn through photoperiod and temperature manipulation. In addition to the culture of rotifers and artemia, GBA will initiate the culture of copepods as an early larval diet. The hatchery employs a sophisticated direct digital control system produced by Allerton Technologies. GBA will provide growers with juveniles and will work with them to assist in the development of this new industry. GBA also plans to establish its own grow-out farm in the near future.

BAY SCALLOP CULTURE IN A VIRGINIA SALTWATER POND.

Michael J. Oesterling and **Laura A. Rose,** Virginia Institute of Marine Science, College of William and Mary, Department of Advisory Services, Gloucester Point, VA 23062.

Efforts at bay scallop culture (*Argopecten irradians*) in Virginia begun in the 1960's by Mike Castagna, experienced a resurgence in 1990. At that time, lantern net technology for grow-out

was not considered feasible, primarily due to regulatory uncertainty surrounding the permitting of large numbers of lantern nets in Chesapeake Bay. As the result of increasing publicity, the owner of a saltwater pond requested the evaluation of his pond for scallop culture. Private ownership of the pond made the use of hanging culture possible without any regulatory constraints.

Periods of growth over the duration of this project can be divided into three phases. Animals with a mean shell height of 4.7 mm were stocked into 1-mm mesh pearl nets on 22 June 1995 and cultured at a very high density for 21 days (Phase I). They were subsequently culled, restocked and grown at a density of 4388–5265 per square meter (406–488/sq ft) for 38 days (Phase II). For final grow-out, they were transferred to either 6-mm mesh pearl nets (439 sq m or 41/sq ft) or 15-mm mesh lantern nets (Phase III). Lantern nets were either 4-tiered or 5-tiered, with each tier holding 100 animals (density of 513 sq/m or 50 sq ft) and were stocked on 25 August and 1 September.

Pearl net growth during Phase I averaged 0.20 mm per day. Growth in pearl nets at mid-density during Phase II averaged 0.32 mm per day. Final grow-out in the pearl nets at a reduced density averaged 0.33 mm per day for 81 days. Lantern-net growth over 110 days averaged 0.34 mm per day. However, during September and early October, growth in lantern nets averaged 0.65 mm per day. In both pearl nets and lantern nets, market-size animals (over 40.0 mm shell height) were produced by the first week of October, approximately 100 days after initial stocking.

LABORATORY CULTURE OF TAUTOG: A PILOT STUDY. **Dean M. Perry, Renee Mercaldo-Allen, Catherine Kuropat,** and **James Hughes,** USDOC, NOAA, National Marine Fisheries Service, Milford Laboratory, Milford, CT 06460.

Spawning of field-captured adult tautog (*Tautoga onitis*) was accomplished in the laboratory. The embryos were cultured to hatching and successfully raised through the difficult larval stages to juveniles. Static culture containers, changed twice a week, proved superior to flow-through seawater systems. Newly hatched larvae were fed protozoans for the first 4 days post-hatch, and then were fed rotifers and artemia that had been enriched with highly unsaturated fatty acids. Larval mortality was high until natural plankton was added to the diet. Laboratory cultured artemia, supplemented with natural plankton and a commercial food made an adequate diet for juvenile tautog.

A SIMPLE SYSTEM FOR LONG-TERM EXPOSURES OF ADULT OR LARVAL BIVALVES TO BACTERIAL PATHOGENS. **Steven Pitchford,** USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford, CT 06460.

A method used for long-term exposures of larvae, juveniles and adult bay scallops (*Argopecten irradians*) to bacterial pathogens is described. The standard 48-hour static screening tests are satisfactory for highly pathogenic larval pathogens, but inadequate when studying slower acting or more opportunistic bacterial infections. Large numbers of test animals were kept alive for up to three months in an inexpensive, easily maintained system using aerated 3-liter plastic jars. In addition, procedures were devised to facilitate feeding, water changes, and observation while at the same time minimizing bacterial contamination of the surroundings. This arrangement would also be suitable for maintaining other species of bivalves and allow other types of studies such as juvenile oyster disease, toxicology, and viral transmission.

This project was funded partially by a University of Connecticut, Marine Sciences and Technology Center Grant.

LESSONS FROM SCALLOP SPAT COLLECTION IN WASHINGTON STATE. **Walter Y. Rhee,** School of Fisheries, University of Washington WH-10, Seattle, WA 98195.

Japanese onion-bag collectors were set in Hood Canal, Washington, to test the feasibility of scallop grow-out culture through natural spat collection. A total of 406 fine-mesh (2×1 mm weave) onion bags were deployed for a period of three months from April to June, each onion bag holding one of three types of substrates: netlon, discarded gill nets, or acetate film. The results and the lessons learned from the collection are discussed.

CHANGING TIMES FOR RHODE ISLAND SHELLFISHERIES: URI's SHELLFISH AQUACULTURE TRAINING PROGRAM FOR SHELLFISHERMEN. **Michael A. Rice** and **Joseph T. DeAlteris,** Department of Fisheries, Animal and Veterinary Science, University of Rhode Island, Kingston, RI 02881.

The shellfishing industry in Rhode Island has been in decline since the middle 1980s. In 1985, about 27 million pounds (shell on) of northern quahogs, *Mercenaria mercenaria*, were harvested from Rhode Island waters, but by 1993 only 11 million pounds were harvested. In the same time period, the number of full-time shellfishermen dropped from about 800 to a present number of 300. Catch per unit effort by remaining fishermen is about the same as in the previous decade, but market prices have remained low leading to depressed fisheries incomes. Traditionally, the Rhode Island shellfishing community has been reluctant to embrace aquaculture activities in the state, but recent political action by the State Legislature has brought aquaculture discussion to the forefront. A legislative commission charged with the promotion and protection of an aquaculture industry in Rhode Island has been formed, and aquaculture-friendly legislation is pending. Although opposition to aquaculture remains among some shellfishermen, other shellfishermen are considering the adoption of shellfish

aquaculture as a supplemental income source. In the summer of 1995, we conducted a course for shellfishermen using the technique of transient gear aquaculture of oysters, *Crassostrea virginica*. A total of 17 students registered for the course, 12 of whom were shellfishermen. Lectures covered issues of permitting, oyster biology, business planning, and marketing. Using the URI vessel R/V Captain Bert, the shellfishermen deployed cages at three sites in Narragansett Bay on June 7, using about 150,000 3–4 mm seed (≈750 ml volume) oysters per site. Monitoring the three sites (Dutch Island Harbor, Hope Island and Fox Island) on a biweekly basis, the students found that oysters grew at varying rates depending on site. At the Fox Island site, a total volume of 217 l of oysters was attained by October 30 with sizes averaging 30–40 mm valve height. At Dutch Island Harbor and Hope Island oyster biovolumes were 69 l and 123 l, respectively, with minimal mortalities. The growth of oysters at Fox Island approaches that of oysters in the more nearly eutrophic Point Judith Pond. Although fouling of enclosures is less in the Narragansett Bay sites than in the pond, starfish predation is greater in the Bay. These results were pumped through a 60-μm plankton net and preserved in cally feasible in Narragansett Bay, but attention must be paid to effective predator exclusion. This is publication number 3210 of the College of Resource Department, University of Rhode Island.

MARTHA'S VINEYARD SHELLFISH GROUP AQUACULTURE TRAINING PROGRAM.

Elizabeth F. Scotten, Gabriella C. Castro, and **Debra L. Colombo,** Martha's Vineyard Shellfish Group, Inc., Oak Bluffs, MA 02557.

Under funding from the National Marine Fisheries Service (NMFS), Fishing Industry Grants (FIG) Program and a NMFS grant to the Nantucket Research and Education Foundation (NREF), the Martha's Vineyard Shellfish Group initiated an Aquaculture Training Program for fishermen who have been displaced by the George's Bank fishing area closures. The program was seen as an opportunity for fishermen to make a transition from offshore fishing to shellfish aquaculture business.

A total of 15 trainees worked closely with the staff of the Martha's Vineyard Shellfish Group and the local shellfish constables learning hatchery and on- and off-shore nursery techniques. The trainees participated in all aspects of larval and juvenile care, from algal culture and spawning to grow-out, both on- and offshore. The trainees assisted with the design, assembly and operation of an on-shore shellfish culture nursery. They also spent time with the local shellfish constables building off-shore rafts and monitoring growth therein.

The program also included a weekly lecture series where science, industry and regulatory people addressed many of the issues facing aquaculturists today. By providing exposure to numerous aquaculture resources and through a hands-on approach to aquaculture, the Martha's Vineyard Shellfish Group developed a potentially effective aquaculture training program.

A PC-CONTROLLED, EXPERIMENTAL MOLLUSCAN REARING APPARATUS FOR STUDIES OF FEEDING STRATEGIES AND NUTRITION.

Barry C. Smith,[1] **Gary H. Wikfors,**[1] **Jennifer H. Alix,**[1] and **Mark S. Dixon,**[1,2] [1]USDOC, NOAA, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460; [2]University of Connecticut, Marine Sciences and Technology Center, Groton, CT 06430.

Production of microalgal feeds has been a major limiting factor in the development of the shellfish aquaculture industry. On a production scale, many grams per week of microalgal biomass may be required to feed shellfish. Knowledge of feeding regimes yielding the highest conversion efficiencies of algal feed to molluscan growth is required to maximize the return on an algal-culture investment.

At the Milford Laboratory, specialized, manually-controlled molluscan rearing chambers have been used to study shellfish nutritional requirements since 1982. The system consists of twelve PVC chambers fitted with screens to hold the shellfish being studied. Contemporary process-control technology now can be used to program such things as feeding time and duration in investigations of shellfish nutrition. A computer-controlled, solenoid-valve system has been added to the existing manual system to control seawater flow, volume of microalgal food, and feeding duration automatically. All components of the system are "off-the-shelf" in that they are readily available. An object-oriented software package controls the outputs of a digital In-Out board; the output signals trigger relays which operate the solenoid valves. Each chamber has a solenoid valve for seawater flow, algal feeding, and chamber draining. The system runs independently until stopped, reducing labor requirements while adding experimental flexibility. Each chamber represents a model for a programmed molluscan nursery system.

Initial results suggest that feeding young post-set bay scallops, *Argopecten irradians*, every six hours yields growth superior to feeding once daily. Subsequent experiments will work toward developing feeding standards for molluscan shellfish analogous to those employed routinely in agricultural animal husbandry.

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AN IMPORTANT NEW DISEASE OF HARD CLAMS, *MERCENARIA MERCENARIA*, IN THE NORTHEAST UNITED STATES.

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In July of 1995, a meeting of hard clam culturists was held with Drs. Leavitt and Smolowitz in Provincetown, MA. The culturists described a 4-year history of chronic, increasing severe clam mor-

tality on the planted flats in Provincetown, MA. In 1995, up to 80% of the seed planted 1–1/2 to 2 years before was dead. Crab predation was a recognized problem on the clam leases, but the additional possibility of a primary disease was considered since losses appeared to be too high for crab predation alone. Clam samples were obtained at that meeting and at a subsequent visit by Smolowitz and Leavitt to the leases in August. Histologic examination of 12 clams showed 6/12 contained an endosporulating protozoal organism similar to QPX (seen in a Canadian hatchery in 1989). These Provincetown animals, however, also showed bacterial infections.

Leavitt and Smolowitz revisited two Provincetown leases in mid-October, 1995. A total of 80 clams were collected. Fifty clams were identified as moribund or "affected" (poor growth over the summer, slight gaping of 1–2 mm and chips in the shell edges). Thirty animals from the same flats were collected as controls (at least 1 cm of new growth over the summer and no gaping or chips). Histopathologic examination showed that 45/50 of the "affected" clams contained the QPX-like parasite accompanied by severe inflammation. The parasite was seen in only 3/30 of the control clams. No other possible disease-causing agent was seen in these clams.

In November 1995, both Smolowitz and Perkins were sent samples of approximately 18-month-old clams from a culture site in Duxbury, MA. One population of clams from that site had experienced heavy mortalities over the preceding 3 months. Examination of these clams showed that the same QPX-like organism was responsible for the mortalities in Duxbury, MA.

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PRELIMINARY INVESTIGATIONS OF GENETICS AND BREEDING OF THE BAY SCALLOP, *ARGOPECTEN IRRADIANS*. **Sheila Stiles,[1] Joseph Choromanski,[1] Daniel Schweitzer,[1,2] and Qin-Zhao Xue,[3]** [1]USDOC, NOAA, National Marine Fisheries Service, Milford Laboratory, Milford, CT 06460; [2]Marine Sciences and Technology Center, University of Connecticut, Groton, CT 06340; [3]Chinese Academy of Sciences, Qingdao, China.

Genetic investigations that comprise various approaches for increasing growth rate of the commercially valuable bay scallop, *Argopecten irradians*, were initiated. Projects included mass-selection, inbreeding, population genetics and strain/field evaluations. Approximately 15 mass spawnings were accomplished to establish foundation crosses for mass-selection. Scallops were measured and the top 10–15% or largest scallops and the bottom 10–15% or smallest scallops were selected and then spawned to produce the next generation. Preliminary results from initial selective breeding for fast growth are indicating success with mean size of scallops in the high or fast growth line larger than that of scallops in the slower-growing line.

Scallops were also sampled for shell color such as stripes or orange, and were self-fertilized to determine inbreeding effects, as well as to explore the potential for improving heritable production traits that might be linked to shell markers. Markings were inherited. However, in contrast to mass-spawned cultures, development to 48 hours and subsequent survival were lower and growth retarded in several inbred scallop cultures, suggesting inbreeding depression.

Breeding results were supported by a population genetics investigation of wild and cultured bay scallops employing enzyme electrophoresis conducted cooperatively under the sponsorship of the U.S.-China Marine and Fisheries Protocol Agreement on Living Marine Resources. Scallops obtained from various populations ranging from Canada to Florida, as well as from Milford Laboratory strains, were measured and characterized for size, age, rib number, and shell and mantle color. Specimens were then sampled for enzyme electrophoresis and eventual DNA analysis. Other samples were brought from China or purchased from a supermarket. Allozyme analysis revealed genetic variation among and within populations, which should be considered in broodstock selection and enhancement and restoration efforts for aquatic organisms in general, including finfish. Milford mass-spawned and inbred lines provided valuable information regarding genetic diversity and stock structure.

Field assessments of habitat suitability and strain performance in stock restoration and enhancement efforts are indicating growth of some scallops to marketable size in less than a year. All of these projects demonstrate that the bay scallop is a suitable model for genetic studies and that it can have excellent responses to selection for improved growth.

This project was funded partially by a University of Connecticut, Marine Sciences and Technology Center Grant.

VELIGER VODOO AND OTHER WITCHCRAFT IN THE WESTPORT RIVER: BAY SCALLOP, *ARGOPECTEN IRRADIANS*, VELIGER ABUNDANCE AND THE VARIABILITY OF SPATFALL RECRUITMENT TO ARTIFICIAL SPAT COLLECTORS IN THE WESTPORT ESTUARY, MASSACHUSETTS, **Karin A. Tammi,[1] Wayne H. Turner,[2] Margaret Brumsted,[3] and Michael A. Rice,[1]** [1]Department of Fisheries, Animal and Veterinary Science, University of Rhode Island, Kingston, RI 02881; [2]Water Works Group, P.O. Box 197, Westport Point, MA 02791; [3]Dartmouth High School, Slocum Road, Dartmouth, MA 02747.

During the past 3 years, the Bay Scallop Restoration Project has pioneered the widespread use of artificial spat collectors (4-mm plastic mesh onion bags containing monofilament) in the Westport River. Researchers have observed a significant variability in scallop recruitment to collectors deployed throughout the estuary. In general, poor scallop recruitment to spat collectors may be attributed to estuarine circulation patterns, crab predation and

fouling. This information has led to an investigation of the bivalve larval dynamics in the Westport River with the goal of optimizing deployment time of spat collectors. From May to September 1995, weekly bivalve larval sampling was conducted within four main channels of the Westport Estuary. At each site, 200 liters of water were pumped through a 60-μm plankton net and preserved in ETOH for later enumeration and identification. Beginning in June, multiple longlines containing 20 spat collectors were deployed weekly to 5 study areas of which 3 were being monitored simultaneously for bivalve larvae. At Corey's Island, an additional collector with a commercial fine mesh (1.5 to 3.0 mm) containing a polyethylene tube (40 × 80 cm) as the settlement substrate was also deployed. During the summer of 1995, 4 major bivalve spawning events occurred coinciding with full and new moons. Larval bivalve identification determined that these spawning events were bay scallops, blue mussels and soft-shelled clams. Monthly scallop recruitment to collectors reflected the larval bivalve profile of that study area. The greatest scallop recruitment for all study areas was observed in longlines deployed in late June and harvested by mid-August. Corey's Island displayed the greatest recruitment (per 60 bags), yielding a total of 887 scallops, followed by Canoe Rock (250 scallops) and Horseneck Channel (230 scallops). Although the Hicks Cove and Jug Rock study areas were not sampled for bivalve larvae, the monthly scallop recruitment occurred at the same time as the other 3 areas. Jug Rock yielded a total of 862 scallops and Hicks Cove 108 scallops for the same dates. Corey's Island had the greatest overall monthly recruitment for the entire study due to the significantly better performance of the fine-mesh collector. This study indicates that *A. irradians* spawns heavily in late June and that the optimal time to deploy spat collectors is in late June with retrieval by mid-August, thus improving collection efforts for the future.

UPDATE ON FEDERAL POLICY AFFECTING MARINE AQUACULTURE IN THE EXCLUSIVE ECONOMIC ZONE OF THE UNITED STATES. **Eric M. Thunberg,** USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Woods Hole, MA 02543.

Marine aquaculture in the United States has been primarily located in coastal near-shore waters. However, for a variety of reasons development of coastal sites continues to be difficult prompting some aquaculture professionals to consider the feasibility of off-shore aquaculture. Recently, the New England Fishery Management Council approved a proposal to allow development of an experimental off-shore sea scallop demonstration project. A proposed salmon operation was considered but not approved. Proposals for ocean ranching of tunas and other species are still in the developmental stages but may eventually become a reality. In most instances, accommodation of aquaculture in the Exclusive Economic Zone of the United States will require modification of existing law and modification of existing fishery management plans. At this time, considerable ambiguities exist concerning the treatment of marine aquaculture in the EEZ which have only recently begun to be taken up by the United States Congress and Fishery Management Councils. This paper provides an update and status report on developing legislation and policy statements that will affect marine aquaculture in waters under Federal jurisdiction. Unresolved issues are highlighted with special emphasis on their economic implications.

WHAT A YEAR TO BE A MUD CRAB! THREE YEARS ON THE BAY SCALLOP RESTORATION PROJECT, WESTPORT RIVER ESTUARY, MASSACHUSETTS. **Wayne H. Turner, Karin A. Tammi, Bart Harrison,** and **Bethany A. Starr,** The Water Works Group, Inc., Post Office Box 197, Westport Point, MA 02791.

The Bay Scallop Restoration Project (BSRP) was launched in 1993 with the goal of generating the interest, involvement, and enthusiasm required to restore and enhance the renewable economic resources of traditional fishing and farming communities. Eighty-three thousand hours of volunteer work enthusiastically invested by teams of people have been channeled into this effort. These people: *students, teachers, parents, graduate students* have left a major impact, not only on the bay scallop, *Argopecten irradians,* but on the way in which communities participate and positively affect the direction of their economic future and environmental quality.

With three years of research on the BSRP, community volunteers, led by graduate students have uncovered several significant clues about bay scallop propagation in the Westport River. In 1993, when the BSRP first began, mud crabs, *Panopeus* spp., went largely unnoticed as very few were found on the river bottom or in the propagation equipment. Green crabs, *Carcinus maenas,* on the other hand, were plentiful and practically every spat bag (propagation equipment used to catch juvenile scallops) had at least one if not two green crabs associated with it.

Strangely enough, in the summer of 1994, green crabs were reduced in number and mud crabs surged. Researchers began counting thousands of mud crabs pouring from nearly every spat bag. Because of the recent prevalence of mud crabs, an experiment using floating rafts was set up in the Westport River, with each raft housing a different combination of four ingredients: mud crabs, green crabs, spat-size bay scallops, and yearling tautog, *Tautoga onitis* (approximately two inches in length).

The conclusions drawn from this study are intriguing: 1) mud crabs ate the bay scallops; 2) green crabs did not eat the bay scallops and instead ate the mud crabs; 3) yearling tautog could not seem to handle a green crab (probably due to size of the crab), but cleverly enough, researchers observed that mud crabs in a raft with two-inch tautog lost one leg per day. By the fourth day of the experiment, the mud crab could no longer move and the tautog ate it. Therefore, 1995 appeared to be a good year to be a mud crab for at least three reasons. First, green crabs have been down in numbers for the past two years. Secondly, yearling tautog, commonly found in spat bags in 1993, were virtually absent during 1994 and

1995. Finally, support of this abundant supply of mud crabs seemed to become the propagation activities of the BSRP by supplying spat-size bay scallops as a preferred food source of mud crabs.

GROWTH OF BAY SCALLOPS, *ARGOPECTEN IRRADIANS*, IN 5 MM MESH LANTERN NETS. **James C. Widman, Jr.**[1] and **Christopher G. Cooper,**[1,2] [1]USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford, CT 06460; [2]Sea Change Foundation, Covington, VA 24426.

One growing scenario for bay scallop culturists is to spawn during the natural warm-weather season, thereby reducing hatchery energy costs. If net-suspension culture is used, one needs to determine when biofouling would require cleaning. It is also important to determine whether any differences in growth occur due to handling, or by position of the scallops in the shelves of the nets. To answer these questions we conducted the following experiment:

Bay scallops, *Argopecten irradians irradians*, were deployed at a density of 750/m^2 in 5 mm mesh, 5-tiered lantern nets in Long Island Sound near Groton CT. Scallops with an initial mean shell height of 17.6 mm (8.1–32.5 mm range) were held from July 11 through November 27, 1995. Fifteen nets were deployed in a modified latin square design to determine the effects of handling, shelf position, and biofouling on scallop growth. Three nets were sampled every month, and the remaining 12 were sampled three per month. Each time a net was sampled the scallops were moved into a new net. Final mean shell heights ranged from 39.0–47.4 mm. Although there was a significant difference in mean shell height among shelves, the difference was not consistent, i.e., shelf one did not always provide optimal growth. Scallops reared in the bottom shelf had lower survival because the bottom of the net often hit the cement anchor due to the high current. Scallops handled on a monthly basis were usually smaller and had lower survival than those handled only once during the experiment. Very little fouling of the scallop shells was observed, although the nets were always fouled. There was no effect of fouling on growth during the study period.

The scallops harvested from this experiment are now enjoying winter at the bottom of Long Island Sound in Groton and Milford, CT. We plan to report the overwintering and final grow-out results next year.

This project was funded partially by a University of Connecticut, Marine Sciences and Technology Center Grant.

FEEDING STRATEGIES FOR POST-SET BAY SCALLOPS, *ARGOPECTEN IRRADIANS:* WHAT? HOW MUCH? HOW OFTEN? **Gary H. Wikfors,**[1] **Barry C. Smith,**[1] **Jennifer H. Alix,**[1] and **Mark S. Dixon,**[1,2] [1]USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford, CT 06460; [2]Marine Sciences and Technology Center, University of Connecticut, Groton, CT 06340.

Bringing bay scallops to market in one growing season in the northeast will improve the economics of scallop farming from two standpoints: 1) losses to winter-kill will be avoided, and 2) the farmer's return on investment will not be deferred. To accomplish this, seed scallops will need to be produced early in the season in a heated, recirculated seawater system; it will take one heck of a lot of algae to feed them. Even more algal feed will be needed to grow scallops to market in such a system. The economics of product value *versus* feed costs will be dependent upon two major factors: 1) cost of algal biomass, and 2) feed conversion efficiency (i.e., how much algal biomass is converted to scallop biomass and how much is wasted?). Maximizing feed conversion efficiency requires answers to the three questions posed in the title.

We have conducted several feeding experiments with young, post-set scallops comparing algal diets and feeding schedules to begin finding answers. "What" to feed post-set scallops seems to include: 1) cells larger than about 6 μm, 2) algal cultures that are not "clumped" in aggregates, and 3) algal strains with high levels of total lipid and essential fatty acids. High-lipid *Tetraselmis* strains that support rapid growth of oysters are also superior scallop diets.

The "How much" question has yielded less clear an answer. Doubling a ration that is only partially consumed results in significantly faster growth. This feeding behavior, however, will result in wasted algal feed if rations are adjusted to maximize growth. One solution for this problem may be to find alternative uses for unconsumed algae from scallop-rearing tanks.

An experiment to determine "How often" to feed post-set scallops yielded better growth when animals were fed every six hours, as compared with feeding less often or feeding every three hours. Differences between good and poor algal diets were less severe when fed at the optimal six-hour regime than when fed only once every 24 hours.

These preliminary findings present logical directions for future research to develop biochemically-based feeding standards and schedules for nursery culture and grow-out of bay scallops.

This project was funded partially by a University of Connecticut, Marine Sciences and Technology Center Grant.

PIONEERING EFFORTS TO PRIVATELY CULTURE QUAHOGS, *MERCENARIA MERCENARIA*, IN THE TOWN OF EDGARTOWN, MA. **Paul Willoughby** and **Jack Blake,** Martha's Vineyard Shellfish Group, Inc., Oak Bluffs, MA 02557.

Fishermen participating in the Martha's Vineyard Private Aquaculture Initiative, an aquaculture training program funded under the Fishing Industry Grants Program of the National Marine Fisheries Service (NMFS) and a NMFS grant to the Nantucket Research and Education Foundation, describe their first attempts to field-culture quahogs in private and cooperative public/private projects in the town of Edgartown.

One-half million small (1.5-mm) seed quahogs purchased on June 13 were successfully cultured to 20-mm in experimental nurs-

ery floats on existing and proposed private aquaculture lease sites. One raft design that performed well in an exposed site subject to 50 mph winds and waves up to two feet is described.

Quahogs (6–10 mm) culled from the rafts at the end of July and planted in a muddy substrate were observed after two weeks to have suffered high (95%) predatory mortality by mud crabs. Larger (19 mm) seed planted in sandy bottom in September showed next to no mortality when sampled two weeks later. In mid-October 280,000 20-mm quahogs from the public/private project were seeded by ten fishermen at various Edgartown sites. Similar growth and survival was achieved in floating nursery trays on a private lease site where 160,000 of an initial 200,000 1.5-mm seed quahogs measured 20 mm at the end of November.

Difficulties experienced in securing private aquaculture lease areas are also presented.

MURPHY'S LAW AND THE RAISING OF ATLANTIC STURGEON. Frederick B. Wishner, Hofstra University, Hempstead, NY 11551.

The U.S. Fish and Wildlife Service Hatchery at Lamar, PA was successful in hatching 150,000 Atlantic sturgeon larvae on 4 July 1995 using a large female which was spawned out of the Hudson River. A total of fifty-eight fingerlings were made available to the Hofstra Aquaculture Laboratory. The first batch of 35 was transported on 14 September 1995 to the campus facility where 30 were placed in a 100 gallon fiberglass tank with air and a powerhead water changer with a submersible pump hooked up to a trickle filter. Another five were placed in an aquarium with an underground filter. The ambient water temperature was 17°C and the pH was 7.2, while concentrations of ammonia and nitrites were negligible. Food was supplied by the hatchery. Biokiowa C100 and C750 were fed at 1% body weight per day by hand. The light cycle was 12-hours light and 12-hours dark. Twenty-three fingerlings were brought to Aqualong Co. in Riverhead, New York on 7 December 1995 where they were maintained in a 6,000 gallon tank at 47±°F.

Fish in the 100-gallon fiberglass tank did not survive more than 2 days. Overfeeding may have been the cause. Fish held in the aquarium were cold-shocked on day 14 and only 2 survived. The 2 that survived grew from 55-mm to 110-mm, similar to those at the hatchery which were kept in a raceway. The fish at Aqualong fared better; eleven of 23 survived the first snow storm and ambient water conditions. Water continues to be changed weekly with no other care. Fish that survived seem to fare better on warm days after well water is exchanged and the temperature heats up to 54°F. They then began to feed on trout chow and became more active. Further growth studies will be conducted with those being raised in parallel at the New York Aquarium by Dr. Dennis Thoney.

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APPLICATIONS OF BIOTECHNOLOGY TO SHELLFISH RESEARCH

101 USES FOR THE SMALL SUBUNIT RIBOSOMAL RNA GENE: APPLICATIONS TO *HAPLOSPORIDIUM NELSONI*. **Eugene M. Burreson,*** Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062.

The small subunit ribosomal RNA (SSU rRNA) gene is a part of the rRNA transcription unit, which is present in 100s to 1000s of copies within the genome. A large part of the SSU rRNA gene sequence is well conserved across all eukaryotes, however there are also hypervariable regions that are species-specific and these areas can serve as targets for molecular probes and primers. We isolated genomic DNA from *Haplosporidium nelsoni* and amplified the SSU rDNA via the polymerase chain reaction (PCR) with eukaryotic universal primers. The gene was sequenced and two species-specific regions were identified to use as a DNA probe and PCR primers for sensitive and specific detection of *H. nelsoni*.

The DNA probe has been used in *in situ* hybridizations for *H. nelsoni* diagnosis in histological samples and the PCR primers have been used for detection of *H. nelsoni* from infected oyster tissue or hemolymph. We plan to use the probe and primers for elucidation of the *H. nelsoni* life cycle. With the use of both the probe and primers, we have determined that *H. nelsoni* was introduced to the east coast of the U.S. by importations of infected *C. gigas*. The amplification products from haplosporidian-infected *C. gigas* were sequenced using the PCR primers and, except for one transition, were identical to the *H. nelsoni* SSU rDNA sequence.

Comparison of the sequence of conserved regions of the SSU rRNA gene across taxa allows inference of phylogenetic relationships. We have sequenced this gene in several other haplosporidians. Multiple alignment and phylogenetic analysis with ciliates, dinoflagellates, and apicomplexans showed that the phylum Haplosporidia has an alveolate ancestry. Within the Haplosporidia, these analyses showed that the genus *Haplosporidium*, as presently defined, is not monophyletic.

WAITER THERE IS A BUG IN MY CLAM: A MOLECULAR ANALYSIS OF SYMBIONT TRANSMISSION IN SEVERAL MARINE BIVALVES. **S. Craig Cary,** Graduate College of Marine Studies, University of Delaware, Lewes, DE 19958.

The power of molecular diagnostic tools has recently been applied to certain areas of shellfish research. Nucleic acid probe technology based on 16S rRNA sequences provided the high resolution necessary to identify the presence and location of extremely low numbers of bacteria in eukaryotic cells. Ribosomal genes offer ideal targets for hybridization probes because. a) they are present in multiple copies, b) they offer a range of variable regions, including some regions that are invariant among all living organisms, and others that are unique to particular organisms or related groups of organisms. In addition, actively growing bacterial cells may contain as many as 104 ribosomes each a potential hybridization target for a complementary oligodeoxynucleotide probe. Oligodeoxynucleotide probes directed against rRNA targets are rapidly becoming one of the most powerful techniques in microbial ecology enabling the detection of ribosomal genes at very low copy number from a mixed natural population at defined levels of phylogenetic specificity. These probes can be utilized in both standard detection analysis using the Polymerase Chain Reaction or for the actual localization of the bacteria within the host organism using highly sensitive in situ hybridization protocols. Collectively these methods provide highly sensitive detection diagnostic capabilities. Recently these methods have been applied to determine the symbiont transmission mechanism in several bivalves inhabiting deep sea hydrothermal vents and cold seep habitats. The resolution has provided insight into the mechanism of symbiont transmission and constraints on larval dispersal and settlement.

PRODUCTION OF TRANSGENIC DWARF SURFCLAMS, *MULINIA LATERALIS*, WITH PANTROPIC RETROVIRAL VECTORS. **Thomas T. Chen,*** Biotechnology Center, Department of Molecular and Cell Biology, University of Connecticut, Stoors, CT; **Jenn-Kan Lu,** Department of Biological Sciences, University of Maryland at Baltimore County, Baltimore, MD; **Standish K. Allen,** Haskin Shellfish Research Laboratory, Institute of Marine and Coastal Sciences, Rutgers University, Port Norris, NJ; **Tomoyo Matsubara,** Department of Pediatrics, UCSD School of Medicine, La Jolla, CA; **Jane C. Burns,** Department of Pediatrics, UCSD School of Medicine, La Jolla,CA.

A pantropic pseudotyped retroviral vector containing the envelope protein of vesicular stomatitis virus was used as a gene transfer vector in the dwarf surfclam, *Mulinia lateralis*. These pantropic retroviral vectors have an extremely broad host cell range and can infect many non-mammalian species. Newly fertilized dwarf surfclam eggs were electroporated at 700 volts in the presence of 1×10^4 cfu of pantropic pseudotyped retroviral particles. Infection was well tolerated and did not affect the survival rate of the embryos. Gametes collected from P_1 presumptive transgenic animals were analyzed for the presence of provirus by PCR, and in different experiments 13–33% of the gamete pools were positive for the transgene. Dot blot hybridization of DNA samples from the F_1 offspring of two different crosses between infected P_1 and wild type individuals revealed that 28% and 31% of F_1 offspring were transgenic, respectively. Southern blot analysis of DNA isolated from PCR-positive F_1 animals confirmed integration of a single copy of the provirus into the host genome. Thus, the germlines of these two P_1 transgenic animals were mosaic for the transgene. Expression of b-galactosidase encoded by the provirus was detected in transgenic but not control surfclam embryos. Pantropic

pseudotyped retroviral vectors provide a useful method for the stable introduction of foreign genetic information into surfclams and may facilitate the introduction of desirable genetic traits into commercially important shellfish and crustaceans.

DEVELOPMENT OF CONTINUOUS MARINE INVERTEBRATE CELL LINES. **Rosemary Jagus,*** Center of Marine Biotechnology, Suite 236 Columbus Center, 701 E. Pratt Street, Baltimore, MD 21202.

We are attempting to develop continuous marine invertebrate cell lines by transfecting sea urchin disrupted gastrula cells with oncogenes of various types. We propose to use the broad host range pseudotyped retroviral vector that has been successfully used in finfish and the dwarf surf clam. Our overall strategy is to transform disrupted gastrula cells with a range of oncogenes such as those for *myc*, *ras*, SV40 large T-antigen, nonfunctional PKR, and eIF4E, using the pseudotype retroviral vector, pLGRNL. In this Moloney murine leukemia virus-based vector, the virus coat protein is replaced by the VSV-G protein giving rise to the vector's characteristic wide host range. The vector also contains the neomycin phosphotransferase gene to allow selection of stable transformants. Our immediate goals are: a) to construct selectable pseudotype vectors expressing growth promoting genes under the control of a suitable sea urchin promoter; b) demonstrate expression in short term disrupted gastrula cultures; c) select neomycin resistant colonies.

Cells of the sea urchin, *Strongylocentrotus purpuratus*, will be used to demonstrate the approach since there is a relative abundance of information on sea urchin molecular genetics, including the characterization of strong promoter elements. The approach will provide a strategy for the development of immortalized cell lines from other economically and therapeutically important marine invertebrates. We have worked initially on Phase a). Because genes in this vector will integrate into the host DNA, and because we will be transforming with oncogenes, there is a potential human health risk. Consequently, we are first assessing the ability of several sea urchin promoters to support transcription in human cells (HeLa). We will begin our studies with promoters that function well in sea urchin cells but poorly in human cells. We have engineered sea urchin promoters into the mammalian expression pCDNA1/neo containing the reporter gene chloramphenical aminoacyl transferase (CAT). Of the sea urchin promoters available, we have chosen those that are expressed uniformly with respect to cell type and which exhibit activity well into gastrulation. These include SpHE, the hatching enzyme promoter, Spec-1, and subregions of the CyIIIa (actin) promoter. Currently, the abilities of these promoters is being assessed for transient expression in HeLa cells. Our next stage will be to determine the stability of the pseudotype vector to a range of sea water concentrations. This [illegible]e does not require sea urchin cells, since we can assess virus viability in HeLa cells.

BIOTECHNOLOGY IS A BUSINESS, NOT A SCIENCE. **Richard K. Koehn,** University of Utah, Salt Lake City, UT 84112.

While scientific research is the fuel of technological innovation and economic growth, the commercial exploitation of scientific discovery is neither straight forward nor simple. Biotechnology is an industry with high economic volatility reflecting the rapidly changing fortunes of a new industry in both rapid growth and consolidation. The climate for investment in biotechnology has been described as having moved from passion to panic.

If agricultural biotechnology is a step-child of medical biotechnology, then marine biotechnology is the orphan. Marine research has the potential to be of economic value, but to date marine biotechnology has been characterized more by poetic sweeps than by concrete economic growth. The reasons for this will be discussed.

Most marine research is performed in universities or other nonprofit organizations. As such, the policies of these institutions on conflict-of-interest, equity position, royalty sharing, management of intellectual property, etc., are critical to the successful commercialization of marine science. How these relate to the financial, legal, managerial, and scientific aspects of marine biotechnology will be discussed.

If marine research is to fuel significant growth of marine biotechnology, the research must become much more market driven. Biotechnology is not just molecular biology research, but science in service of technological innovation and commerce.

BIOTECHNOLOGY AND SHELLFISH: APPLYING NEW SCIENTIFIC TECHNIQUES TO AN OLD ENVIRONMENT. **Kennedy T. Paynter,*** Department of Zoology, University of Maryland, College Park, MD 20742.

The advent of molecular biology, the manipulation of RNA, DNA and associated molecules, has revolutionized the scientific world in the last decade. It is now possible to add specific genes to the genome of almost any species, to detect a single abnormal cell among tens of thousands of normal ones, and to determine genetic identity with great accuracy. Needless to say, the medical community wasted no time in embracing these advances and applied them to detect and/or cure a variety of diseases. At present, these techniques are also being applied in the agriculture industry to improve production and the quality of farmed products. While these advances will likely result in direct improvement of industrial output, the application of molecular tools to conduct research on important ecological or environmental issues has been more slow.

There are a few examples of molecular techniques being employed in marine research 10 to 15 years ago but, for the most part, molecular techniques have been commonly employed by marine scientists only in the last few years. However, the promise of molecular techniques to marine research is great. For aquaculture, the construction of fast-growing transgenic fish and shellfish might

vastly improve production rates. Comparison of various segments of DNA among aquatic species could improve detection capabilities for important pathogens by several orders of magnitude. This might allow for the detection of unknown life cycle stages of certain parasites, clarify relationships between disparate groups of pathogens and establishing phylogenetic relationships between pathogens. Most importantly these tools will enable us to better understand the biology and ecology of marine and estuarine species.

A BACTERIOPHAGE P1 HIGH MOLECULAR WEIGHT GENOMIC LIBRARY FROM THE OYSTER, *CRASSOSTREA VIRGINICA*. **James C. Pierce**,* Department of Biological Sciences, Philadelphia College of Pharmacy and Science, Philadelphia, PA 19104–4495.

The bacteriophage P1 cloning system is able to generate bacterial clones with insert sizes up to 100 kb. These clones have a number of unique features including, positive selection, single copy plasmid replication and a inducible high copy number replicon. Rare cutting restriction sites and T7 and Sp6 promoters border the cloning site and facilitate analysis and characterization of P1 clones. My laboratory is currently constructing a P1 genomic library from the eastern oyster, *Crassostrea virginica*. To minimize DNA contamination from algal and microbial cells we have obtained relatively pure sperm cells from male gonads by direct puncture with a capillary tube. High molecular weight (HMW) genomic DNA in the megabase (Mb) range was isolated by cell lysis and purified by sucrose gradient centrifugation. Genomic inserts were generated by Sau3A1 partial digest followed by sucrose gradient fractionation. P1 clones containing HMW oyster genomic inserts are being constructed using a two stage, in vitro P1 packaging reaction. Our goal is to construct a genomic library that contains 20,000 individual P1 clones with an average insert size of about 80 kb. Since the *C. virginica* haploid genome is estimated to be 3.8×10^8 bp, a genomic library of this size will give a library coverage of approximately 4 fold. Specific clones are isolated using PCR screening of clone pools and by a nonradioactive colony purification protocol. A P1 library for *C. virginica* will significantly improve our ability to perform genetic physical mapping studies and in the isolation and study of specific genomic regions. P1 clones have proven to be excellent substrates for genome targeting in mammalian systems and may prove useful in the genetic engineering of the oyster genome. Construction of transgenic oysters which have novel and commercially important traits will be facilitated by access to a P1 oyster library.

GENETIC ENGINEERING ABALONE: GENE TRANSFER AND PLOIDY MANIPULATION. **Dennis A. Powers**,* **Vicky Kirby**, and **Marta Gomez-Ghiarri**, Hopkins Marine Station, Stanford University, Pacific Grove, CA.

We are genetically engineering abalone with enhanced growth by gene transfer and ploidy manipulation. We have: (i) developed efficient methods for simultaneously transferring genes into thousands of abalone eggs, (ii) cloned the first abalone promoter, (iii) coupled various promoter to reporter genes and the coho salmon growth hormone, (iv) transferred these constructs into abalone, (v) determined integration and expression, and (vi) developed triploid abalone with enhanced growth. The abalone β-actin promoter was cloned and sequenced. This promoter and others were coupled to luciferase, β-galactosidase and coho salmon growth hormone. These recombinant plasmids were linearized and introduced in fertilized eggs of the red abalone (*Haliotis rufescens*) by electroporation. When the conditions were optimized, the majority of the embryos became transgenic and retained the constructs for more than a year. Southern hybridization analyses suggested head-to-tail concantermers integrated in the genome and these genes were expressed. Since it takes several years to reach sexual maturity, the transmission of these transgenes to the next generation is being evaluated. In addition to our transgenic work, we have also used pressure, temperature and chemical treatment to manipulate the ploidy of abalone. Although these treatments had different efficiencies and the results varied depending upon time after fertilization, we have successfully generated triploid abalone that grow significantly faster than their diploid counterparts. We are using triploid manipulation of transgenic abalone to create unique strains of abalone for aquaculture purposes.

DEVELOPMENT OF MOLECULAR MARKERS FOR POPULATION GENETIC ANALYSIS OF *PERKINSUS MARINUS*. **Kimberly S. Reece*** and **John E. Graves**, Virginia Institute of Marine Science, College of Willima & Mary, Gloucester Point, VA 23062; **David Bushek**, Baruch Marine Field Laboratory, University of South Carolina, Georgetown, SC 29440.

We are investigating the population structure of the oyster pathogen *Perkinsus marinus* to help identify mechanisms of dispersal and migration. Infected oysters collected from Connecticut to Texas have been used to produce *in vitro* cultures of the parasite for genetic analysis. We have constructed a genomic library, identified regions of the DNA which show intra-specific variability, and designed primers for these regions. Using universal primers for polymerase chain reactions (PCR), fragments of *P. marinus* actin, 18S rRNA, the internal transcribed spacer (ITS) region of rRNAs, ATPase 6 (mitochondrial) and serine protease genes were amplified and cloned. Amplified gene fragments were labeled with digoxigenin and used as probes to screen the genomic library for lambda phage clones containing the genes and flanking sequences (avg. insert = 18 kbp). Phage clones containing the genes were isolated and the inserts containing *P. marinus* DNA were mapped with restriction enzymes. Regions flanking the genes were subcloned into plasmid vectors, sequenced, and non-coding regions identified by computer searches of the DNA sequence for coding regions. Based upon these results, PCR primers were designed to amplify 1–3 kbp non-coding fragments of the flanking regions. DNA isolated from geographically distinct *P. marinus* cultures was amplified using the newly designed primers and surveyed with a suite of restriction endonucleases to assess fragment length poly-

morphisms between isolate cultures (RFLP analysis). Genetic variation has been observed at several loci. Many of the fragments are also being sequenced to assess variation at the nucleotide level. As more isolates become available, RFLP and sequence analysis will continue and both phenetic and cladistic analyses will be employed to determine relationships among isolates from different areas.

PROTEIN-CARBOHYDRATE INTERACTIONS FOR SELF/NON-SELF RECOGNITION IN SHELLFISH. **Gerardo R. Vasta,*** The Center of Marine Biotechnology, University of Maryland Biotechnology Institute, Columbus Center, 701 E. Pratt St., Baltimore, MD 21202.

Viral, bacterial, fungal and protozoan epizootic diseases are recognized as significant detrimental factors for the successful exploitation of natural and cultivated stocks of marine shellfish, such as crabs, shrimps, oysters and clams. Because shellfish do not synthesize immunoglobulin antibodies, those established methods for the control of disease in vertebrates, such as rational vaccination programs, can not be applied to molluscan and crustacean species. Therefore, the elucidation of their internal defense mechanisms in shellfish is of utmost importance. In the long term, the identification and thorough characterization of structure and function of the recognition/effector gene products and the detailed understanding of how their expression is regulated, will enable us to enhance disease resistance through their adequate stimulation and to further apply transgenic approaches to the development of disease-resistant shellfish species. The participation of certain carbohydrate-binding proteins (lectins) in non-self recognition/defense mechanisms is supported by ample evidence obtained in a variety of animal models, including man. Within the invertebrates, at least three major lectin categories can be identified: one group includes lectins that show significant homology to membrane-integrated or soluble C-type vertebrate lectins, such as homologues of the mannose-binding receptor. The second includes β-galactosyl-specific lectins homologous to the S-type vertebrate lectins (Galectins). The third group is constituted by lectins that show homology to acute phase reactants from vertebrates, such as C-reactive protein and serum amyloid P. The multiplicity in lectin specificity and the nature and distribution of the carbohydrate moieties recognized suggest that serum lectins may contribute as a carbohydrate-based recognition system for potentially pathogenic microorganisms. One approach for the application of transgenic technology to disease resistance may be at the level of promoting the efficient recognition of the pathogen in the organism in question. Therefore, lectins may be considered as suitable target gene(s) for transgenesis since upon recognition of the pathogen, the complex lectin/pathogen would be phagocytosed and trigger the organism's natural defense mechanisms. *In vitro* manipulation of the lectin specificity aimed at the recognition of the appropriate pathogen target carbohydrate moiety, could be accomplished by site-directed mutagenesis and the modified gene incorporated into the germline of the invertebrate of interest. Clearly, an exception to this approach would include those obligate or facultative intracellular pathogens that have developed strategies for blocking or evading the host's intracellular killing mechanisms.

BIVALVE FISHERIES

A PROFILE OF THE EAST HAMPTON TOWN SHELLFISH HATCHERY AND RESEEDING PROGRAM. **John Aldred,*** Town of East Hampton, 159 Pantigo Road, East Hampton, NY 11937.

The East Hampton Town Shellfish Hatchery is located in Montauk, the easternmost hamlet on New York's Long Island. Built in 1989–90 with seed money from New York State, it is operated by the Town for the enhancement of local shellfish stocks on public bottomlands. Ten percent of yearly production is made available to the state for regional distribution.

The motivation for a facility of this kind was provided first by the closure of the local striped bass fishery in 1984 and in following years by the virtual elimination of the bay scallop from the region due to recurrent smothering algal blooms known as the brown tide. These two fisheries had historically furnished the largest earning potential for local inshore commercial fishermen.

The hatchery annually produces in the neighborhood of ten million hard clam, oyster and bay scallop seed suitable for field distribution. A seven thousand square foot former U.S. Navy warehouse on Fort Pond Bay houses static water larval and pediveliger rearing systems, a flowing water upwelling nursery, temperature controlled as well as mass culture algal systems, shop, lab, and office space. A field nursery in Napeague Harbor, Amagansett contains rafted tray and pearl net systems for final grow out to planting size. A second upwelling nursery is located in Three Mile Harbor, East Hampton to take advantage of warmer harbor waters and to provide alternatives in the eventuality of unanticipated water quality problems in one or another site.

Along with Town officials, the hatchery has also organized oyster relays from uncertified waters to provide harvest potential and participated in designating management areas to protect spawning stocks. Projects underway include an overwinter survival study of hard clam seed in different sediment types, a pilot oyster aquaculture project for local fishermen, a demonstration of bay scallop spat collection techniques for the Peconic estuary, and a comparative assessment of clam seed survival using hand and machine planting techniques.

LEASE SITE CONSIDERATIONS FOR HARD CLAM AQUACULTURE IN FLORIDA. **W. S. Arnold,* H. A. Norris,** and **M. E. Berrigan,** Florida Department of Environmental Protection, Florida Marine Research Institute, 100 Eighth Avenue S.E., St. Petersburg, FL 33701.

Hard clams of the genus *Mercenaria* support an important commercial fishery in the Indian River lagoon on the eastern coast of central Florida. That fishery has supported landings with an esti-

mated annual ex-vessel value of as much as $15 million. Unfortunately, because of the extreme variability in abundance of natural hard clam stocks in the lagoon, the annual value of the fishery fluctuates drastically although market demand remains strong.

Hard clam aquaculture provides a viable means of meeting market demand while avoiding the vagaries of natural clam supply. The hard clam aquaculture industry is burgeoning in the Indian River and is expanding statewide. It is more expedient to culture hard clams in Florida than it is in northern states because of the more rapid shell growth of Florida clams and the abundance of sites with suitable water quality in Florida. However, conflicts over resource allocation are arising between hard clam aquaculturists and the harvesters of natural clam beds and between aquaculturists and the resource managers concerned with the impact of aquaculture on the natural environment. The harvesters are concerned that lease sites, generally 5–10 acres in size, are being located in areas that support productive natural clam beds, thus denying harvesters access to those beds. Resource managers are concerned about the impact of aquaculture operations on the natural benthic assemblage, including seagrass beds, and on the aesthetics of the local environment. Potential lease sites are surveyed prior to letting, but that effort is conducted on a case-by-case basis, requires considerable effort by the lessee with no guarantee of success, and provides no coherent framework for lease allocation.

To mitigate these conflicts, we are developing Geographic Information System (GIS)-based tools to map the distribution of hard clam beds and other natural features. Information on the distribution of clams and seagrass beds, water depth, proposed lease boundaries, physical features, and any other available and pertinent information is fed into GIS map overlays. These map overlays can then be plotted and presented to all concerned user groups for consideration. The maps can also be applied *a priori* to guide potential culturists in the selection of a suitable site, and to guide the State of Florida in the macroscale development of hard clam aquaculture in Florida.

RELATIVE EFFICIENCY OF 3.5″ DREDGE RINGS IN THE OFFSHORE SEA SCALLOP FISHERY. **Jeffrey C. Brust,* William D. DuPaul,** and **James E. Kirkley,** Virginia Institute of Marine Science, College of William and Mary, Gloucester Point VA 23062.

The use of an average meat count restriction in the original sea scallop (*Placopecten magellanicus*) fishery management plan (SSFMP) was not effective in protecting small scallops from being harvested. In March 1994, Amendment #4 to the SSFMP was implemented. The major focus of the amendment was to decrease overall effort in the fishery. Age at first capture was to be controlled by a mandatory increase in the ring size used in the gear. Initially, ring size increased from 3.0″ to 3.25″ for 1994 and 1995. In 1996, the ring size increased to 3.5″. This study used paired tows of the standard, 3.25″ ring and experimental, 3.5″ ring dredges on four commercial trips taken over a 10 month period during 1994 and 1995. The recruitment of a very large year class (1990) in late 1993 and early 1994 made it possible to assess the performance of the 3.5″ ring dredge on a single year class as the scallops grew and more fully recruited to the gear. Our data indicate that the larger ring size will decrease the efficiency of the scallop dredge. This will effectively delay recruitment of an incoming year class by as much as one year relative to the 3.25″ ring dredge. The effects of this delayed entry have been evaluated relative to yield per recruit (YPR), spawning stock biomass (SSB), and age class structure of the resource.

SHELLFISH MANAGEMENT PROGRAM—CLAM PRODUCTION. **T. Jeffrey Davidson,** Atlantic Veterinary College, University of P.E.I., 550 University Avenue, Charlottetown, PEI, C1A 4P3, Canada; **Gef Flimlin,** New Jersey Sea Grant Marine Advisory Service, 1623 Whitesville Road, Toms River, NJ 08755.

In support of the hard shelled clam (*Mercenaria mercenaria*) aquaculture industry in New Jersey, Shellfish Management Program—Clam Production has been developed. This computer-based program will enable aquaculture producers to more effectively manage their nursery and grow out plots in their leases. Parameters affecting productivity will be identified and evaluated allowing the producer to select those factors yielding the best economic benefits. Some of these parameters include: seed source and size, screen and bottom dynamics, water quality indicators (temperature, salinity, etc.) and relevant management factors.

The inventory section will provide the producer with estimates of the number of animals in a plot, their movement within the lease, and mortality figures.

Although Shellfish Management Program—Clam Production is a stand alone on farm program, data assimilated from a number of producers could assist in identifying and evaluating factors affecting the New Jersey clam aquaculture industry as a whole.

LARVAL AND JUVENILE GROWTH OF STIMPSON'S SURFCLAM—A NEW CANDIDATE SPECIES FOR AQUACULTURE DEVELOPMENT? **Christopher V. Davis,*** Darling Marine Center, University of Maine, Walpole, ME 04573; **Sandra E. Shumway,** Southampton College, Long Island University, Southampton, NY 11968.

Stimpson's or Arctic surfclam (*Mactromeris polynyma*, Stimpson 1860) is a cold water circumboreal species, distinguished from other surfclams by its purple colored foot, siphon and mantle edge which turn brilliant orange-red when cooked. Increased demand for wild surfclams to supply the Japanese sushi market prompted an investigation of the aquaculture potential for rearing this species in Maine waters.

On four occasions over two years, adult clams were naturally conditioned and induced to spawn using temperature shock, sperm suspension and flowing seawater. Larvae were reared in either 40

or 400 liter conical tanks and fed daily a mixture of cultured microalgae. Prodissoconch I larvae developed in 24 or 96 hours depending on culture temperature (15.0 and 8.5°C respectively). Metamorphosis occurred in 24 to 42 days at 15.0 and 10.0°C respectively. Size of metamorphosing pediveligers varied from 320 μm (SD = 22) shell length at 15.0°C to 271 μm (SD = 26) shell length at 10.0°C. Juvenile growth was strongly influenced by substrate. Individuals reared on Nitex plastic screening in ambient sea water grew significantly slower than those reared in a silty/sand sediment. Growth of juveniles occurred year round, varying from 0.009 mm · day^{-1} in the winter months to 0.102 mm · day^{-1} in late spring. Clams grew to 12.5 mm shell length, 0.29 g. live weight and 23.5 mm, 2.86 g. at one and two years respectively. After 29 months of growth, clams measured 31.4 mm in shell length and 4.93 g. live wt. These growth rates are approximately twice those seen in populations harvested from the wild and may be further optimized through improved culture methods. This is the first report on the culture of Stimpson's surfclam beyond the larval stage. Given the rapid growth rates we observed, we believe this species may be amenable to aquaculture development in Maine, the Canadian Maritimes and the Pacific Northwest.

A COMPARISON OF THE EFFECTS OF THREE DIFFERENT HABITAT MODIFICATIONS ON INTERTIDAL CLAM POPULATIONS IN PACIFIC NORTHWEST COASTAL ESTUARIES. **Brett R. Dumbauld*** and **Martin Peoples,** Washington State Department of Fish and Wildlife, P.O. Box 190, Ocean Park, WA 98640; **David A. Armstrong,** School of Fisheries, University of Washington, Seattle, WA 98195; **Stephen G. Ratchford,** North Carolina State University, Raleigh, NC 27650.

A review of several field studies on the influence of epibenthic structural modifications to intertidal habitat in Pacific Northwest coastal estuaries suggests that several species of clams display similar responses to each. Three contemporary habitat modification issues are: invasion of the introduced cordgrass *Spartina alterniflora*, the distribution of large quantities of oyster shell on intertidal tideflats to enhance the population of juvenile Dungeness crab, *Cancer magister*, and application of the pesticide carbaryl to remove thalassinid shrimp prior to oyster cultivation. Settlement measured as initial density of *Mya arenaria*, *Tapes japonica* and *Macoma* spp. has been shown to be little affected by the physical structure of *Spartina* and shell with the exception of the large barrier presented by *Spartina* shoots in the later summer and fall. Subsequent survival of small clams however, is negatively influenced by the presence of epibenthic shell, which attracts predators such as juvenile Dungeness crab *Cancer magister*. Although tide height may preclude Dungeness crab from utilizing *Spartina*, similar declines in clam density were observed within cordgrass zones. After clams reach a size threshold, survival is less affected, but growth may be influenced depending on size of the clam, size of the structure, and hydrodynamic scaling factors.

NATURAL AND EX-VESSEL MOISTURE CONTENT OF SEA SCALLOPS (*PLACOPECTEN MAGELLANICUS*). **William D. DuPaul,*** **Robert A. Fisher,** and **James E. Kirkley,** Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062.

Most of the sea scallop (*Placopecten magellanicus*) fishery is conducted on the continental shelf where scallops are shucked at sea. The adductor muscle, or scallop meats, are landed as the commercial product form. Scallop meats are generally stored in linen bags packed in ice for the duration of the fishing trip and are further processed at shore side facilities. Exposure of the scallop meats to ice melt or fresh water during storage or processing causes an increase in moisture content with concomitant increases in weight. During 1992, the U.S. Food and Drug Administration (FDA) became increasingly concerned with vessel handling and on-shore processing practices that substantially increased the moisture content of scallop meats. Subsequently, FDA ruled that a 'natural' scallop could not have a moisture content that exceeded 80% by weight. In 1995, Canada established a moisture standard not to exceed 81%. France has defined a moisture standard for scallops based on a moisture/protein ratio not to exceed 4.99:1.

Prior to the present study, data on the natural moisture content of sea scallops in the northwest Atlantic Ocean has been limited and inadequate to establish regulatory provisions. The goal of this study was to assess natural and ex-vessel moisture content over an extended period of time (1990–1995) to account for latitudinal, depth, seasonal and individual variability. This more quantitative and verifiable study establishes statistically generated ranges of moisture contents and moisture/protein ratios and is discussed in view of input and output based regulatory strategies, international trade and implications for the industry.

CHANGES IN HARD CLAM, *MERCENARIA MERCENARIA*, FISHERIES OF THE MID-ATLANTIC REGION IN RESPONSE TO STOCK FLUCTUATIONS. **Gef Flimlin,* NJ Sea Grant Marine Advisory Service, 1623 Whitesville Rd., Toms River, NJ 08755.**

The processes by which the Hard Clam, or Northern Quahog, *Mercenaria mercenaria*, are being produced in the Mid-Atlantic region have changed dramatically in the past twenty years. Wild harvest in approved waters is often replaced by relay programs, shellfish depuration, and clam aquaculture. These activities are the response by the commercial industry to stock reductions in approved water. Since participation by the commercial sector is linked to landings and harvest price, the number of clammers fluctuates also. Reasons for changes in relative abundance in coastal areas are speculative, but increased human populations, use of estuarine waters for cooling of electric generating plants, increased use of copper in dock building materials and antifouling paint, and the rise of outboard engine use are most often identified by the commercial industry as the most probable causes. Even in

areas of intense clam aquaculture, there are no significant examples of new clam sets.

Since there are no obvious reasons for stock reductions, it is imperative that an ad hoc group of industry members, extension personnel and shellfish researchers be established to focus effort on identifying causes of stock reductions and ways to improve them, whether through stock enhancement, public aquaculture, or habitat improvement.

POLYCULTURE OF SEA SCALLOP SUSPENDED FROM SALMON CAGES. **Alexander Gryska,** New England Fisheries Development Association, 451 D Street, Boston, MA 02210; **G. Jay Parsons,*** Aquatic Industries Ltd., P.O. Box 294, St. Andrews, NB E0G 2X0; **Sandra E. Shumway,** Natural Science Division, Southampton College, L.I.U., Southampton, NY 11968; **Kristin Geib,** P.O. Box 103, West Boothbay Harbor, ME 04575; **Ian Emery,** Snug Harbor Scallop Farm, P.O. Box 17200, Pembrooke, ME 04666; **Sue Kuenstner,** New England Fisheries Development Association, 451 D Street, Boston, MA 02210.

Commercial culture of the sea scallop, *Placopecten magellanicus,* is an expanding industry in Atlantic Canada and New England. In an experiment designed to examine the commercial feasibility of polyculturing scallops with Atlantic salmon, we measured the growth and survival of sea scallops grown in suspension on two salmon aquaculture sites in northeastern Maine. One site was in Johnson Cove, Passamaquoddy Bay and the other was located off Treats Island, near Lubec. Sea scallop spat (11 months of age and 10.2 mm shell height) were grown in standard pearl nets and were deployed on drop lines containing ten nets in August 1994. One drop line of ten nets was sampled about every four months and scallops were counted, measured for shell height, and tissues weights determined. Water samples for chlorophyll and scallop tissue samples for PSP phycotoxin content were also obtained. Scallop growth at the two sites was 35.2 and 38.5 mm shell height after four months and survival was >90%. After one year, shell heights were about 49 and 57 mm, wet adductor muscle weights were 2.8 and 4.5 g, and growth rates were 0.11 and 0.13 mm per day. These growth rates were comparable to sea scallops cultured in Atlantic Canada. Reduced rates of survival were found during the latter part of the experiment and were attributable, in part, to heavy fouling by blue mussels. The potential for supplemental income, diversification of the salmon aquaculture industry, and logistics of culturing scallop in conjunction with salmon will be discussed.

THE 1995 STATUS OF THE SHELLFISHERIES FOR THE NORTHERN QUAHOG, *MERCENARIA MERCENARIA* (L.) IN NEW ENGLAND. **Michael A. Rice,** Dept. of Fisheries, Animal and Veterinary Science, University of Rhode Island, Kingston, RI 02881.

Fisheries for northern quahogs in the southern New England region have been in existence since pre-colonial times, and as recently as the middle 1980s the major fishery market source area. Since the late 1980s, there have been declining catches in New England and increasing market supplies of quahogs from the Middle Atlantic and Southern States due to relay, depuration, and aquaculture programs. Commercial quahog landings in the three major producing states in New England, Massachusetts, Connecticut and Rhode Island were 34,659 bu (188 metric tons meat weight), 187,240 bu (1017 mt) and 134,417 bu (730 mt) respectively in 1994. Since 1990, landings of quahogs in Connecticut have increased largely due to introduction of containerized and bag relaying of product from conditional pollution closure areas. There has been a decline in the landings in Massachusetts and Rhode Island over the same period of time. The condition of quahog stocks in Massachusetts and Rhode Island are not particularly poor, as catch per unit effort has been steady. Lowered catches in these states has been largely driven by the economics of the fishery. The low capitalization required by the quahog fishery (mostly bullrakes) allows fishermen to leave the profession as quickly as they can enter. For example during the mid-1980s when quahog prices were relatively attractive, there were about 800 full time shellfishermen in Rhode Island, but now there are only about 200. These remaining shellfishermen are individually catching as many quahogs as they had previously, but their overall income is down. It is recommended that greater attention to cooperative marketing by the fishermen can lead to greater economic returns.

DEVELOPMENT OF THE HARD CLAM, *MERCENARIA MERCENARIA*, FISHERY IN THE SOUTH ATLANTIC REGION. **Jack M. Whetstone,*** Marine Extension Program, Clemson University, P.O. Drawer 1100, Georgetown, SC 29442-1100; **William D. Anderson,** SCDNR, P.O. Box 12559, Charleston, SC 29442; **Philip S. Kemp, Jr.,** UNC Sea Grant College Program, P.O. Box 3146, Atlantic Beach, NC 28512; **Randal L. Walker,** University of Georgia Marine Extension Service, 20 Ocean Science Circle, Savannah, GA 31411.

The hard clam, *Mercenaria mercenaria,* fishery in the South Atlantic has a historical background to colonial times. Landings remained relatively low until the late 1970's when the commercial fishery adopted mechanized harvesting, relaying and depuration techniques. The landings dramatically increased in the late 1970's but have stabilized since the early 1980's.

Commercial landings for 1994 in the South Atlantic region were: North Carolina–607 metric tons (mt) valued at $6,756,631; South Carolina–133 mt valued at $1,068,260; and Georgia–4.9 mt valued at $73,158.

Variation in landings between states can best be attributed to differences in gear and leasing regulations. Any increases in landings in the future will depend upon the development of hard clam aquaculture in the region, since the fishery appears to be at its maximum sustainable yield levels on open shellfish grounds. Since the late 1980's hard clam aquaculture has received increased interest in the South Atlantic region. Hard clam aquaculture has developed to a varying degree within each state due to the regulation of leases and seed importation requirements.

BIVALVE SEED PRODUCTION

A COST-BENEFIT EVALUATION OF HATCHERY-PRODUCED OYSTERS IN MARYLAND. **Mark L. Homer, Robert Bussell,** and **Chris Judy,** Maryland Department of Natural Resources, Piney Point Aquaculture Center, P.O. Box 150, Piney Point, MD 20674.

In 1994, a pilot program was developed for the Maryland Department of Natural Resources' hatchery located at Piney Point, MD. The goal of the program was to determine gross and net operating production costs and compare these with the State's Natural Seed Repletion Program. The hatchery production timeline began in March, with the acquisition and conditioning of brood stock, continued through spawning and larval development larval setting, and ended with the planting of two lots of spat on shell, one in September and the other in November. All costs, labor, materials, and utilities, were carefully tracked with a total of $37,500 spent on production, site preparation, and planting. Gross production cost, including site preparation and planting, was estimated to be $0.0056 per spat.

In September 1994, 3.4 million spat on shell (550 bushels) were transported to a 1 acre site, previously planted with 5,000 bushels of fresh shell, in Breton Bay (a tributary of the Potomac River). In mid-November 1994, 3.2 million spat on shell (350 bushels) were planted on a 2 acre portion of a natural oyster bar in the upper Wicomico River (a tributary of the Potomac). Monitoring of these oysters for growth and survivorship began immediately after planting and has continued on a bimonthly basis. Initial mortality was extremely high, over 30%, attributed primarily to damage associated with transporting the oysters. As of September 1995, survivorship of the spat planted on the Breton Bay site stood at 8.6% and on the Wicomico River site 13.5%. The current net cost of the combined plantings stands at $0.049 per spat with these oysters needing about 9 more months to attain market size.

The hatchery results were compared with cost and production estimates from the State's Natural Seed Repletion Program. Costs were estimated from experimental plots located near the mouth of the Chester River. Gross production cost was similar to the hatchery value, $0.0058 per spat, even though counts were unusually low for the natural seed that were moved to this site in November 1994. As of March 1995, survivorship exceeded 93%, giving a net production cost of $0.0063 per spat. This site will be surveyed again in November 1995.

HATCHERY AND FIELD CULTURE TECHNIQUES FOR THE GIANT SEA SCALLOP *PLACOPECTEN MAGELLANICUS*. **Richard C. Karney,*** Martha's Vineyard Shellfish Group, Inc., Oak Bluffs, MA 02557; **Frank A. Dutra,** Nantucket Education and Research Foundation, Nantucket, MA 02554; **David Dutra** and **Judy Dutra,** Truro Aquaculture Project, North Truro, MA 02652.

Under funding from the National Marine Fisheries Service, Fishing Industry Grants Program, the Martha's Vineyard Shellfish Group adapted hatchery culture methods for bay scallops to the successful culture of the giant sea scallop. Field collected broodstock were sufficiently ripe in early March (sea water temperature, 4–5°C) to spawn just over seven million eggs. The fertilized eggs were transferred to a 400 liter larval conical, with one micron filtered, aerated, sea water at 12C. After 48 hours, the conical was drained, and about three million scallops (ciliated blastulae and trochophores) were recovered and resuspended. Straight hinge larvae were not observed until the second drain down on Day 4. Larval culture protocol throughout the almost 40 day larval period included a daily feeding of *Isochrisis galbana* (T-ISO) and/or *Chaetoceros neogracili* with a drain down and sizing every other day. The larvae were cultured in three 400 liter conicals of five micron bag filtered sea water, heated to about 15C (range 13–17C). Between Days 28 and 38, 1,350,000 pediveliger larvae (about 250 microns) were moved to downweller sieves. The first fully set juvenile was observed on Day 32. Set scallops were cultured on downweller sieves (130–300 microns) with a flow of bag filtered water (10–50 microns) at sea water temperatures of 8–16C. By June 8 (Day 90) the largest seed measured 2 mm and were moved to 1 mm mesh Korean spat bags in a cage anchored off the shellfish hatchery. By July 3 a total of 519,000 2 mm seed were successfully transferred to the inshore field culture systems.

All of several potential off-shore growers were frustrated in their attempts to secure proper permits from the regulatory agencies. Over 80 percent of the 519,000 seed scallops were lost during the month of July when water temperatures reached 22C and regulatory delays prevented transfer of the seed to cold water growout sites. At the end of July, the remaining 90,000 seed were finally permitted to be moved to the deep water (65′) site of the Truro Aquaculture Project in Cape Cod Bay. The seed were stocked in 130 2.5 mm mesh pearl nets (600 seed/net) and ten 18″ by 24″ ⅛″ mesh plastic bags (1,200 seed/bag), which were set into 75 tiers in five vinyl-coated, wire bottom cages. On September 29, after two months on the Cape Cod site, 66 percent of the seed scallops (X = 16 mm) were still alive and further thinned. The seed was sampled again on November 10 and averaged 22.4 mm.

HOW IMPORTANT IS THE TIME OF TRANSPLANT IN THE SUCCESS OF OYSTER (RELAY) FARMING? **Eric Powell*** and **Susan Ford,** Haskin Shellfish Lab., Rutgers Univ., Port Norris, NJ 08349; **John Klinch** and **Eileen Hofmann,** CCPO, Old Dominion Univ., Norfolk, VA 23529.

The oyster industry in New Jersey transplants oysters from seed beds to leases and then later harvests them for sale. During their time on the leased grounds, oysters grow to market-size, but also suffer increased mortality from disease. At one time, oysters were left on the leased grounds for more than one year. Recently, losses to disease have forced the industry to rely on oysters kept on leases for only a few months. Normally, transplant occurs in May/early June and harvest from September to December. Poor survival over the summer led to the recommendation that transplanting occur one month earlier, in April, to permit harvest before disease mor-

tality becomes significant. Results of a one year trial by the industry suggest an improved fall harvest. The earlier transplant was likely successful because the spring bloom is stronger over the leased grounds than over the seed beds. The degree to which the spring bloom can be advantageously used may be crucial.

We used an oyster population dynamics model to investigate the timing of transplant. Simulations were run for transplants in November, January, March, April, and May. The simulations agree with observation in suggesting that an increased harvest results from transplanting oysters in April rather than May/June. The simulations also indicate that earlier transplants are even more advantageous. A November transplant nearly doubles the available harvest. A March transplant is only moderately less advantageous than November. In both cases oysters take advantage of the majority of the spring bloom and this increased growth in the spring increases the abundance of market-size oysters in the spring and fall. Mortality rates from *P. marinus* decline, but not dramatically. Thus the simulations suggest that the principle effect of a change in transplanting time is to change the abundance of market-size oysters prior to the initiation of disease mortality in July, and, thus, at the same mortality rate, more oysters are available for harvest in the fall.

A POSSIBLE OPTION FOR ENHANCEMENT OF THE WILD FISHERY FOR THE SEA SCALLOP, *PLACOPECTEN MAGELLANICUS*. **Shawn M. C. Robinson,* Jim D. Martin,** and **Ross A. Chandler,** St. Andrews Biological Station, Dept. Fisheries and Oceans, St. Andrews, New Brunswick, Canada, E0G 2X0; **Don Bishop,** Fukui North America, P.O. Box 119, Island View Drive, Golden Lake, Ontario, Canada, K0J 1X0.

To date the wild scallop fishery in eastern Canada has relied on the natural cycles of recruitment in the various beds of sea scallops to sustain their fishing activity. As with other populations of scallops around the world, the sea scallop is subject to variable recruitment rates which are most likely due to biological and physical factors in the larval and early juvenile stages. With increasing fishing pressure on the scallop stocks, the industry is beginning to look at methods of stabilising and enhancing their production. The goal of our research was to test the feasibility of enhancing the recruitment rate of scallops to the bottom by collecting scallop larvae using a suitable substrate for settlement which would also allow the resulting spat to detach and settle to the bottom at a later date.

We deployed replicates of five different types of Biocord (resembling a fuzzy type of rope made for biological filters) in September 1995 using divers and monitored the settlement and survival of the early juveniles until early December. Results indicated all five substrates were attractive as a settlement substrate although there were differences between the different types. Settlement densities ranged from 100 to 400 spat/m of biocord. Two waves of settlement were also observed.

SELECTIVITY AND ECONOMIC ANALYSIS OF DIFFERENT CULTCH MATERIALS FOR OYSTER SETTING IN HACKBERRY BAY, LA. **Mark Schexnayder, Randall Pausina, Ron Dugas,** and **David Lavergne.**

Setting rates of eastern oyster, *Crassostrea virginica* (Gmelin, 1791), on six kinds of cultch, were compared from data collected on the "Public Seed Ground" in Hackberry Bay and from a tray experiment in southern Barataria Bay, LA. Crushed concrete, shucked oyster shell, reef shell, mixed shell, Kentucky limestone, and Bahamian limestone were placed in large volumes in Hackberry Bay during August 1994. Thus producing an actual applied experimental situation. Square meter samples of these planted materials were taken in November 1994 and July 1995. On an equal volume basis, crushed concrete produced the most seed oysters; shucked shell production was a close second. In the trays, crushed concrete again produced the most seed oysters per volume. Calculation of cost per sack of seed oysters for each of the six materials indicated that crushed concrete, shucked oyster shell, and mixed shell were the most economical, while Bahamian and Kentucky limestones were the least. On silty-clay bottoms shucked oyster shell as cultch offers several advantages over crushed concrete, including more exposed surface for setting and less density, hence slower sinking rates.

CONSERVATION OF FRESHWATER MUSSELS

FACTORS INFLUENCING THE GROWTH AND SURVIVAL OF JUVENILE *VILLOSA IRIS* (BIVALVIA: UNIONIDAE) IN AN ARTIFICIAL STREAM SYSTEM. **Braven B. Beaty*** and **Richard J. Neves,** Virginia Cooperative Research Unit, Department of Fisheries and Wildlife Sciences, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061.

The propagation of freshwater mussels is being promoted to assist in the recovery of declining wild populations. This project investigated some of the properties that influence the success of a juvenile mussel rearing system. Newly transformed juvenile mussels (*Villosa iris*) were placed in a flow-through artificial stream system supplied with natural river water from the Clinch River in Carbo, VA. Juvenile mussels were held in the troughs in small containers loaded to one of two fixed depths with substrate of two size fractions; less than 120 μm and between 120 μm and 600 μm. Individual containers of juveniles were removed at intervals to assess the survival and growth of the animals. Mean survival rates were 40.8%, 17.3% and 19.1% at days 30, 74 and 98, respectively. Size, as approximate area of the valve, was 0.839 mm^2, 1.496 mm^2 and 1.313 mm^2 at days 30, 74 and 98, respectively. Little growth or mortality occurred after the day 74 sampling interval in mid-October. Below about 14°C, the juveniles ceased

growing and suffered little mortality. The effect of flow on survival and growth was determined at each time increment. Flow had no effect on survival after 30 days or on growth at any time, but a significant effect on survival after 74 and 98 days. Increased flow caused a decrease in the survival rate after 74 and 90 days (R^2 of 37.9% and 27.2%, respectively). Initial substrate size and depth had no effect on either survival or growth at any sampling time. This is likely because a layer of fine silt settled from the overlying water upon the substrate, and 75–94% of the mussels were found in this fine silt. Research results could be used to design more effective rearing systems for juvenile mussels.

DECLINE AND DECIMATION: THE EXTIRPATION OF THE UNIONID FRESHWATER BIVALVES OF NORTH AMERICA. Arthur E. Bogan, Freshwater Molluscan Research, 36 Venus Way, Sewell, NJ 08080.

North America north of Mexico historically was home to the most diverse freshwater bivalve fauna in the world with approximately 300 species. The first real attempt to assess the status of freshwater bivalves began in 1970 and by 1971, 11 freshwater bivalve taxa were presumed extinct and generally, 120 taxa were considered rare or endangered. In 1973, the Endangered Species Act was passed and the U.S. Fish and Wildlife Service began evaluating the status of species and listing some as threatened or endangered. As a result of increased interest and the federal listing of species, various states began listing freshwater bivalves as locally extirpated, threatened or endangered. The American Fisheries Society, in the Common and Scientific Names of Aquatic Invertebrates from the United States and Canada: Mollusks (1988), listed 13 taxa as extinct and 30 taxa as federally endangered. Today at least 35 freshwater bivalve taxa are presumed extinct, 52 taxa endangered, 5 taxa threatened at the federal level and an additional 70 taxa as candidates for either threatened or endangered status. At this time 12% of this fauna is presumed extinct, 42% is listed or to be listed and an additional 25% of the fauna is declining. Less than 25% of the freshwater bivalve taxa appear to be maintaining stable populations today.

The problems with the fouling and pollution of the freshwaters of North America were recognized early. S. N. Rhoads (1895) documented the decimation of the freshwater bivalve fauna in the lower Monongahela River. A. E. Ortmann reported the decimation of the unionid fauna in western Pennsylvania (1909) and in the Pigeon River in East Tennessee (1918). The central problem leading to the decline and decimation of the freshwater unionid fauna is the modification and destruction of their aquatic habitat with sedimentation as the single major factor. Sources of sedimentation include poor agricultural and timbering practices. Damming of major rivers has had a dramatic impact on this fauna with the loss of obligate host fish due to changes in water quality and loss of habitat. In-stream gravel mining, dredging, channelization and the often associated headcutting has eliminated stable mussel habitat. Acid mine drainage, and various point and non-point pollution sources all continue to decimate local unionid populations. A new threat to the continued survival of unionid taxa is the introduction in the mid-1980's of the zebra mussel (*Dreissena polymorpha*). These small, bysally attached bivalves cover and smother the native mussels.

GEOGRAPHIC VARIATION IN UNIONID GENETIC STRUCTURE: DO MANAGEMENT UNITS EXIST? David J. Berg,* Dept. of Zoology, Miami University, Hamilton, OH 45011; **Sheldon I. Guttman,** Dept. of Zoology, Miami University, Oxford, OH 45066; **Emily G. Cantonwine,** Savannah River Ecology Laboratory, Aiken, SC 29802.

Unionid populations contain significant levels of within population (w-p) genetic variation. Little is known about levels of among population (a-p) variation and the degree of difference among geographically separated populations. We used allozyme electrophoresis to: 1) describe w-p variation for populations of *Quadrula quadrula* from the Ohio (OH), Tennessee (TN), and Mississippi (MI) river basins; 2) compare a-p variation among basins; 3) consider the results in the context of freshwater mussel conservation biology. All populations contained significant w-p variation (1.8–2.6 alleles/locus; >62% polymorphic loci, mean H = 0.25 to 0.32). OH and TN did not contain significant levels of a-p variation, but a population from the smaller Tensas River, LA (TR) had different allele frequencies at several loci. Genetic distance was positively correlated with geographic distance ($r^2 = 0.721$, $p < 0.001$, $n = 19$); TR was distinct from the other populations. We examined these results using the concept of Management Units (MU)—populations that are functionally independent and exhibit significant levels of mitochondrial or nuclear a-p variation. In the MS basin, at least 2 MUs are present. These may be based on geographic distance or river size. Successful conservation of unionids requires that issues of genetic structure be considered when developing management plans for threatened and endangered taxa.

CONTAMINANT IMPACTS ON NATIVE FRESHWATER MUSSELS—LETHAL AND SUBLETHAL RESPONSES RELATIVE TO WATER QUALITY CRITERIA. Anne E. Keller, National Biological Service, Gainesville, FL 32653.

Native freshwater mussels (Family: Unionidae), the most imperilled fauna in North America, attain their greatest diversity in the southeastern United States. These sedentary animals occupy a unique niche in aquatic systems because they have a parasitic stage during early development, become free-living filter feeders, dwell at the water/sediment interface, and live for up to 90 years. Many environmental impacts have been identified as contributors to the loss of mussel fauna, including habitat destruction, loss of host fish and competition with nonindigenous mollusks. However, contaminants are believed to present one of the most serious challenges to the continued survival of many unionid species.

While unionids have been used as indicators of environmental quality, little was known about their sensitivity to contaminants until the last five to ten years. Most information currently available is on acute toxicity. Little is known about the effects of long term (chronic) exposure. Their decline relative to contaminant toxicity needs examination because the input of pollutants into our aquatic systems can be regulated. Water quality criteria, risk assessments, and current research on sublethal impacts of contamination on unionids will be discussed. In addition, a review of available EC50 and LC50 data will be provided. The utility of such information in addressing conservation issues will be emphasized.

MALATHION TOXICITY TO THREE LIFE STAGES OF UNIONID MUSSELS. **Anne E. Keller*** and **Shane Ruessler**, National Biological Service, 7920 NW 71st St., Gainesville, FL 32653.

The acute toxicity of malathion to clochidia, juveniles or adults of seven species of freshwater mussels was determined in soft or moderately hard reconstituted test water at either 25°C or 32°C. Glochidia tests were conducted for 4, 24 or 48 h, while juvenile (cultured by *in vivo* and *in vitro* methods) and adult exposures lasted 96 h. LC50 values for glochidia of *Utterbackia imbecilis* and *L. teres* tested at 25°C in soft water were 447 mg/L (48 h) and 28 mg/L (4 h) respectively, while *Villosa lienosa* glochidia had an LC50 or 119 mg/L (48 h) at 32°C in moderately hard reconstituted water. Tests with juvenile mussels produced 96 h LC50s ranging from 40 mg/L for *U. imbecilis* in 32°C soft reconstituted water to 219 mg/L at 25°C in moderately hard water. No LC50s were calculable for adult mussels of three species in concentrations up to 350 mg/L. These values are considerably higher than the 48 h LC50 of 1 μg/L for *D. magna*.

THE IMPORTANCE OF HABITAT HYDRAULICS IN THE RESTORATION OF NATIVE FRESHWATER MUSSELS. **James B. Layzer,** National Biological Service, Tennessee Cooperative Fishery Research Unit, Tennessee Tech. Univ., P.O. Box 5114, Cookeville, TN 38505.

Freshwater mussel populations in North America have been devastated by a wide array of physical and chemical perturbations. In some cases, habitat destruction and the loss of mussel populations is essentially permanent as in the case of the construction of dams which inundate riverine habitat, change water quality, and eliminate hosts fish populations. In many other cases, the factors responsible for the extirpation of mussel populations have largely been corrected and conditions may now be suitable for the reestablishment of mussels; however, it is suggested that during the intervening time between the extirpation of mussels and improvement in stream conditions other factors affecting stream hydraulics may prevent the successful reintroduction of mussels. In particular, land-use practices within watersheds may have profoundly affected stream hydrographs by increasing peak discharges following precipitation and decreasing base flows during dry periods. Lower base flows may expose mussel beds, eliminate settlement of juveniles from otherwise suitable habitat, and affect host fish population dynamics and movements. Conversely, results of recent research indicate that high shear stress associated with peak discharge is likely responsible for unsuccessful settlement of juvenile mussels in a headwater stream. Measuring or modelling simple hydraulic variables such as mean water column velocity is inadequate for assessing the affects of altered stream hydrographs on potential mussel habitat. In contrast, complex hydraulic variables such as shear stress and Reynolds boundary number are potentially better predictors of hydraulically suitable sites for mussel reintroductions.

DELAYED REPRODUCTION OF THE FRESHWATER MUSSEL *ELLIPTIO COMPLANATA* THROUGH TEMPERATURE AND PHOTOPERIOD CONTROL. **William A. Lellis*** and **Connie S. Johnson**, National Biological Service, Wellsboro, PA 16901.

Elliptio complanata is a species of freshwater mussel common to streams and rivers of the Atlantic slope. Egg fertilization, larval brooding, and glochidial release are reported to occur within a period of several weeks during early- to mid-summer. In this study we tested the ability of photoperiod and water temperature manipulation to prolong the availability of glochidia for use in artificial propagation. Brood mussels were collected from Pine Creek, Tioga County, PA in late December 1994 when water temperature (0.5°C) and photoperiod (9L:15D) were seasonally low. Mussels were housed in groups of 46 within eight 1.2-m diameter circular fiberglass tanks containing 25 cm of gravel substrate and subjected to one of four environmental treatments. In the first treatment, photoperiod and temperature matched natural conditions. In the second and third treatments, winter conditions were prolonged for periods of 6 and 12 weeks beginning January 1. The fourth treatment matched natural conditions except that winter temperature was held constant at 10°C. Mussels subjected to natural conditions released glochidia between 16 and 19°C while photoperiod and temperature were rising. Initial conglutinates were white and leaf-shaped containing mostly immature glochidia. Subsequent conglutinates were composed of fully developed glochidia packaged within a clear matrix, while final release occurred as free individuals extruded on thin mucus-like strands. Prolonged winter conditions delayed reproduction proportional to length of treatment, whereas elevated winter temperature had no effect on timing of glochidial release. Data indicate that the seasonal availability of *Elliptio complanata* glochidia can be extended three-fold using photoperiod and temperature manipulation.

FRESHWATER MUSSEL CONSERVATION AND THE ENDANGERED SPECIES ACT. Debbie C. Mignogno, U.S. Fish and Wildlife Service, 300 Westgate Center Drive, Hadley, MA 01035–9589.

The repercussions of nearly unprecedented losses in biodiversity are explicitly depicted in North American freshwater mussel fauna. The continent exhibits the richest array of freshwater mussel fauna in the world and 70% of recognized species are considered endangered, threatened, or of special concern. The authorities conferred by the Federal Endangered Species Act (ESA) and various state inacted Endangered Species Acts mandate the development of programs for the conservation of listed and candidate freshwater mussel species by the U.S. Fish and Wildlife Service and other Federal and State management agencies. I will examine various components of the ESA and outline recent research, preservation, and recovery activities conducted by Federal and State agencies under the auspices of the ESA for freshwater mussels, with particular emphasis on the northeastern United States. In addition, I will discuss threats to the continuation of these programs from the Congressionally-directed budget cuts and moratoriums placed on activities conducted pursuant to the ESA.

THE EXOTIC ZEBRA MUSSEL IN NORTH AMERICA: A DIRE PROGNOSIS FOR NATIVE FRESHWATER MUSSELS (UNIONIDAE). Richard J. Neves, National Biological Service, Virginia Cooperative Fish and Wildlife Research Unit, Virginia Tech, Blacksburg, VA 24061; **Catherine Gatenby** and **Bruce Parker,** Biology Department, Virginia Tech, Blacksburg, VA 24061.

The zebra mussel (*Dreissena polymorpha*) is running rampant in North American waters, infesting and exterminating native unionids from much of Lake St. Clair, western Lake Erie, Detroit River, St. Lawrence River, and other localized areas. Its escape in 1991 from Lake Michigan to the Illinois River and spread throughout the Mississippi River Bason now jeopardizes the native mussel fauna in many major tributaries. Populations expand rapidly and achieve densities of greater than $50{,}000/m^2$ in 2 years of colonization. The species readily colonizes living unionids by byssal threads, with densities exceeding 10,000 individuals and weights up to four times that of the colonized unionid. Negative effects on unionid populations include inhibition to feeding and respiration, reduction in glycogen reserves, and ultimately death within 5 years of infestation.

To anticipate possible extirpations or extinctions of native mussel species, a project was initiated in 1992 to evaluate the feasibility of using small ponds and headwater rivers as refugia for unionids in the Ohio and Tennessee rivers. Most unionid species confined in cages and pocket nets in ponds from 1992–1995 have exhibited good survival ($>70\%$), and studies are evaluating reproductive success in ponds and a river refugium. A geographic network of natural and artificial refugia may be necessary to prevent an unprecedented spasm of extinctions of native riverine unionids.

CRUSTACEAN BIOLOGY AND FISHERIES

SOME RECENT TRENDS IN MARYLAND BLUE CRAB POPULATIONS. George R Abbe,* Estuarine Research Center, Academy of Natural Sciences, 10545 Mackall Road, St. Leonard, MD 20685; **Cluney Stagg,** Fisheries Administration, Maryland Department of Natural Resources, Tawes B-2, 580 Taylor Avenue, Annapolis, MD 21401.

With major reductions in the size of many Maryland Chesapeake Bay fisheries, added pressure has been exerted on the blue crab in recent years. In an effort to understand some of the consequences of this increased pressure, we have analyzed data collected along 12.5 km of western Chesapeake Bay in Calvert County from 1968 to 1995. Commercial peeler crab pots of 25-mm (1-in) mesh, baited daily with menhaden, were used to sample crab stocks at three locations with up to sixty pots fished during alternate weeks from June through November. Station catches were sorted, measured and weighed by sex. From 1968 through 1995 112,994 crabs were caught in 18,106 pots, of which 73% were legal size. Although the annual mean catch per unit effort (CPUE) showed considerable variation, this appeared to be normal. Crabs per pot ranged from 0.85 in 1968 to 20.01 in 1981 while Maryland commercial landings ranged from 10.3 million pounds to 59.7 million pounds during the same years. From 1968 to 1980 legal CPUE averaged 3.60, from 1981 to 1985 it averaged 8.14, and from 1986 to 1995 it was 3.66. Thus the legal CPUE of the most recent period is little different from that of the earliest period. There are, however, several trends that have become apparent in recent years indicating that fishing pressure may be putting a severe strain on the blue crab population. Significant correlation between this fishery independent data and Maryland DNR's fishery dependent data demonstrate the relevance of these trends.

From 1968 to 1982 the annual male percentage decreased significantly from 66% to 38% ($r^2 = 0.79$; $p < 0.01$). Since 1983 this percentage has fluctuated more, but it has not shown any further decrease. Mean carapace width and weight of females have not changed significantly over time, but width of males ($r^2 = 0.47$) and weight of males ($r^2 = 0.34$) have both decreased significantly ($p < 0.01$). Legal size, which constituted 64 to 86% of the annual catch between 1968 and 1991, has had its three lowest years since just 1992; and the percentage of legal males in the catch decreased from 56% in 1968 to 19% in 1995 (13% in 1994) ($r^2 = 0.75$; $p < 0.01$). These most recent downward trends related to size of males indicate that they are being removed from the population shortly after reaching legal size. With only small males available to crabbers, even more pressure may exerted on females which could result in continued decreases in population size and stability. Further regulations may be necessary.

THE GONOPOD TEGUMENTAL GLANDS OF SNOW CRAB, *CHIONOECETES OPILIO:* A CLOSER LOOK YIELDS EVIDENCE FOR SEXUAL FUNCTION. **Peter G. Beninger*** and **Annie Ferguson,** Département de Biologie, Université de Moncton, Moncton N.B. Canada E1A 3E9; **Carole Lanteigne,** Centre Marin de Shippagan, C.P. 1010, Shippagan, N.B. Canada E0B 2B0.

The role of the tegumental glands found in the first gonopod of brachyuran crabs has hitherto been a matter of conjecture. In order to elucidate the nature and ultimate function of these glands, histological and histochemical studies were performed on 17 male snow crabs. Mature (M) and immature (IM) individuals were differentiated based on the carapace width (CW): cheliped height ratio. Immature crabs were subdivided into 3 groups: small immature (<40 mm CW), medium immature (40–70 mm CW), and large immature (70–100 mm CW). The precise distribution of the glands within the first gonopod was determined via serial sections (7–10 μm), and histochemical tests were performed for lipids, aminated substances, acid mucopolysaccharides, and neutral mucopolysaccharides. The volume fraction of the gonopod glandular region occupied by glands was assessed using stereologic counts.

The glands were determined to be of the rosette type, and restricted to a specific region at the base of the endopodite. Ducts leading from these glands to the cuticle of the ejaculatory canal only were clearly visible in medium immature to mature individuals; these ducts connected to pores in the cuticle. Cuticular pores and ducts were not observed in small immature crabs. The volume fraction of the glands increased in each successive maturity category, with a mean of 0.8% in small immature crabs and a mean of 8% in mature crabs. The glands contained either acid or neutral mucopolysaccharides, or a mixture of both. The pores of the ejaculatory canal contained similar secretions. These observations support the conclusion that the first gonopod tegumental glands in *C. opilio* are accessory sex glands.

THE EFFECT OF WATER VOLUME AND SURFACE AREA OF CULTURE CONTAINERS ON WEIGHT GAIN OF JUVENILE FRESHWATER PRAWNS, *MACROBRACHIUM ROSENBERGI.* **Louis R. D'Abramo,* Curtis G. Summerlin, William H. Daniels,** and **H. J. Wan,** Department of Wildlife and Fisheries, Mississippi State University, Mississippi State, MS 39762.

Juvenile freshwater prawns (mean weight = 0.225 g) were individually held in containers under conditions of different volume, surface area, and flow rate (turnover rates of 44 and 86 minutes), (2 × 2 × 2 factorial design) and fed a semi-purified diet. Reduced volume or reduced area was associated with a significant reduction in weight gain after 60 days. No significant effect of turnover rates was observed. The density effect on growth rate occurs even in the absence of other conspecifics and may be due to the attainment of a threshold level of a particular metabolite. The freshwater prawn exhibits the same density dependent growth relationship evidenced by other aquatic organisms.

FIELD EXPERIMENTS ON THALASSINID SHRIMP CONTROL FOR OYSTER CULTURE IN WASHINGTON STATE. **Brett R. Dumbauld,*** Washington State Department of Fish and Wildlife, P.O. Box 190, Ocean Park, WA 98640; **David A. Armstrong** and **Kristine L. Feldman,** School of Fisheries, University of Washington, Seattle, WA 98195; **John R. Skalski,** Center for Quantitative Sciences, University of Washington, Seattle, WA 98195.

Field experiments are being conducted to examine use of the pesticide carbaryl and the placement of oyster shell as a physical barrier to control mud shrimp *Upogebia pugettensis* and ghost shrimp *Neotrypaea californiensis* on intertidal oyster culture grounds in Washington State coastal estuaries. Survival and growth of juvenile oysters *Crassostrea gigas* and re-invasion of shrimp were monitored on 100 m^2 carbaryl treated plots and untreated controls from 1990–1993. Addition of a thick layer of oyster shell to carbaryl treated and untreated mudflat is currently being investigated on 64 m^2 plots. Results of these experiments suggest marked differences between the effects of each species of shrimp on oyster seed and epibenthic shell. Ghost shrimp cause immediate loss of oyster seed as well as the oyster shell barrier, while mud shrimp have a lesser effect. All shrimp are killed when carbaryl is applied in July including small mud shrimp which settle as post-larval recruits in May and June. Ghost shrimp can reinvade treated plots almost immediately as post-larval recruits settling in fall and preferentially select open mud, while mud shrimp re-invade the following spring and appear to preferentially seek shell. Despite obvious deleterious effects of shrimp on oyster seed survival, no significant effects have been detected on oyster growth.

RELATIONS AMONG FIXED STATION BLUE CRAB POT SAMPLING RESULTS, REPORTED CHESAPEAKE BAY LANDINGS AND WINTER DREDGE SURVEY RESULTS. **Cluney Stagg,*** Fisheries Administration, Maryland Department of Natural Resources, Tawes B-2, 580 Taylor Avenue, Annapolis, MD 21401; **George R. Abbe,** Estuarine Research Center, Academy of Natural Sciences, 10545 Mackall Road, St. Leonard, MD 20685.

Declining trends in available measures of apparent abundance have motivated a renewed interest in the status of the blue crab stock in Chesapeake Bay. One of the longest, and potentially most useful, data sets available for assessing the current status of the Chesapeake Bay blue crab stock was begun in 1968 and has continued to the present. Catch per unit effort (CPUE) and biological data (size and sex composition) were collected in commercial peeler pots of 25-mm mesh at three stations along 12.5 km of Chesapeake Bay in Calvert County. Correlation analysis was conducted to examine the relation between Calvert Cliffs

CPUE and reported (1) Maryland pot catch, (2) Maryland total catch, (3) Chesapeake Bay pot catch, and (4) Chesapeake Bay total catch. From 1968 to 1981, correlation coefficients (**r**) of (1) 0.91*, (2) 0.94*, (3) 0.82*, and (4) 0.82* resulted (* $= p < 0.001$). By the end of the 1994 season, **r**s had declined by as much as 25%. It was hypothesized that the post-1981 divergent trend could be largely explained by the sharp increase in crab pot effort in Maryland having compensated for changing blue crab abundances, such that catch did not move in parallel with Calvert Cliffs CPUE. Maryland reported commercial crab pot effort increased by about 50% from 1984 (first year available) through 1994. Following effort-standardization of Maryland reported crab pot catches from 1985 to 1994, there were improvements in **r** of as much as 18%.

In an unrelated study supported by the National Marine Fisheries Services's Chesapeake Bay Stock Assessment Committee, a randomized winter dredge blue crab survey has been conducted over all of Chesapeake Bay since the winter of 1988–89. More than 1000 samples have been taken each winter while crabs are dormant. A close relation between the dredge survey data and the Calvert Cliffs data would further support the representative character of the Calvert Cliffs data. To assess the relation between the two, Calvert Cliffs annual CPUE was regressed against the prior winter nominal age-1 crab index (60–120 mm), with the following result: dredge survey age-1's predicted the Calvert Cliffs abundance index for the following summer with a coefficient of determination, r^2, $= 0.90$ ($p < 0.0001$). The results from these two analyses imply that Calvert Cliffs data is representative of the baywide blue crab stock, and thus, is a very useful long-term times series for evaluating the Chesapeake Bay blue crab stock status. Given the previous statement, Calvert Cliffs CPUE results indicate that current (1990–94) abundance levels more closely resemble those of the late-1960s and 1970s, and that early-to-mid 1980s abundances were probably anomalous.

CRUSTACEAN HEALTH PROBLEMS

IMPACT OF "BUMPER CAR" DISEASE ON THE NORTH AMERICAN LOBSTER FISHERY. **Richard J. Cawthorn,** Atlantic Veterinary College, University of Prince Edward Island, Charlottetown, P.E.I. C1A 4P3.

Although 1993 landings of lobsters were valued at $300 million in Canada and $210 million in the United States, postharvest losses are 10–15% annually. "Bumper car" disease of lobsters caused by the scuticociliate *Anophryoides haemophila*, can be significant in coldwater impoundments. Although outbreaks occur more frequently and with greater severity, the epidemiology and economic impact of "bumper car" disease are not well documented. The ciliates are maintained in cell-free, defined medium at 5°C. Cultured ciliates require longer and more parasites to kill lobsters than those transmitted by intrahaemocoelic injection from lobster to lobster. Horizontal transmission likely occurs across gills of lobsters. Several licensed disinfectants and chemotherapeutants are efficacious against *A. haemophila in vitro*. Additional to indirect fluorescent antibody testing utilizing monoclonal antibodies prepared to sonicated ciliates, parasites are detected with oligonucleotide probes based on ssu-rDNA of *A. haemophila*. The prevalence of *A. haemophila* in wild-caught lobsters should be reevaluated with more sensitive and specific diagnostic tools. A definition of healthy *versus* ciliate-infected lobsters is being prepared, based on haematology and clinical chemistry of haemolymph. Our novel bar-coded labelling system for aquatic organisms facilitates experimental design and randomization protocols of lobsters. The model of "bumper car" disease will aid study of health and infectious disease processes of lobsters. (Funded as subcontracts from the Canadian Atlantic Lobster Promotion Association (CALPA) and Diagnostic Chemicals Limited (DCL). CALPA and DCL were supported in part by the Industrial Research Assistance Program of the National Research Council of Canada.)

AN OVERVIEW OF PENAEID SHRIMP PATHOGENS IN U.S. WATERS. **John A. Couch,** Sr. Res. Scientist, Gulf Ecology Division Laboratory, U.S. Environmental Protection Agency, 1 Sabine Island Dr., Gulf Breeze, FL 32561.

Intensive efforts, over the last 20 years, to culture penaeid shrimp in North America and worldwide have increased the focus on pathogens that restrict or limit success in shrimp survival. Since the first discovery and description of a pathogenic baculovirus in 1970–71 (*Baculovirus penaei*-BP) in penaeid shrimps, many other BP-type baculoviruses have been reported from at least six geographic areas as pathogens of over 15 species of penaeid shrimps in 5 of the six subgenera of the genus *Penaeus* and in other related penaeioid genera (Lightner, 1993, and unpubl. Data). High larval shrimp mortalities are reported frequently caused by these and other baculoviruses in shrimp hatcheries.

Apart from the dynamic role of newly discovered viruses in penaeids, other pathogens including bacterial species have been found to cause severe losses. Texas necrotizing hepatopancreatitis (a rickettsial form) and Vibriosis epizootics in North and South America are recently more clarified threats, with a longer history.

These newly recognized pathogens are silhouetted against the background of known fungal, and protozoan pathogens that have been encountered for decades in penaeid shrimp in North America. These include, particularly, the microsporidian protozoan and suspect ciliate ecto-commensals whose specific roles are still unresolved in shrimp/crustacean health. Future direction in research on viral, bacterial and protozoan pathogen of shrimp, particularly molecular probe use for diagnostics are noted.

TOXICANT EFFECTS ON GRASS SHRIMP EMBRYOS.
William S. Fisher* and **Lee A. Courtney,** National Research Council, Environmental Protection Agency, Gulf Breeze, FL 32561; **Patricia S. Glas** and **James R Rayburn,** Gulf Ecology Division, National Health and Environmental Effects Research Laboratory, Environmental Protection Agency, Gulf Breeze, FL 32561.

Embryos of the grass shrimp *Palaemonetes pugio* have been used successfully to characterize toxicity of oil products and efficacy of remediation. Externally-brooded embryos require 12 d incubation to hatch at test temperatures (27°C) and developmental endpoints are easy to detect through the transparent embryonic coat. By 3 d after oviposition embryo organ systems are beginning to develop, by 5 d the heart is visibly contracting, and by 8 d the compound eyes are fully developed. Developmental abnormalities due to exposure to solvents include abnormal eye formation and pigmentation, disfigured telson, diminished hepatopancreas and developmental delay. Abnormalities are seen as early as 4 d after oviposition and as late as 2 d post-hatch. The first layer of the outer envelope of the embryonic coat is secreted by the pleopods of the female. During development, three additional envelopes (comprised of at least 7 new layers) are generated from the embryo. By day 9, the outer envelope begins to erode as if in preparation for hatching. Concomitantly, the permeability of the coat is increased and the embryos become more sensitive to toxicants. This can be traced by the penetration of fluorescent beads of different sizes. Partly due to this increased permeability, acute 96 h exposures to toxicants at this late stage of development (initiated on d 9) result in LC_{50} values relatively close to those generated by the 12 d tests. For example, the 12 d LC_{50} for water soluble fraction of fuel oil is 15.2% v/v and the 96 h LC_{50} is 22.3%. Comparatively close values have also been obtained for ethanol, DMSO and acetone.

HISTOPHAGOUS CILIATE DISEASES OF CRUSTACEA.
J. Frank Morado, National Oceanic & Atmospheric Administration, National Marine Fisheries Service, Alaska Fisheries Science Center, 7600 Sand Point Way NE, Seattle, WA 98115.

In 1888 the first histophagous ciliate infection of a crustacean was reported in Europe. At the time of this discovery, the complete absence of circulating host hemocytes was noted; this observation eventually became the hallmark of histophagous ciliate infections of crustaceans. It was also noted that the host was a recently molted crustacean, but this observation was consider of lesser importance. Histophagous ciliate infections have since been sporadically reported in both wild and captive crustaceans from the eastern North Pacific, and the western and eastern North Atlantic Oceans. Until recently all histophagous ciliate infections were restricted to marine crustaceans.

Until 1980, the sporadic and infrequent reports of systemic ciliate disease of crustaceans and their low prevalences suggested that ciliate infections could be of importance in aquaculture, but not in the population dynamics of wild Crustacea. Since 1980, the evidence now clearly indicates that systemic ciliate disease is a major problem in the culture of the Australian crayfish, *Cherax quadricarinatus,* and in the captive maintenance of the American lobster, *Homarus americanus,* and Dungeness crab, *Cancer magister.* The evidence further indicates that systemic ciliate disease may play an important role in the population dynamics of wild Dungeness crab and the American lobster.

Almost from the time of their initial discovery, the taxonomic identity of the disease causing agents has been a major point of confusion. Over the last couple of years however, considerable effort has been directed toward the identification of the respective ciliated protistans. This presentation will discuss the taxonomic affinities of the respective disease causing agents, some aspects of disease pathogenesis, and review the history and present a current perspective of histophagous ciliate infections of crustaceans.

EFFECTS OF DIMILIN ON THE BLUE CRAB, *CALLINECTES SAPIDUS* IN SHALLOW WATER HABITATS.
Steve Rebach, Department of Natural Sciences, University of Maryland Eastern Shore, Princess Anne, MD 21853.

Diflubenzuron (Dimilin) is used as an insecticide for Gypsy Moth control. Because it is a chitin synthetase inhibitor, it can be a potential threat to other arthropods including crustaceans. We used a static renewal testing paradigm to determine the LC_{50} of Dimilin WP-25 to juvenile blue crabs (carapace width: 25 mm–60 mm). Both molt stage and dose frequency affected toxicity. When we exposed the crabs at random molt stages, $LC_{50} = 3.5$ mg l^{-1}. When crabs were exposed on the day of molt, $LC_{50} = 300$ μg l^{-1}. If initial exposure occurred on the day of molt and the crabs were subsequently exposed to repeated doses, $LC_{50} = 18.5$ μg l^{-1}. Effects were age and molt stage sensitive.

THE PARASITIC DINOFLAGELLATES OF MARINE CRUSTACEANS.
Jeffrey D. Shields, Chesapeake Bay National Estuarine Research Reserves in Virginia, Virginia Institute of Marine Science, Gloucester Point, VA, 23062.

Dinoflagellates are often thought of as free-living, autotrophic protistans that live in pelagic or neritic surface waters. There are, however, many heterotrophic and parasitic forms. The latter include some unusual parasites of crustaceans that are relatively unknown. There are two orders that parasitize crustaceans: the Blastodinida, with two families, and the Syndinida, with one family. Parasitic dinoflagellates infect copepods, amphipods, mysids, euphausiids, and decapods. They inhabit the eggs, stomach, soft tissues and the hemal sinuses of their hosts. Their pathogenicity varies with their invasiveness in the host. The gut-dwelling blastodinids are relatively benign, while the chytriodinids kill their host egg. Members of the pervasive Syndinida are overtly pathogenic and insidiously ramify throughout the hemal sinuses and organs of their hosts. Host castration, feminization, lethargy, and eventually death are common results of infection. Past work ex-

amined the taxonomy, nuclear organization and division, and host-parasite relationships of these parasites. More recently studies have compared ultrastructural differences between parasitic and free-living forms. With the advent of major epizootics of *Hematodinium* sp. on commercially important crustaceans, and outbreaks of *Syndinium* and *Blastodinium* spp. on copepod populations, attention has shifted to the economic and ecological impacts of these parasites on their host populations. Recent epizootics have caused significant financial losses to the afflicted commercial fisheries. These epizootics appear associated with host-parasite systems that occur in regions with unique hydrological features, such as fjords or poorly draining estuaries with shallow sills. These regions are ideal for the application of a "landscape ecology" approach that could lead to a better understanding of the epizootiology of parasitic dinoflagellates and other marine pathogens.

ECOLOGICAL FUNCTION OF BIVALVES

INTERTIDAL OYSTER REEFS AS CRITICAL ESTUARINE ENVIRONMENTS: EVALUATING HABITAT USE, DEVELOPMENT AND FUNCTION. Loren D. Coen,* Elizabeth L. Wenner, David M. Knott, Bruce W. Stender, Nancy H. Hadley, and **M. Yvonne Bobo,** Marine Resources Research Institute, South Carolina Department of Natural Resources, Charleston, SC 29412.

In South Carolina, over 95% of the oysters grow intertidally, forming extensive "biogenic" reefs that are very different from oyster reefs studied elsewhere. Whether these intertidal habitats are functionally equivalent to other structurally complex habitats, such as seagrasses or salt marshes, is an important question. We have constructed three replicate experimental reefs (each 23 m^2) at each of two sites: a "pristine" oyster flat and a "degraded" area adjacent to a marina. A novel flume net system was developed for quantifying transient species, and last spring we initiated sampling of the transient fauna and the resident reef community on each experimental reef and on an adjacent natural reef of equivalent size. We are also collecting continuous environmental data and comparing contaminant levels, oyster diseases (Dermo and MSX) and other life history parameters (e.g., growth, condition indices, reproduction) on the experimental and natural reefs. To date we have collected over 38 species of transient fishes and decapod crustaceans (dominant genera, *Penaeus, Palaemonetes, Anchoa, Leiostomus, Gobiosoma*), with peak densities exceeding 2,400 individuals/reef. Samples of resident fauna have been sorted and identified only from the first season; preliminary analyses show no differences between sites or between experimental and natural reefs, with regard to overall abundance or species richness. Individual species, however, showed marked differences in abundance patterns. The oyster parasite *Boonea impressa*, for example, numerically dominated the natural reef resident community, but was not found in the initial sampling of the experimental reefs. The complete temporal series of samples will be used to explore changes in the status of reef habitat and function throughout its succession.

OYSTER PRODUCTION—A LARGE SCALE PERSPECTIVE. Jerry McCormick-Ray,* Dept. Environmental Sciences, Clark Hall, University of Virginia, Charlottesville, VA 22903.

Oysters have evolved with the Chesapeake Bay ecosystem. Historical evidence supports their widespread distribution and abundance, forcing consideration of the intergration of the oysters' biology with the physical system. Ecological science suggests the critical role of *Crassostrea virginica* in shoreline changes; in marsh development, erosion, and sediment deposition; in food chains and nutrient transfers; in water quality, hydrodynamics, and benthic function. These ecological roles are important to a diverse array of species, including other species of commercial importance, and they are important to ecosystem change. A large scale perspective of the oyster in the Chesapeake Bay highlights the need to focus oyster productivity and sustainment on watershed management and ecosystem processes.

PHYSICAL AND BIOLOGICAL SCALING OF BENTHIC-PELAGIC COUPLING IN COASTAL ECOSYSTEMS: THE ROLE OF BIVALVE SUSPENSION FEEDERS. Elka T. Porter,* Roger I. E. Newell, and **Lawrence P. Sanford,** Horn Point Environmental Laboratory, University of Maryland, Box 775, Cambridge, MD 21613.

It is increasingly recognized that benthic suspension-feeding bivalves are an important component of estuarine ecosystems because they increase the transfer of seston and particle-bound toxicants from the water column to the benthos and because of their role in nutrient regeneration. Water flow is crucially important for supplying seston to the bivalves and controlling benthic-pelagic nutrient fluxes at the sediment-water interface.

Previous studies on seston removal by bivalves and nutrient regeneration have been performed either in flumes in the laboratory or field flumes, and in laboratory mesocosms such as the Marine Ecosystems Research Laboratory at the University of Rhode Island. A disadvantage of flumes is that water column processes are not adequately scaled although benthic boundary-layer processes are well represented. Mesocosm water column processes may be represented well but processes at the sediment-water interface are not accurately scaled. Minimal mixing at the sediment-water interface in mesocosm tanks is problematic for accurately studying benthic processes because low water flow directly affects seston uptake by bivalves and may lead to refiltration, enhanced sedimentation, reduced resuspension, and a change in nutrient regeneration processes. Therefore, results from such studies in flumes or mesocosms cannot be scaled up to ecosystem level with confidence.

We utilized coupled mesocosm and flume systems to study nutrient and particle fluxes at the benthic boundary layer in order

to obtain benefits inherent in both experimental systems. We studied the effect of the interaction of benthic bivalve suspension feeders and water flow on seston quantity and quality and on nutrient regeneration in systems of two different sizes and with different water flows at the sediment-water interface. Preliminary results of replicate experiments indicate that oysters (*C. virginica*) decrease Chl *a* and seston concentrations in systems of both sizes, but water flow did not appear to have a substantial effect. However, water flow at the sediment-water interface did significantly affect nutrient regeneration in systems with bivalves and in systems of both sizes. These results emphasize the importance of considering water flow in ecosystem studies with benthic bivalve suspension feeders.

TROPHIC COMPETITION BETWEEN THE PACIFIC OYSTER *CRASSOSTREA GIGAS* AND THE POLYCHAETE *LANICE CONCHILEGA* IN THE BAY OF VEYS (FRANCE).

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The Bays of Veys leasing grounds (i.e., 406 acres), representing a yearly oyster production of 10,000 metric tons, are fully exploited. Strongly spatially correlated to these leases and interfering with the oyster industry, a population of the polychaete *Lanice conchilega* has drastically increased since 1986 to reach 8,000 individuals.m^{-2} in several areas. The trophic competition between the Pacific oyster *Crassostrea gigas* and the polychaete *L. conchilega* was studied by assessing the polychaete suspension feeding activity at the laboratory. Particle size distributions were compared at the input and output of an experimental design to estimate the polychaete retention efficiency. Although particles ranging from 4 μm to 12 μm were kept by *L. conchilega*, no upper threshold and maximum retention rate were reached. In contrast, *C. gigas* showed a retention efficiency at 2 μm and reached a 6 to 8 μm upper threshold. Based on our results, a trophic competition is likely to occur between *C. gigas* and *L. conchilega*, therefore affecting the oyster industry. Standardized filtration rates reached $0.27 l.h^{-1}.dmw^{-1}$ and $2.41 l.h^{-1}.dmw^{-1}$ for (1 g) *L. conchilega* and *C. gigas* respectively. Polychaete assimilation rates (0.37) were significantly lower than those of *C. gigas* (0.49). Respiration rates were estimated to 0.21 and 0.62 $ml.O_2.h^{-1}.dmw^{-1}$ for *L. conchilega* and *C. gigas* respectively. Therefore, polychaete scope for growth (SFG) ($2.17 J.h^{-1}.dmw^{-1}$) was significantly lower compared to the $63.7 J.h^{-1}.dmw^{-1}$ *C. gigas's* SFG. Based on these results and both species field stock assessments, *L. conchilega* was responsible for 21% carrying capacity decrease. However, *L. conchilega's* SFG represented only 5% of *C. gigas* population's SFG. Several hypothesis regarding both populations interactions and further management are discussed with regards to physical and biological assumptions.

ECONOMICS OF THE AQUACULTURE INDUSTRY

CHARACTERISTICS OF ON-BOTTOM OYSTER RACK STRUCTURES IN THE CHESAPEAKE BAY.

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The drastic decline in Maryland's oyster harvests over the past several decades has resulted in a change of direction in the management of oyster stocks including the implementation of experimental off-bottom oyster culture leases. This project incorporates the results of previous disease research and off-bottom rack studies to develop the characteristics of an on-bottom rack culture system.

This system was designed to be worked using traditional commercial fishing vessels and techniques. Results from this study found that there was no significant difference in growth between oysters raised in a rack structure and those grown in floating trays. This study was performed at a low salinity (<10 ppt) site on the Wye River. Two summers of disease analysis showed zero prevalence of *Perkinsus marinus*.

The racks, each holding ten ADPI OBC series bags and extending from the bottom 1.1 m, were evaluated for bottom break free force, air weight of rack-fouled and air weight of rack after mechanical cleaning. Bottom break free loads were in the order of twice the fouled air weight however the first spring haul was typically four times the fouled air weight. Fouling loads, composed of sediment, feces, pseudo-feces and biofouling communities, comprised 25% and 30% of the rack air weight, or roughly 45 kg per rack.

LARVAL PROCESSES

USE OF MESOCOSMS FOR 'IN SITU' CULTURE OF MARINE INVERTEBRATE LARVAE.

Sandra Brooke* and **Roger Mann**, Virginia Institute of Marine Science, Gloucester Pt., VA 23062.

Larvae have been cultured for descriptive and experimental purposes for over 100 years; however, there has never been a critical comparison of growth and survival of laboratory cultured larvae with sibling larvae maintained "in situ" in the natural environment. Consequently, there remains the question of to what extent we can extrapolate laboratory generated data on growth rate and nutritional status to field situations. In order to address this deficiency we developed an in situ mesocosm for comparative growth studies in the field with mollusc larvae. The mesocosm is a small, submersible larval culture chamber. It is self contained with respect to power supply to enable maintenance of an oscillating flow of water through the larval chamber. Larvae from two molluscan classes were used to test the application of the mesocosm to different ecosystems. The first test organism was the American Oyster, *Crassostrea virginica* in the high nutrient environment of the Chesapeake Bay; the second was the Queen conch,

Strombus gigas in the oligotrophic waters of the Exuma Sound, Bahamas. Sibling larvae were exposed to natural ambient water conditions in the mesocosm or maintained in laboratory culture. Growth, development and nutritional status were evaluated for both treatments.

SIZE AND DEPTH DEPENDENT LARVAL MORTALITY: A MODELING STUDY. Margaret M. Dekshenieks,* Eileen E. Hofmann, and **John M. Klinck,** Center for Coastal Physical Oceanography, Old Dominion University, Norfolk, VA 23529; **Eric N. Powell,** Haskin Shellfish Research Laboratory, Rutgers University, Port Norris, NJ 08349.

The rates of mortality for larval stages of most marine organisms are difficult or impossible to measure, and as a consequence are largely unknown. This is problematic since mortality, in particular predation loss, is an important process determining recruitment and the structure of marine communities. Simulation models provide one means of testing the effects of different mortality losses on population structure. Thus, a time and depth-dependent model of the growth and behavior of larvae of the Eastern oyster, *Crassostrea virginica*, was developed to investigate the effects of different predation strategies on the survivorship of this species. Simulations that included a size-dependent mortality showed that the largest number of larvae survive to settlement size if predation loss decreases with larval size. Simulations that included a depth-dependent predation loss showed that more larvae survive to setting size when predation is concentrated in surface waters. However, few larvae survive if benthic predation rates are high or if predation rates are constant with depth. Simulation results clearly illustrate the differences in larval survivorship resulting from variable size and depth dependent predation mortality.

METAPOPULATION DYNAMICS OF OYSTERS IN A SUBESTUARY OF THE CHESAPEAKE BAY: ESTIMATING EARLY LIFE HISTORY MORTALITY RATES. Roger Mann,* Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062.

Metapopulation dynamics studies by Mann and Hamrick suggest that physical processes contribute significantly to observed distribution of post settlement patterns of oysters in the James River, Virginia. I seek to address the estimation of mortality rates in that system as progressive losses from egg production and spatially (density dependent) related fertilization efficiency, to larval development rate and survival, to metamorphic competency and success (the latter in relation to spatial substrate variability), and finally to post settlement mortality as young-of-the-year (spat). Literature and derived values can be combined to develop defensible explanations for differences in egg production, at values between 2.4×10^7 and 3×10^9 m^{-2}, and observed spat densities of 10–15 m^{-2}.

METAPOPULATION DYNAMICS OF OYSTERS IN A SUBESTUARY OF THE CHESAPEAKE BAY: THE ROLE OF PHYSICAL TRANSPORT. Roger Mann* and **John Hamrick,** Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062.

Metapopulation dynamics seeks to examine quantitative contributions of parent populations (sources) to a pool of progeny and the eventual fate and distribution of those same progeny (sinks). Such exercises are complicated in marine invertebrate studies by larval mortality and potentially large losses and gains to the larval pool caused by emigration and immigration respectively. We present a status report of a case study—oysters in the James River, VA—in which immigration of larvae is negligible, defined populations of reproductively active oysters exist and have been quantified within the river to provide spatial egg production maps (sources), recruitment of larvae is indicated by spatial distribution and abundance of early post settlement stages (sinks), and larval dispersal is examined using a three dimensional finite difference water flow model. We pose three questions: (a) does larval distribution, as predicted from egg production and passive drifter simulations with appropriate time steps, resemble distribution of early post settlement stages, (b) if so, can the designation of source and sink populations be attributed predominantly to larval dispersal processes, and (c) if not, then what are we missing?

GENETIC VARIATION IN TIME AND SIZE OF METAMORPHOSIS IN THE BIVALVE, *MULINIA LATERALIS*. Marguerite C. Pelletier, Department of Zoology, University of Rhode Island, c/o USEPA NHEERL-AED 27 Tarzwell Drive, Narragansett, RI 02882.

Metamorphosis allows organisms to exploit two discrete environments. Variation in age and size at metamorphosis can enable organisms to increase their survival, growth, and future reproductive success in changing environments. This study was done to determine the presence and amount of genetic variation in age and size at metamorphosis in the bivalve, *Mulinia lateralis*. *Mulinia lateralis* is an important food item for bottom dwelling and bottom feeding organisms. It has been used both as a model species in genetic studies and as a toxicity test organism for regulatory agencies. An initial feeding study showed that a diet of a 50:50 mixture of *I. galbana* (T-ISO) and *P. lutheri* resulted in the best larval survival, larval growth, and percent metamorphosis. Three reciprocal cross experiments were performed using a total of nine different males and nine different females. Genetic variation accounted for 15.49% to 23.07% of the variation in age at metamorphosis and for 17.09% to 23.19% of the variation in size at metamorphosis. The variance levels were low enough that using several individuals and randomizing their offspring throughout the treatments should remove any genetic bias in toxicity testing with this species. The large amount of variance not accounted for by cross is probably due to individual variation. Variation in age and

size at metamorphosis is conserved as a way to deal with uncertain, changing environments.

LOBSTER FISHERIES I AND II

A RAPID FIELD-TEST FOR THE DETECTION OF CHEMICALLY STRIPPED EGG-BEARING LOBSTERS.

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A field-test was developed to detect berried lobsters that have been illegally stripped of their eggs by being dipped in chlorine bleach. This field-test is simple in concept, easy to use, and is based on the detection of residual chlorine on the pleopods of suspect animals. A swimmerette is clipped from a suspect lobster and placed in a glass or plastic vial containing 20 ml of deionized water and 1.0 gram of Potassium iodide. When a non-dipped swimmerette is placed in this solution it remains clear. If a swimmerette has been dipped in chlorine, however, the solution instantaneously changes to a bright yellow. The intensity of this color change is directly proportional to the length of time since the lobster had been dipped. Animals most recently dipped have the most intense color change. The intensity of this temperature dependant color change falls off over time. Fishing trips for lobster are generally from 1–10 days in duration. Lobsters are usually held in running seawater systems during transport to shore. This field-test is able to detect chlorine residual under these conditions up to 12 days post-dipping. In addition, it is simple to use, easy to interpret, and cost effective. Sample vials can be prepared ahead of time and stored at a cost of less than 10 cents per vial in quantity. The color change is rapid and easy to interpret with the naked eye. If necessary, interpretations can also be documented with a laboratory spectrophotometer that can measure and record this color change (350 nm) for use as evidence of wrongdoing.

This test was developed with a grant from the Woods Hole Oceanographic Institution Sea Grant Program (R/E-20) and the generous support of the Massachusetts Division of Marine Fisheries, the University of Pennsylvania Research Foundation, the National Fisheries Institute, and the Lobster Institute of the University of Maine.

RECRUITMENT STRATEGIES IN MARINE DECAPODS: A COMPARATIVE APPROACH.

Michael Clancy and **J. Stanley Cobb,** Dept. of Biological Sciences, University of Rhode Island, Kingston, RI 02881.

The lobster, *Homorus americanus*, the rock crab, *Cancer irroratus*, and the Jonah crab, *C. borealis*, are found sympatrically in rocky areas along the northeast North American coast. The presence of three sympatric species of large decapods whose adult habitat choice is the same presents an opportunity to make a comparative approach to understanding recruitment in crustaceans. We wish to determine how recruitment tactics differ, and to use the differences to deduce the nature of the factors which influence recruitment strategies. In this paper we will report our research on *Homarus* and *C. irroratus*, and refer to the literature on *C. borealis* and other *Cancer* species.

Homarus americanus females begin to reproduce at 5–7 years and are moderately fecund (7,000–90,000 eggs) while *c. irroratus* begin to reproduce at 1 year and are very fecund (50,000–1,000,000 eggs). *H. americanus* carries eggs 9–11 months; *C. irroratus* approximately 4 months. In both species the larvae are present in the water column for 4–8 weeks during June–August, thus are subjected to similar environmental and hydrographic conditions. In the plankton, the annual abundance over an 8-year period of lobster postlarvae ranged from 0.004 to 0.068/m^3, while the average for *irroratus* megalopae was 1.0 to 159/m^3. Hydrographic transport models suggested that postlarval (megalopal) abundances of both species are influenced significantly by wind driven transport and that this may be an important delivery mechanism to coastal waters.

In both species, the final postlarval (megalopal) stage is a competent swimmer which makes the transition from surface to bottom. Laboratory experiments and field sampling showed that lobster postlarvae select cobble or rock-on-sand bottoms to settle on, while *C. irroratus* megalopae settle in equal numbers on sand, rock and mud. Benthic suction sampling showed abundances of newly settled lobsters to be 1–3/m^2 in rocky habitats, while newly settled *C. irroratus* abundance was 10–100/m^2 in both sand and rocks. Several months after settlement, the distributional pattern of *Homarus* recruits is unchanged. *Cancer irroratus* recruits are found only in cobble/rocky areas, suggesting either high post settlement mortality in sandy areas, or rapid movement into the adult habitat. Unlike early benthic lobsters, which experience significantly higher survival in rocks when compared to other habitats, field experiments suggest quite low survival rates among the smallest rock crabs and that mortality is equal among sand rock habitats. Larger crabs suffer significantly lower mortality rates in rocks than in sand. Despite many ecological and behavioral similarities the lobster and rock crab use quite different tactics during their early life history.

THE EFFECT OF DIET ON WEIGHT GAIN, SHELL HARDNESS, AND FLAVOR OF NEW SHELL LOBSTERS.

Darrell Donahue,* **Robert Bayer, Terry Work,** and **John Riley,** University of Maine, 5710 Bio-Resource, Orono, ME 04469–5710.

Harvested newly shed lobsters placed in pounds for long-term storage are prone to damage resulting from high density storage. It is beneficial for the new shell to harden as soon as possible after shedding. The recent development of artificial diets offers the potential for adding supplements to increase weight and mus-

cle gain and shell hardening. This paper reports on the effects of Vitamin D supplements on these parameters, and also considers the effects of the formulated diets on flavor of the lobsters. Two hundred newly-shed lobsters were tagged, weighed and placed in floating crates. They were fed 5 different diets, herring, fish racks, and three levels of Vitamin D supplemented pelleted feed for a period of 38 days. At this time they were removed from the water and re-weighed. Samples of carapace and claw shell were removed and their thickness measured. These were then subjected to a compressive force/deformation test to measure shell strength. Results indicated a significant difference in weight gain, with the highest level of Vitamin D actually inhibiting this gain. Shell thickness and strength were affected by treatments but there was no measurable relation to weight gain. Subsamples of the lobsters were cooked, and their taste evaluated by a trained flavor panel. Results showed a significant difference in flavor between the lobsters fed herring, and the other two diets but no significant differences between those fed fish racks and the pelleted feed. No off-flavors were detected in lobsters fed any of the three diets.

SIZE AND TIMING OF SETTLEMENT IN POSTLARVAL LOBSTERS: IS THERE A GROWTH ADVANTAGE? **Mary-Jane James-Pirri*** and **J. Stanley Cobb**, Department of Zoology, University of Rhode Island, Kingston, RI 02881; **Richard A. Wahle**, Bigelow Laboratory for Ocean Sciences, West Boothbay Harbor, ME 04575.

Classic metamorphic models derived from insect and amphibian systems indicate that size at, and timing of, the transition from larval to adult habitat can have significant consequences on future growth and survival, however these models have not been applied to marine crustaceans. Here we investigate the effect of size and timing of settlement of postlarval lobsters and the subsequent effect on growth attained by the end of the growing season. Early, middle and late settling postlarvae were collected from the plankton off southern coastal Rhode Island. A postlarvae were held in the field in cages suspended off a dock in Narragansett Big Lobsters were reared from mid-June through November 1, 1995. Carapace length and intermolt duration were recorded for each instar for early, middle and late settlers. A repeated measures profile analysis showed that for instars IV–VIII, ear and middle settlers were larger and had significantly different growth curves than late settlers. Our size and intermolt duration estimates were then applied to planktonic postlarval data from 1988–1994 to generate estimated growth trajectories during the first growing season for each year. We observed that for all years, early settling postlarvae were estimated to be 30–50% larger and 2–3 instars older than l settling postlarvae by the end of the growing season. We show that the combined effect of size and timing of settlement of postlarval lobsters does influence the amount of growth attained by the end of the growing season, and postulate that this may influence survival. In this manner, lobsters do fit the predictions of classic metamorphic models that have been derived from other systems.

OVERVIEW OF THE CANADIAN LOBSTER (*HOMARUS AMERICANUS*) FISHERY: RECENT TRENDS IN LANDINGS AND MANAGEMENT AND THE OUTLOOK FOR THE FUTURE. **Douglas S. Pezzack**, Invertebrate Fisheries, Maritime Region, Dept. of Fisheries and Oceans, PO Box 550, Halifax, Nova Scotia, Canada, B3J 2S7.

The lobster fishery is the most valuable fishery on the east coast of Canada, with landed value exceeding $315 million. While most groundfish stocks declined during the 1980's, lobster landings increased from 15,000 t in the late 1970's to over 48,000 t in 1991, the highest this century. The increases occurred over most of the western North Atlantic, and were due mainly to increased abundance and a resulting increase in fishing effort. To date no single parameter can explain the increases, but the large scale nature of the increases suggests an environmental rather than a management or fishery related factor. Landings peaked in 1991 and have since declined. In traditionally productive areas of the Gulf of Maine and much of the Southern Gulf of St. Lawrence landings remain well above long term means and the decline has been slow. In the more marginal lobster fishing areas that have a history of wide swings in abundance, the landings have dropped sharply. Data indicate that while abundance has been high the reproductive potential as measured by eggs per recruit (E/R) has been very low, in most areas less than 2% that of an unfished population. Fishing power has never been higher and it continues to increase while at the same time the regulations controlling the fishery have remained substantially unchanged for 30 years. The combination of low E/R, high exploitation rates, increasing fishing power, increased expectations, and high prices is one of high risk with the population dependent upon continued above average survival to maintain itself. The future stability of the fishery requires new measures that will reduce risk of recruitment over fishing. These new measures must include the fishers as an integral part of the management system. Proposals are underway to develop greater comanagement of the fishery but these require a change in thinking by all participants. The changes needed and methods of obtaining them are discussed.

EXPERIMENTS TO EXTEND THE SURVIVAL OF LOBSTERS AIR SHIPPED TO DISTANT MARKETS. **John Riley*** and **Gulni Ozbay**, University of Maine, 5710 Bio-Resource, Orono, ME 04469–5710.

An improvement in the survival rate of lobsters (*Homarus Americanus*) held in an aerial environment is needed to allow shipment by commercial flights to potentially lucrative distant markets in Pacific rim countries. Survival times of 3–5 days out of water are normal, but commercial airlines will not guarantee delivery in less than 4 days to these destinations, and after this time, percent mortality and loss of condition make this economically

unattractive. Despite some degree of facultative breathing out of water, the reduction of gill functions reduces the animals' respiratory and excretory capacities, resulting in potentially lethal high blood levels of NH_3 and/or low levels of pH due to acidosis. Investigations into these phenomena indicated that hemolymph acid-base level is of great importance to the animals' survival response to an aerial environment. Research was then conducted on the effects and practicality of buffering this acid-base level by either immersion for several days in seawater with varying concentrations of $CaCO_3$ and Na_2CO_3 or direct injection of these agents prior to shipment. Results of these tests are reported.

A COMPARISON OF THE OSMOREGULATORY CAPABILITIES OF COASTAL AND ESTUARINE LOBSTERS. **C. M. Rockel** and **W. H. Watson III**, Zoology Department and Center for Marine Biology, UNH, Durham, N.H. 03824.

The American lobster, *Homarus americanus*, is a limited osmoregulator, maintaining hemolymph osmolarity just above ambient levels. In order to determine if estuarine lobsters differ from coastal lobsters in their ability to osmoregulate we examined how groups of animals from each habitat responded to gradual (5 ppt/day, at 15°C) drops in salinity from 30 ppt to 5 ppt. Specifically, we determined: 1) survivorship; 2) ventilatory rate, as an indirect measure of oxygen consumption and; 3) Na^+/K^+ ATPase activity. All lobsters survived until the salinity level reached 5 ppt. Lobsters were then maintained at 5 ppt until death, at which point their survival was measured in hours. On average, estuarine lobsters survived 130 hours while coastal animals survived only 49 hours. In addition, male coastal lobsters survived twice as long as female coastal lobsters (66.3 hours versus 31.1 hours). In order to obtain an indirect assessment of the energetic cost of osmoregulation at each salinity level, scaphognathite pumping rate (ventilation) was measured during the last hour at each salinity level. Estuarine lobster respiratory rates showed no change until the salinity dropped below 10 ppt, while coastal lobsters exhibited elevated respiratory rates as soon as 15 ppt. Female coastal lobsters demonstrated significantly elevated respiratory rates when compared to coastal males. Estuarine lobsters exhibit no sex-related differences in survivorship or in ventilatory rate. In order to determine if the differences observed between coastal and estuarine lobsters is due to acclimation, two additional studies have been initiated. In the first, we measure Na^+/K^+ ATPase in the gill tissue of lobsters acclimated to normal (30 ppt) and reduced salinity water (15 ppt). Our preliminary data indicates that exposure to low salinities increases activity of these ion pumps by nine fold in coastal animals and three fold in estuarine. In the second study, lobsters were "transplanted" from one habitat to the other and held at that new salinity for >6 weeks. Ventilation and survivorship were then measured as described above. Our preliminary data indicates that coastal animals held at estuarine salinity levels show improved osmoregulatory capabilities. These data suggest that differences in the osmoregulatory capabilities of coastal and estuarine lobsters are probably due to acclimation, which in part, induces expression of increased Na^+/K^+ ATPase activity.

TEMPERATURE CONTROL OF RECRUITMENT IN THE AMERICAN LOBSTER. **S. L. Waddy** and **D. E. Aiken**, Invertebrate Fisheries, Maritime Region, Dept. Fisheries and Oceans, St. Andrews, NB Canada E0G 2X0.

The American lobster experiences a wide range of thermal conditions within its natural range and experimental studies have led to many advances in the understanding of how temperature controls growth and reproductive functions. Temperature control mechanisms are complex as the various environmental factors regulating lobster biology often act synergistically, resulting in responses that are difficult to predict if the various factors are studied in isolation. Several intriguing mechanisms involving temperature and other environmental regulators have been identified. It is known that temperature, season and daylength interact to control larval development, juvenile and adult growth, and maturation and reproduction. Dramatic physiological changes occur at the autumnal equinox and the winter solstice (and probably the spring solstice and summer equinox as well) and many biological processes appear similarly affected by real calendar time. For example, the temperature required to induce premolt and ovarian maturation is considerably lower in the spring than in the autumn and a difference of only a few days at critical times of the year can produce diametrically different responses to temperature. The rate of ovarian maturation varies from a few weeks to a few months depending on the time of year, and larval development at a given temperature can vary by more than 50% between late spring and late summer. Many growth and reproductive processes require that the temperature reach a certain threshold and the threshold changes with both daylength and time of year. Accumulating evidence indicates that the thermal requirements for development, growth and reproduction are not cumulative (as in degree-days) but rather are a combination of threshold and cumulative phenomena.

MOLLUSCAN DISEASE I

STATUS OF OYSTER DISEASES IN MARYLAND'S OYSTER RECOVERY AREAS. **Gustavo W. Calvo*** and **Stephen J. Jordan**, Maryland Department of Natural Resources, Cooperative Oxford Laboratory, 904 S. Morris St., Oxford, MD 21654.

Oyster recovery areas (ORA) currently regulate the placement of seed and harvest of oysters by zoning Maryland's rivers. We report on the status and variability of diseases in Buoy Rock (CHBR) and Old Field (CHOF) in Chester River-zone "C"; Cabin Creek (CRCA), Oyster Shell Point (CROS), and Irish Creek (BCIC) in Choptank River-zones "A", "B", and "C", respectively; and Wilson Shoals (NRWS) in Nanitcoke River-zone "C".

Sampling was conducted once seasonally in spring, summer, and fall of 1995 by collecting two replicate samples of 30 oysters

in each oyster-bar. Temperature and salinity were recorded at the time of sampling. Diagnosis for *P. marinus* was by Ray's fluid thioglycollate medium assay of hemolymph and diagnosis for *H. nelsoni* was by hemolymph analysis and paraffin histology.

As expected, prevalence and intensity of *P. marinus* infections increased as the seasons progressed. In the fall, prevalence was 73% in CHOF (12 ppt), and 30% in CHBR (12 ppt); 30% in CRCA (10 ppt), 97% in CROS (12 ppt), and 98% in BCIC (15 ppt); and 76% in NRWS (14 ppt). The above results represent a 19%–50% increase in salinity and a 30%–85% increase in fall prevalence relative to values recorded in 1994. Variation in prevalence between the two samples collected from each oyster-bar was <30% (N = 20). *Haplosporidium nelsoni* was present in Nanticoke River oysters. We will discuss these results in relation to changing environmental conditions and management implications.

PERKINSUS MARINUS TRANSMISSION DYNAMICS IN CHESAPEAKE BAY. **Lisa M. Calvo*** and **Eugene M. Burreson,** Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062; **Christopher F. Dungan,** Cooperative Oxford Laboratory, Maryland DNR, Oxford, MD 21654; **Bob S. Roberson,** University of Maryland, College Park, MD 20742.

This study is the first to systematically examine the seasonality of *Perkinsus marinus* transmission in eastern oysters, *Crassostrea virginica*, in relation to water column abundance of *P. marinus* cells, host oyster mortality, and temperature. The study was conducted for a one year period in the lower York River, VA. Flow cytometric immunodetection methods were employed to determine *P. marinus* cell abundance in water samples collected 3 times a week. Uninfected sentinel oysters were deployed biweekly to determine infection transmission rates and local host oyster mortality rates were estimated biweekly by monitoring a captive population of native oysters.

Environmental abundance of *P. marinus* cells, infection acquisition by sentinel oysters, and mortality of *P. marinus* infected oysters varied seasonally. Distinct peaks of all three parameters occurred during the month of August, following maximal summer temperatures. Water column parasite cell abundances, infection pressure, and oyster mortalities decreased from summer maximums as temperatures decreased in September and October and remained at low levels from October through the termination of the study in March. Strong and significant positive correlations were found between water column parasite cell abundance and temperature, water column parasite cell abundance and oyster mortality, oyster mortality and temperature, and oyster mortality and *P. marinus* prevalence in sentinel oysters. *Perkinsus marinus* prevalence in sentinel oysters did not significantly correlate with water column cell abundance. These results support the currently accepted hypothesis that infective stages of *P. marinus* originate from dying oysters and are most abundant in August. The occurrence of elevated cell abundances in early summer, immediately before epizootic oyster mortalities, suggests that pathogen cells may also originate from other sources.

USE OF SPECIFIC-PATHOGEN-FREE (SPF) OYSTERS TO MEASURE GROWTH, MORTALITY, AND ONSET OF MSX AND DERMO DISEASE IN SOUTH CAROLINA. **Nancy H. Hadley,* M. Yvonne Bobo, Donnia Richardson,** and **Loren D. Coen,** Marine Resources Research Institute, Charleston, SC 29412; **David Bushek,** University of South Carolina, Belle Baruch Institute, Georgetown, SC 29440.

The SCDNR's Marine Resources Research Institute has recently initiated a long-term study of intertidal oyster reef ecology, utilizing two field sites, a "degraded" marina and a "pristine" tidal creek. Native oysters at these sites have been monitored monthly since September 1994 and both Dermo and MSX have been detected at prevalences as high as 100% (Dermo) and 42% (MSX). As part of this study we planted Specific-Pathogen-Free (SPF) oysters at these sites to monitor growth, mortality, and onset of MSX and Dermo diseases. These SPF juveniles were produced from native intertidal stocks in our hatchery and reared under strict quarantine. All seawater was filtered to 0.45 μm and UV irradiated to remove or destroy infective stages present in our local water supply. Juveniles spawned in March 1995 were subsampled (n = 50) at 4 months of age to determine disease status. Assays for MSX (histological method) and Dermo (RFTM body burden method and polyclonal antisera reactions) were negative. Individuals averaging 12.3 mm shell height (SH) were planted at the two field sites in July 1995 in plastic mesh bags (200 oysters/bag, 3 bags per site). Subsamples (10 oysters/bag, 30/site) were assayed after 1, 2, 4, 8 and 12 weeks of exposure. Dermo was first detected at the marina site at 8 weeks and at the tidal creek site at 12 weeks. MSX was not detected in the deployed oysters during the first 3 months of the study, which will continue through spring 1996. After 4 months of field exposure, oysters averaged 47.8 mm SH at the tidal creek site and 43.4 mm at the marina site ($p = 0.07$) and mortality was low (6.5–11.5%).

DISTRIBUTION AND POPULATION DYNAMICS OF A HYDROZOAN INQUILINE SYMBIONT OF THE EASTERN OYSTER. **Dale S. Mulholland*** and **Frank E. Friedl,** Department of Biology, University of South Florida, Tampa, FL 33620.

My investigations of a cnidarian symbiont lightly attached to the gills and mantle of the eastern oyster, *Crassostrea virginica*, raise questions of their impact on oyster health and human consumers. A survey of intertidal oysters in the Gulf of Mexico from Grand Terre, LA, to Tampa Bay, FL, and in the Atlantic from Charleston, SC, to Palm Beach County, FL, found no cnidarians in oysters north of Tampa bay or north of St. Augustine, FL. Many locations in Tampa Bay and along the central Atlantic coast of Florida yielded oysters with these symbionts.

Previously, immature specimens from Ft. Pierce, FL, were placed in the genus *Eutima* by Kubota and Larson (Proc. of Japanese Soc. of Syst. Zool, no. 41, 1990). More details of the morphology of polyps and medusae are now available.

Monthly collections from intertidal oyster populations in Tampa Bay were made for a year, and the results suggest some explanations for the apparent geographic distribution. Cnidarian numbers dropped drastically after oysters were exposed to freezing temperatures during low tide. The cnidarian population was also severely depressed during a summer of unusually heavy rainfall and reduced water osmolality.

Population dynamics of this hydrozoan suggest they are capable of rapid asexual reproduction when conditions are favorable; hundreds, even thousands, can be found in any one oyster. These large numbers raise concerns about the use of affected oysters as food. Sexually reproducing medusae soon appear if conditions remain favorable. However, the number of oysters with cnidarians does not increase rapidly, suggesting the cnidarians have limited or vulnerable means of distribution.

***PERKINSUS MARINUS* TISSUE DISTRIBUTION AND SEASONAL VARIATION IN OYSTERS (*CRASSOSTREA VIRGINICA*) FROM FLORIDA, VIRGINIA AND NEW YORK.**

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Perkinsus marinus infection intensity was measured in American oysters (*Crassostrea virginica*) that were collected in October 1993, December 1993, March 1994, May 1994 and July 1994 from Apalachicola Bay (Florida), Chesapeake Bay (Virginia), and Oyster Bay (New York). Gill, mantle, digestive gland, adductor muscle, hemolymph and other remaining tissue (including gonadal material) were dissected from 20 oysters from each site at each collection time, and were separately diagnosed for *Perkinsus marinus* infections by incubation in Ray's Fluid Thioglycollate Media and subsequent microscopic quantification of enlarged prezoosporangia. At all sampling times and sites, average *P. marinus* infection intensity (# parasites gm^{-1} wet weight tissue or ml^{-1} hemolymph) was consistently lowest in hemolymph samples, and the greatest density of the parasite was generally found in the digestive gland. *P. marinus* prevalence was 100% at all sites for each of the 5 collection times. Seasonal infection intensity patterns and mean total oyster body burdens differed among the sites. The highest average body burdens were measured in Virginia oysters in September 1993 but were lower by December probably due to mortality of heavily infected oysters and diminution of parasite activity typically associated with colder temperatures. During the same time period, infection intensities remained at about the same level or slightly higher for Florida and New York oysters, respectively. The lowest infection intensities were measured in March for Florida oysters, and in May for oysters from Virginia and New York. The retention of relatively high *P. marinus* infection levels in New York oysters compared to Virginia oysters may have been due to constant high salinity in Long Island Sound plus a very rapid decline in temperature in the fall which may have prevented epizootic mortalities such as those often associated with *P. marinus*.

PHYLOGENETIC POSITION OF THE GENUS *PERKINSUS* BASED UPON ACTIN GENE SEQUENCES.

Kimberly S. Reece,* **Mark E. Siddall,** and **John E. Graves,** Virginia Institute of Marine Science, College of William & Mary, Gloucester Point, VA 23062.

Perkinsus species are presently classified within the phylum Apicomplexa. This placement, however, is controversial. Based upon both morphological observations and nucleotide sequence data of the small subunit rRNA gene some have suggested that *Perkinsus* species may be more closely related to dinoflagellates than to apicomplexans. To reevaluate the phylogenetic position of *Perkinsus*, we obtained nucleotide sequence data for actin genes from *Perkinsus marinus*, a haplosporidian and three species of dinoflagellates. DNA was isolated from *in vitro* cell cultures of *P. marinus*, cultures of three dinoflagellates, *Gyrodinium uncatenum*, *Gymnodinium sanguineum* and *Prorocentrum minimum* and from the spores of *Haplosporidian louisiana*. Actin gene fragments (622 bp) were amplified from each of the genomic DNA isolations using "universal" actin gene primers in the polymerase chain reaction (PCR). Genomic Southern blot analysis indicated that there are two closely related actin genes in *P. marinus*. The two actin fragments amplified from *P. marinus* genomic DNA have been sequenced and demonstrate 97.4% nucleotide sequence identity. All of the 16 observed nucleotide differences occur at the third position of codons and the encoded protein fragments of these two genes are identical. Amplified fragments from the dinoflagellates and the haplosporidian are presently undergoing DNA sequence analysis. For phylogenetic analyses, several protozoan actin gene sequences, in particular those from apicomplexans and ciliates, were downloaded from the Genbank (NCBI) sequence database. Parsimony analysis with these actin gene sequences will be done to constuct phylogenetic trees.

***PERKINSUS MARINUS*, FLOW CYTOMETRIC IMMUNOASSAY AND INTERANNUAL ABUNDANCE IN CHESAPEAKE BAY ESTUARIES.**

Bob S. Roberson* and **Tong Li,** University of Maryland, Department of Microbiology, College Park, MD 20742; **Christopher F. Dungan,** Cooperative Oxford Laboratory, Oxford, MD 21654; **Eugene M. Burreson,** Virginia Institute of Marine Sciences, Gloucester Point, VA 23062.

The development of flow cytometric immunodetection methods for enumerating *Perkinsus marinus* in the diverse mix of particulates found in water samples from estuarine waters of the Ches-

apeake Bay, has offered a means for monitoring the seasonal environmental abundance of this oyster pathogen. The method employs antibody produced in rabbits against cultured forms of the organism, to permit specific fluorochrome labeling of *Perkinsus* sp. cells. The immunolabelled cells in water sample particulates were detected by flow cytometry. By adjusting trial count ranges of fluorescence, size, roughness, nucleic acid content or autofluorescence, followed by sorting from a sample pool made from the previous year's set of samples, it was possible to select ranges for each parameter that would exclude contaminating non-*Perkinsus* particulates arising or anticipated during the year. Parameters were adjusted until the sort was judged by microscopic examination to be free of contaminating particulates. This technique has been used to monitor environmental abundance of the pathogen in the Tred Avon River at Oxford, Maryland for three years, and from a second, higher salinity site, the mouth of the York River, during the last year. Although no period of the year yields samples free of *Perkinsus* organisms, abundance peaks occur during the summer months from June through August.

HEMATOLOGICAL PATHOLOGY OF WASTING SYNDROME IN BLACK ABALONE. **Jeffrey D. Shields*** and **Frank O. Perkins**, Virginia Institute of Marine Science, The College of William and Mary, Gloucester Point, VA 23062; **Carolyn S. Friedman**, Calif. Fish & Game, Fish Health Laboratory, Bodega Marine Laboratory, P.O. Box 247, Bodega Bay, CA 94923.

Withering syndrome is a debilitating and fatal disease of the black abalone, *Haliotis cracherodii*. The etiological agent for the disease is presently unknown. Statistical analyses of hemocyte counts, and various parameters from the stained blood smears of over 600 abalone indicate a pattern of disease in the blood of the host. We tentatively identify three hemocyte types as representatives of a single class of lymphocyte. Small lymphocytes are most likely stem cells, that develop into hyalinocyte-type cells, that grow further into large fibrocyte-like cells. Small lymphocytes were not abundant in the lymph of healthy abalone (35.4% prevalence), but were common in wasted animals (72.9% prevalence). Hyalinocytes and fibrocytes declined sharply in abundance in wasted animals. In wasted animals, fibrocytes dehisce almost immediately upon contact with a microslide. Fibrocytes from healthy animals typically dehisce after 10+ minutes. In addition, healthy abalone had a mean of 1.73% dead cells in their blood compared to a mean of 4.69% in slightly shrunken abalone, and 10.32% in the blood of wasted abalone. Cellular inclusions and an unusual cell type were also significantly more prevalent in wasted than in healthy hosts. Cellular inclusions were found in 40% of wasted abalone compared with 15% in nonsymptomatic hosts. (Note that the prevalence in the healthy animals may represent subclinical infections.) The unusual cell type was found in 28% of wasted abalone compared with 15% in nonsymptomatic hosts. There was also a strong positive relationship between the presence of the unusual cell type and the presence of cellular inclusions. Our results support the view that the infectious agent may be blood borne, and that it causes lysis of hemocytes.

COMPARISON OF *HAPLOSPORIDIUM NELSONI* DIAGNOSTIC TECHNIQUES: POLYMERASE CHAIN REACTION OUTPERFORMS HISTOLOGY. **Nancy A. Stokes,*** **Juanita G. Walker**, and **Eugene M. Burreson**, Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062.

Histological examination of preserved, sectioned, and stained oyster tissue has been the method of choice for detection of the protistan pathogen *Haplosporidium nelsoni* for the past 30 years. We recently developed primers for polymerase chain reaction (PCR) which are sensitive and specific for the small subunit ribosomal DNA of *H. nelsoni*. Detection of *H. nelsoni*-infected oysters by the techniques of histological examination and PCR were compared in monthly samples of oysters held in trays in the York River, VA over an eight month period beginning May 1995. PCR was conducted on DNA extracted from both hemolymph and gill and mantle tissue and histological examination was conducted on transverse sections through the visceral mass including gill and mantle.

Haplosporidium nelsoni was not detected by histology or PCR of tissue DNA until July, whereas PCR of hemolymph DNA detected low prevalence levels in May and June. In the July, August, and September samples, PCR of tissue DNA detected almost twice as many infections as histological examination, while PCR of hemolymph DNA detected as many or more infections as histological examination, but fewer than PCR of tissue DNA. Of the 122 oysters already screened by both techniques, 42 *H. nelsoni* infections were detected by PCR but not by histological examination, whereas 2 local infections were detected by histological examination but not by PCR.

MOLLUSCAN DISEASE II

THE EFFECT OF PENTACHLOROPHENOL ON NADPH PRODUCTION IN OYSTER HEMOCYTES: IMMUNOMODULATORY CONSEQUENCES. **Cal Baier-Anderson*** and **Robert S. Anderson**, Chesapeake Biological Laboratory, University of Maryland, P.O. Box 38, Solomons, MD 20688.

The generation of reactive oxygen species (ROS) by *Crassostrea virginica* hemocytes is thought to be an important line of defense against pathogens. ROS are produced by NADPH oxidase during the respiratory burst, when an electron from reduced β-nicotinamide adenine dinucleotide phosphate (NADPH) is transferred to oxygen, forming the superoxide anion. Since increased production of NADPH is required for ROS generation, one possible

mechanism of immunotoxicity is to inhibit NADPH production. The effects of a putative environmental immunotoxicant on NADPH production and the concomitant effects on ROS modulation are reported here.

Oyster hemocytes were exposed *in vitro* to sublethal concentrations of pentachlorophenol (PCP), a biocide and uncoupler of oxidative phosphorylation that inhibits ROS generation, and assayed for increased NADPH production following stimulation with phorbol myristate acetate. NADPH production was estimated using a colorimetric method that measures intermediate metabolism in terms of the reduction of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) via phenazine methosulfate (PMS). In this assay, electrons are transferred from NADPH to MTS in a series of redox couples where PMS is the intermediary. Preliminary results indicate that PCP partially inhibits the production of NADPH in a dose-dependent manner, which could explain decreased ROS production. The implications of decreased NADPH production in terms of toxicity and immunocompetence will be discussed.

ISOLATION OF A cDNA CLONE FROM *MERCENARIA MERCENARIA* THAT CODES FOR A PROTEIN RELATED TO THE CYTOCHROME P450 III SUBFAMILY OF ENZYMES. **David Brown,*** Duke University Marine Lab, Beaufort, NC 28516; **George Clark,** National Institute of Environmental Health Safety, Research Triangle Park, NC 27709; **Rebecca Van Beneden,** University of Maine, Orono, ME 04469.

We are using molecular techniques to identify possible factors in the high prevalence of gonadal tumors observed in *Mya arenaria* from Washington County Maine and *Mercenaria mercenaria* from the Indian River in Florida. The etiology of the high prevalence of tumors is not known, but genetic factors and exposure to agricultural chemicals seem to be involved. It appears that gender might also play a role. In both of the bivalve populations the prevalence of tumors was highest in females and female clams tended to have more aggressive, invasive tumors.

As part of our investigation into the molecular mechanisms for the etiology of these tumors, we have isolated several cDNA clones from a *M. mercenaria* cDNA library. One clone was sequenced and the predicted amino acid sequence aligns with known sequences for vertebrate cytochrome P450 enzymes. The highest amino acid homology was to enzymes in the cytochrome P450 III subfamily. This is one of the older cytochrome P450 subfamilies, and in vertebrates this family of enzymes is involved in the metabolism of steroids. Steroids have been implicated in the development of tumors in vertebrates and information from the epizootic studies suggested sex hormones or at least gender could be involved in the development of gonadal tumors in *M. arenaria* and *M. mercenaria*. Therefore, the isolation of this cytochrome P450 clone has opened up several new areas of investigation into the development of these gonadal tumors as well as the role of cytochrome P450 enzymes.

INFECTIVITY AND PATHOGENICITY OF *PERKINSUS MARINUS*. 3. FECAL ELIMINATION. **David Bushek*,** Baruch Marine Laboratory/USC, PO Box 1630, Georgetown, SC 29442; **Susan E. Ford** and **Marnita M. Chintala,** Haskin Shellfish Research Laboratory, Rutgers University, IMCS, Box B-8, Port Norris, NJ 08349.

The rate at which hosts shed live parasites has numerous implications for understanding a host-parasite relationship. It may reflect 1) the initial host response to an experimentally delivered dose; 2) differential reaction to qualitative parasite differences; 3) infection development rate; 4) actual infection intensity; and 5) transmission potential. We examined the interaction between *Perkinsus marinus* and *Crassostrea virginica* by measuring the rate at which parasites appeared in the feces and pseudofeces of experimentally infected oysters at various times post challenge. The daily discard rate decreased during the week following challenge. Initially, more cells were found in the pseudofeces than in the feces, but by day 7 relatively few parasites were found in the pseudofeces. After day 7, the rate of discard in feces increased exponentially, indicating that parasites in the original dose were probably shed in decreasing number for about 1 week; thereafter, increasing numbers mirrored the intensification of infections over time. This explanation is supported by the finding that the number of parasites shed was roughly correlated with time to death. The continuous release of viable parasites from live oysters represents an important potential transmission mechanism in nature and may provide a nondestructive method of estimating infection intensity. When oysters were challenged with equal numbers of cultured or wild parasites from naturally infected oysters they eliminated 10 times more cultured than wild cells in the feces during the first two weeks post-inoculation. The pseudofeces ratio was 50:1 (cultured: wild) suggesting that some qualitative difference between the parasite types favored retention of wild cells or elimination of cultured cells by the gills and palps. There was no difference in the number of lag, log, and stationary phase cultured parasites eliminated during the first day post-challenge, indicating that size or stage are unlikely to account for the differences between wild or cultured cells.

INFECTIVITY AND PATHOGENICITY OF *PERKINSUS MARINUS*. 1. PARASITE CHARACTERISTICS. **Marnita M. Chintala,*** Haskin Shellfish Laboratory, Rutgers University, IMCS, Box B-8, Port Norris, NJ 08349; **David Bushek,** Baruch Marine Laboratory/USC, PO Box 1630, Georgetown, SC 29442; **Susan E. Ford,** Haskin Shellfish Research Laboratory, Rutgers University, IMCS, Box B-8, Port Norris, NJ 08349.

To help define the physiological characteristics of *in vitro* cultured *Perkinsus marinus*, we assessed the infectivity and pathogenicity of the cultured parasites 1) in comparison to wild parasites isolated from naturally infected oysters, 2) after increasing culture passage, and 3) in different stages of cultures [lag, log, and stationary]. Oysters were inoculated via the shell cavity with a weight

standardized dose of parasites and held for 12 weeks at 28°C and 25 ppt. Mortality was assessed daily and total parasite burdens were determined in all dead and surviving oysters. Most oysters inoculated with wild *P. marinus* died with heavy infections before the end of the experimental period while mortality of oysters given an equal dose of cultured parasites was very low (10–13% of the wild challenge). Many oysters surviving the challenge with cultured parasites had infection intensities approaching those in the dead oysters, suggesting that they would have died in a longer experiment. There were no consistent differences in mortality among oysters challenged with parasites in the first, fifth, ninth, and fiftieth + culture passages (5–15% mortality). These results indicate that cultured cells are considerably less virulent than wild cells and that they lose most of their virulence during the initial culturing process. As would be expected from this, repassage of cultured *P. marinus* through oysters failed to restore virulence. Mortality attributable to cultured *P. marinus* occurred only in oysters inoculated with log phase parasites. Parasite burdens were highest in those challenged with log phase cells, an order of magnitude lower in those inoculated with lag phase parasites, and lowest in oysters dosed with stationary phase cells.

IDENTIFICATION OF *PERKINSUS MARINUS* PORTALS OF ENTRY BY HISTOCHEMICAL IMMUNOASSAYS OF CHALLENGED OYSTERS. **Christopher F. Dungan*** and **Rosalee M. Hamilton,** Cooperative Oxford Laboratory, Oxford, MD 21654; **Eugene M. Burreson** and **Lisa M. Ragone-Calvo,** Virginia Institute of Marine Science, Gloucester Point, VA 23062.

Since focal *P. marinus* lesions are commonly found within digestive tract epithelia of infected oysters, such epithelia have been proposed as primary portals through which infective pathogen cells invade oyster hosts. Untested mechanistic hypotheses propose that waterborne *P. marinus* cells are ingested by feeding oysters, invade connective tissues via gut epithelia, and subsequently disperse systemically to colonize all oyster tissues. In spite of available methods for experimental infection of oysters by exposure to a variety of *P. marinus* cell types, neither the proximate infective cell type(s), their invasive mechanisms, nor their portals of entry have been documented to date.

We challenged uninfected oysters in the laboratory and in estuarine waters endemic for dermo disease. Challenged oysters were fixed, systematically subsampled histologically, and sections fluorescence-immunostained for microscopic localization of *P. marinus* cells and lesions. Pathogen cells were routinely observed associated with external epithelia, and within gut lumina of both experimentally- and naturally-challenged oysters. At 21 days post-exposure, experimentally-challenged oysters consistently showed lesions containing proliferating pathogen cells in epithelia and connective tissues of gill, mantle, and labial palp, and in visceral connective tissues, but not in digestive tract epithelia. These data do not support prevailing views of *P. marinus* infection dynamics, but suggest possible alternative mechanisms of gut epithelium colonization which may strategically enhance parasite fitness.

PROTEASE BLOCKERS INHIBIT *PERKINSUS MARINUS IN VITRO* AND *IN VIVO*. **Mohamed Faisal,*** and **Jerome F. La Peyre,** Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, Virginia 23062; **Craig D. Wright,** Novavax Inc., Rockville, MD 20852.

Recently, we demonstrated that *Perkinsus marinus* produces serine proteases that are important for its pathogenicity. Protease blockers are used in the control of protease-producing protozoa. In this study, we examined the effects of *Bacillus lichenformis* protease blockers on *P. marinus*.

The propagation of *P. marinus* was reduced by coincubation with the protease blockers *in vitro*. The degree of growth suppression, however, differed between isolates. At a concentration of 10 mg/ml, the propagation of LMTX-1 isolate was totally suppressed and viability was reduced by 72%. In contrast, this same concentration caused a remarkable suppression in the growth rate of *Perkinsus*-1 isolate but no mortalities.

We then investigated the effects of the bacterial protease blocker on the initial phase of *P. marinus* infection. Oysters were injected with an overdose of *P. marinus* cells (10^7/oyster) and then administered liposomes containing the protease blockers daily for 6 weeks. A significant reduction in the parasite body burden occurred in oysters that received the treatment (33,491 parasite/g) as compared to control oysters (328,863 parasite/g). These results suggest that protease blockers may be promising in the control of *P. marinus*.

Further investigations have revealed that oyster plasma contains protease inhibitors that suppress *P. marinus* and other proteases. The involvement of plasma protease inhibitors in the defense against *P. marinus* remains to be elucidated.

INFECTIVITY AND PATHOGENICITY OF *PERKINSUS MARINUS*. 2. DOSING METHODS AND HOST RESPONSE. **Susan E. Ford*** and **Marnita M. Chintala,** Haskin Shellfish Research Laboratory, Rutgers University, IMCS, Box B-8, Port Norris, NJ 08349; **David Bushek,** Baruch Marine Laboratory/USC, PO Box 1630, Georgetown, SC 29442.

Host-parasite interaction studies are facilitated by the ability to infect hosts with known parasite doses under controlled conditions. *In vitro* propagation of *Perkinsus marinus*, a protozoan parasite of the eastern oyster, supplies unlimited quantities of parasites for which many characteristics are defined. To provide guidelines for infection experiments using *P. marinus* and to help evaluate their results, we investigated the effect of 1) 4 different inoculation methods, 2) single vs. multiple dose feeding, and 3) the physiological condition of hosts. Oysters were given a weight standardized dose of parasites and held for 12 weeks at 28°C and 25 ppt. Mortality was assessed daily and total parasite burdens were determined in all dead and surviving oysters. Both mortality

and parasite burdens in surviving oysters followed a pattern that roughly corresponded to the number of barriers to infection breached by the delivery method: intramuscular injection > intravalvular injection > intubation > feeding. This pattern was found for both cultured and wild parasites, although infections from wild parasites developed much more quickly regardless of dosing method. Oysters fed cultured *P. marinus* in one large dose had somewhat heavier total mortality (11%) than did oysters fed an identical dose that was split into 24 aliquots over 8 weeks (3%), suggesting that continuously delivered small doses (the natural situation) are easier to deal with than a single large dose (experimental challenge). Within two weeks of intravalvular injection, oysters with ripe gonads suffered mortalities that were much higher than in unripe oysters. The deaths were attributed to a combination of the injection, spawning, and water that became fouled with dense gamete concentrations. We found no evidence that holding oysters for a prolonged period (6 weeks) before challenge altered *P. marinus*-caused mortality.

OYSTERS, OXYGEN METABOLISM, AND HEMOCYTES. **Frank E. Friedl,*** Department of Biology, University of South Florida, Tampa, FL 33620–5150.

Bivalves in general are considered to be succinate-producing facultative anaerobes able to accommodate hypoxia. They have metabolisms with both aerobic and anaerobic components. Using microelectrode techniques similar to those reported for the freshwater clam *Elliptio* (Vitale and Friedl 1994, J. Shellfish Res. 13(1):301), we have found surfaces and tissues of monovalve *Crassostrea virginica* preparations in normoxic environments deeply hypoxic with ambient surface gradients indicating oxygen uptake. The animals appear to be dependent on whole body diffusion for gas exchange, and have a "tissue-animal" character. We have shown that *Crassostrea* hemocytes take up oxygen and produce H_2O_2, and also phagocytose under anoxic conditions. Luminol-dependent chemiluminescence, well-known for this animal, can be eliminated by N_2 purging, indicating a direct oxygen dependence and that stored, metastable reactants are not involved. The presence of a hydroperoxide metabolism in a diffusion dependent animal with hypoxic tissues raises questions about the utility of H_2O_2 production. It is suggested that oxygen-derived molecules detected by luminol-dependent chemiluminescence may be a consequence and measure of a routine, activity related, oxygen metabolism.

EMERGING EVIDENCE OF EXTRACELLULAR PROTEASES AS IMPORTANT VIRULENCE FACTORS OF *PERKINSUS MARINUS*. **Jerome F. La Peyre,* Kathleen A. Garreis, Heather A. Yarnall,** and **Mohamed Faisal,** Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062.

Our recent studies have shown that *Perkinsus marinus* secreted multiple serine proteases. Current investigations are being performed to determine the role these proteases may play in the survival and pathogenicity of this deadly protozoan.

Serine proteases constitute the major part of the protozoan's extracellular proteins and are secreted by all *P. marinus* isolates so far examined. At physiological pH, *P. marinus* proteases (PMP) hydrolyzed a wide range of proteins including extracellular matrix proteins (e.g. fibronectin). PMP appeared essential for the development of *P. marinus* since protease inhibitors suppressed the parasite propagation.

Recent findings have also shown that hemocyte motility is reduced in a dose dependent manner by exposure to PMP. Moreover, lysozyme activities and hemagglutinin titers of oyster plasma were suppressed as a result of coincubation with purified PMP.

Investigations done *in vivo* suggested that PMP may be important for these establishment of infection and propagation of the parasite. For example, we have recently found that the parasite body burden in oysters administered liposomes containing *P. marinus* extracellular proteins and then challenged with *P. marinus*, increased significantly compared to oysters fed liposomes containing only seawater.

There is thus increasing evidence suggesting that PMP play a role in the invasion and growth of the parasite in host tissue and also in counteracting both cellular and humoral host defenses of the oyster.

MOLLUSCAN FEEDING STUDIES

KINETICS OF DIARRHETIC SHELLFISH TOXINS IN THE BAY SCALLOP, *ARGOPECTEN IRRADIANS*. **Andrew G. Bauder*** and **Jon Grant,** Department of Oceanography, Dalhousie University, Halifax, NS, Canada, B3H 4J1; **Allan D. Cembella** and **Michael A. Quilliam,** Institute for Marine Biosciences, National Research Council, Halifax, NS, B3H 3Z1.

Diarrhetic shellfish poisoning (DSP) is a worldwide public health risk which also constitutes an economic threat to the commercial harvest of shellfish. Bivalve molluscs can acquire DSP toxins by ingesting toxic dinoflagellates from the water column and from benthic seston. Although there have been field studies relating the incidence of DSP toxins in shellfish to toxic dinoflagellate blooms, few studies have described DSP toxin kinetics in bivalves under either natural or controlled laboratory conditions.

In the present study, the dynamics of DSP toxins were examined in juvenile and adult bay scallops by feeding cells of the epibenthic dinoflagellate *Prorocentrum lima*, a known producer of DSP toxins, to scallops in controlled laboratory microcosms. Liquid-chromatography combined with ion-spray mass spectrometry (LC-MS), a powerful new analytical method for marine toxin detection, was used to analyze for DSP toxins in dinoflagellate cells and scallop tissues. Toxin kinetic parameters, including rates of toxin uptake, biotransformation, anatomical compartmentalization and detoxification were determined. The physiological effects of

toxic dinoflagellates on scallop feeding activities and survival were also established.

Juvenile and adult clearance rates were not inhibited by exposure to toxigenic *P. lima* cells and no scallop mortalities were observed. Relatively high weight-specific ingestion rates demonstrated that scallops could exceed regulatory toxin limits (0.2 μg DSP toxin g^{-1} wet wt.) in less than one hour of exposure to high *P. lima* cell densities. Toxin saturation levels (2 μg g₁ wet wt.) were attained within the first two days of exposure, however toxin retention efficiency was very low (<5%). Although most of the total toxin body burden was associated with visceral tissue, weight-specific toxin levels were also high in gonads of adult scallops. Rapid toxin loss from gonads within the first two days of depuration indicated that the toxin was derived primarily from a labile (unbound) component within the intestinal loop section through the gonads. Detoxification of visceral tissue, however, followed a biphasic pattern of rapid toxin release within the first two days of depuration, followed by a more gradual toxin loss over a two week period, suggesting that fecal deposition may be an important mechanism for rapid release of unassimilated toxin and intact dinoflagellate cells.

MUCOCYTE DISTRIBUTION AND RELATIONSHIP TO PARTICLE TRANSPORT ON THE PSEUDOLAMELLIBRANCH GILL OF *CRASSOSTREA VIRGINICA* (BIVALVIA: OSTREIDAE). **Peter G. Beninger*** and **Suzanne C. Dufour,** Département de Biologie, Université de Moncton, Moncton, N.B. Canada E1A 3E9.

To gain a more complete understanding of particle transport on the bivalve pseudolamellibranch gill, the mucocyte secretion types and distribution were determined for this organ in the Eastern oyster *Crassostrea virginica*, and related to previous endoscopic data. Three adult oysters were collected from Shediac Bay (New Brunswick, Canada) in July 1994, immediately fixed, then dissected and processed for histology and whole mount mucocyte mapping using a modification of the periodic acid—Schiff—alcian blue protocol. One type of mucocyte contained acid-secretion mucopolysaccharides (AMPS), while the other type contained neutral mucopolysaccharides (NMPS). A clear gradient in mucocyte density was observed from the plical crest to the trough; for all but the anteriormost 15 plicae the proportions of each mucocyte type remained constant; the 15 anteriormost plicae presented an increased proportion of AMPS. These proportions would produce a relatively viscous acid-dominant mucus after mixing on the ciliated gill surface. The principal filament troughs contained relatively few mucocytes, aligned on the median ridge. This arrangement could account for the observed range of particle velocities in these filaments. The results of the present study conform to a pattern of specialization of mucus types and functions on the diverse types of bivalve gill, depending on particle trajectory, transporting surface architecture, and dominant current flow.

DIFFERENTIAL SENSITIVITY AND PSP TOXIN ACCUMULATION IN TWO CLAM SPECIES, *SPISULA SOLIDISSIMA* AND *MYA ARENARIA*. **Monica V. Bricelj*** and **David Laby,** Marine Sciences Research Center, State University of New York, Stony Brook, NY 11794-5000; **Allan D. Cembella,** Institute for Marine Biosciences, National Research Council, 1411 Oxford Street, Halifax, NS B3H 3Z1, Canada.

Differences among bivalve species in the rate of accumulation of paralytic shellfish poisoning (PSP) toxins are generally correlated with their *in vitro* nerve sensitivity to saxitoxin (STX) and ability to maintain active feeding during toxic dinoflagellate blooms. In this study, the relative sensitivity of juvenile surfclams, *Spisula solidissima*, and softshell clams, *Mya arenaria*, to PSP toxins was determined from their burrowing activity in sediment, and siphon responsiveness (retraction). These two behavioral components are likely to influence survival of natural populations. Long-term (11 d) exposure to the high-toxicity dinoflagellate *Alexandrium tamarense* (*cf. excavatum*) (clone PR18b) had no effect on burrowing or siphon retraction in *Spisula*, but both behaviors were severely impaired in *Mya*, within a few hours of exposure to toxic cells. Although overall, both behavioral responses showed similar temporal patterns, burrowing was more severely affected and provided a more reliable and consistent sensitivity index than siphon flaccidity. The *Mya* test population, which had no prior history of exposure to PSP toxins, was characterized by high individual variability in toxin sensitivity: *ca.* 27% of clams tested repeatedly during toxification generally showed no burrowing inhibition. This provides the first evidence of intrapopulation variability in sensitivity to PSP toxins in a bivalve species.

Both species accumulated most of their body burden of toxin (82 to 89%) in viscera during toxin uptake, but *Spisula* showed a 23-fold higher rate of toxin accumulation and maximum toxicity than *Mya* under identical experimental conditions. The toxin composition of ingested dinoflagellates exhibited no significant changes in *Mya* tissues. In contrast, *Spisula* showed a high capacity for *in vivo* metabolic transformation of individual PSP toxins, as evidenced by the production of potent decarbamoyl gonyautoxins (dcGTXs) from weakly potent N-sulfocarbamoyl (C-) toxins, as well as gonyautoxins, present in ingested cells. Enzymatic conversion of PSP toxins to decarbamoyl derivatives has been previously confirmed in only three Pacific clam species. This study identifies likely pathways for *de novo* production of these toxins (dcGTXs and dcSTX) in *Spisula*, and discusses the implications of our laboratory results in explaining the differential retention of PSP toxins in field populations of these two bivalve species.

TEMPORAL PERSPECTIVES ON FEEDING AND DIGESTION BY SUSPENSION-FEEDING BIVALVES.

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Food acquisition by bivalves in the natural environment is driven by a complex interplay between the different time scales of variation in oceanographic variables, time constraints on acclimation capabilities and temporal variations in energy demands. To provide empirical data to help predict temporal changes in bivalve food acquisition, sea scallop (*Placopecten magellanicus*) and mussel (*Mytilus edulis*) feeding behaviour was monitored *in situ* over time-scales ranging from hours to months.

Food acquisition was monitored at several coastal sites in Nova Scotia using a new sediment trap method that provides time-series data on the ingestion, absorption and egestion rates of bivalve populations over hourly to daily intervals. Replicate trap data demonstrate the high precision of the method. Short-term feeding responses (integrated over hourly intervals) of scallops to environmental changes show that periodic feeding activity can account for a high proportion of daily food intake and that food quality (organic content of seston) explains (93% of the variation in absorption efficiency. Seasonal variations in scallop and mussel feeding responses (integrated over daily intervals) were monitored concurrently over four 40 day sampling periods during the spring, summer, and fall of 1995. Data are currently being analyzed and results will be discussed in the context of inter-specific variations and the relative importance of possible exogenous forcing functions (food supply, flow, and temperature) and endogenous controls (energy demands and acclimation capabilities) on bivalve feeding physiology.

FIELD AND MODELLING STUDIES OF BIVALVE CULTURE IN A BOREAL ENVIRONMENT.

Jon Grant* and **Conrad A. Pilditch,** Department of Oceanography, Dalhousie University, Halifax, Nova Scotia, Canada B0N 2S0.

In northern climates, the spring phytoplankton bloom inevitably occurs during periods of low water temperatures. Based on temperature functions of feeding, bivalves cultured under these conditions (e.g. *Mytilus edulis* in suspended culture) thus face possible temperature-inhibition of ingestion during the spring bloom. Field studies of particulate food supplies and mussel growth as well as field estimates of feeding rates indicate that (1) primary production is inhibited by ice cover, (2) winter food supplies have low organic content despite high concentrations, (3) mussels display reduced absorption efficiency with this food supply, (4) and mussels have a negative energy balance during winter. Because mussels are in poor condition at the end of winter, the spring bloom is essential in promoting summer growth. Simulation modelling of mussel scope for growth in early spring suggests that acclimation of feeding to low temperatures allows animals to take advantage of the spring bloom, with accelerated growth during this period. The timing of the spring bloom is also critical to the growth trajectory at this time. Further implications of food and temperature limitation for bivalve growth are discussed.

DELIVERY OF LOW MOLECULAR WEIGHT, WATER-SOLUBLE NUTRIENTS TO MARINE SUSPENSION FEEDERS.

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A major problem in formulating artificial diets for marine suspension-feeders is the development of microparticle types that will effectively deliver low molecular weight, water-soluble nutrients to these organisms. Retention of nutrients, such as amino acids, by alginate, carrageenan or zein microgel particles is poor, with more than 80% being lost within two minutes after suspension in seawater.

Lipid-walled microcapsules retain amino acids and other water-soluble, low molecular weight nutrients with greater efficiency than microgel particles. Lipid-walled microcapsules with walls prepared with tripalmitin retain more than 90% riboflavin after 24 h suspension in seawater. Feeding experiments indicate that shrimp (*Panaeus vannamei*) mysids are capable of breaking down tripalmitin capsule walls and liberating capsule contents for assimilation.

Unfortunately, we found that tripalmitin-walled capsules were not efficiently digested in feeding experiments with clams (*Tapes philippinarum*). Softening the capsule walls, by inclusion of 40% w/w of the triacylglyceride fraction of fish oil, improved capsule digestibility but reduced retention of encapsulated nutrients; for example, after 24 h suspension is seawater, lipid-walled capsules with walls composed of 60% w/w tripalmitin and 40% w/w fish oil retained only 21% of encapsulated oxytetracycline. Clearly, optimal microparticles for the delivery of encapsulated nutrients to suspension-feeders should show both efficient retention and digestibility of encapsulated material.

FEEDING ACTIVITY IN THE SEA SCALLOP *PLACOPECTEN MAGELLANICUS:* COMPARISON OF FIELD AND LABORATORY DATA.

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Suspension-feeding bivalves are exposed to a food supply that fluctuates unpredictably in both concentration and quality of the particles. Many studies have attempted to understand how these

bivalves are adapted to exploit their food resource, through feeding processes such as capture, selection and absorption, and maintain the flow of energy into growth and reproduction. The objective of this study was to measure the variety of feeding responses sea scallops (*Placopecten magellanicus*) exhibit over the range of environmental conditions they encounter in their natural environment. To accomplish this we exposed scallops to a complete range of temperature conditions and the natural assemblage of particles by mooring a small research vessel at various experimental sites throughout the year. To complement the field work we also studied feeding responses in the laboratory where the concentration (1, 3, 7 and 14 mg l^{-1}) and quality (25, 50, and 80% organics) of the diet were manipulated, by varying the proportions of organic and inorganic components, to simulate field conditions. Clearance rates typically decline with increases in seston concentration but appear to be independent of seston organics and ambient temperatures (0–15°C). Absorption efficiency increases proportionately with increasing seston organics, is independent of seston concentration and appears, at least initially, to be negatively related to increasing ambient temperatures. There was often close agreement between results from the field and the laboratory suggesting that fairly realistic feeding rates may be obtained using controlled diets in the laboratory.

IN SITU MEASUREMENTS OF MUSSEL (*MYTILUS EDULIS*) ENERGY ACQUISITION IN RELATION TO SESTON CONCENTRATION IN A SUBTIDAL MAINE ESTUARY: HOW IMPORTANT IS THE SHELL GAPE RESPONSE? Carter R. Newell,* Great Eastern Mussel Farms, Inc., Tenants Harbor, ME 04860, and Department of Biology, University of New Brunswick, St. John, New Brunswick, Canada E2L 4L5.

Mussel scope for growth was estimated using water pumped 100 m to a flow-through apparatus where filtration rates and respiration rates were measured during low, flood, high and ebb stages of the tide. Repeated measures ANOVA indicated significant differences in filtration rates ($p > 0.001$) between tidal stages. Time-lapse video records of pumping rate estimated by shell gape (as % maximum per individual) in situ showed a similar pattern. A sustained 2–3 hour period of reduced gape during low ambient food was observed around low tide. This was accompanied with a drop in oxygen consumption.

Under ambient conditions of 1–25 million particles per liter, filtration rate was significantly positively correlated with concentration at low concentrations, and was significantly negatively correlated with concentration at high concentrations.

Control of pumping rate using the shell gape response is a mechanism by which subtidal mussels couple maximum energy gain with maximum seston supply. Daily filtration rate is not simply a response to average daily conditions, but rather represents a dynamic response to a fluctuating seston regime.

SESTON SUPPLY TO SCALLOPS IN SUSPENDED CULTURE. C. A. Pilditch* and **J. Grant,** Oceanography Department, Dalhousie University, Halifax N.S., Canada B3H 4J1; **A. L. Mallet** and **C. E. A. Carver,** Department of Fisheries and Oceans, Biological Sciences Branch, PO Box 550 Halifax N.S., Canada B3J 2S7; **P. J. Cranford,** Department of Fisheries and Oceans, Habitat Ecology Division, Bedford Institute of Oceanography, PO Box 1006, Dartmouth N.S., Canada B2Y 4A2.

The filtration activity of dense aggregations of bivalves can locally deplete the water of seston resulting in food limited growth. Under these conditions, the currents that supply seston cannot offset seston removal due to feeding. We conducted a field and modelling study that examined the tidally driven supply of seston to a culture of suspended scallops (*Placopecten magellanicus*) in Whitehaven Harbour, Nova Scotia. The goal of the research was to predict optimal stocking densities and lease geometries which minimize food limitation.

Measurements of seston concentration, diet quality and current velocity were made over six consecutive tidal cycles inside and outside the lease. Scallop ingestion rates were estimated bihourly from biodeposition rates and changed in response to environmental forcing. Current velocities in the centre of the lease were 50% lower than those measured at the reference site. Despite the reduction in flow, seston concentrations inside the lease were not significantly lower than those measured outside the lease. Results suggest that at present stocking densities food limitation did not occur. The field data were used to parameterize a scallop feeding sink term in a one dimensional advection-diffusion model of changes in seston concentration in a lease. The model is used to suggest a optimal scallop density for the Whitehaven lease. The possibility of extending this model to two dimensions to show the effect of lease orientation relative to the predominant flow direction will be discussed. Modelling results suggest that an increase in the size of the lease, and/or stocking density, could reduce the supply of seston to growth limiting levels in the centre of the lease.

SEDIMENTING PHYTOPLANKTON AS A MAJOR FOOD SOURCE FOR SUSPENSION-FEEDING QUEEN SCALLOPS (*AEQUIPECTEN OPERCULARIS* L.) OFF ROSCOFF (WESTERN ENGLISH CHANNEL)? Gérard Thouzeau,* Frédéric Jean, and **Yolanda Del Amo,** URA 1513 CNRS, UFR Sciences et Techniques, 6 avenue Le Gorgeu, B.P. 809, 29285 BREST Cédex France.

The chlorophyll and phaeopigment content of bottom-water, sediment and gut of *Aequipecten opercularis* (Mollusca, Bivalvia) was measured together with seasonal changes in weight and reproduction of the pectinid, offshore Roscoff (western English Channel). The study site (site no. 1 of the PNOC; 48°51′00 N, 3°54′00 W; 71 m depth) belongs to the sand-gravel *Venus fasciata* community which extends on clean coarse sediments of the western English Channel, in areas generally deeper than 65–70 m.

There is no vertical stratification of the water column in summer (mixed waters); water temperature ranges from 9°C in February to 15.4°C in August. Chlorophyll concentration in surface and bottom waters is typically 2.5–3.0 $\mu g \cdot l^{-1}$ during spring bloom (May–June), vs. less than 1.0 $\mu g \cdot l^{-1}$ otherwise. Sediment organic matter content ranged from 2.5 to 6.4% in 1992 and 1993. Seasonal variations of sediment organic matter content were correlated with phytoplankton sedimenting events, emphasizing pelagic-benthic coupling. High seasonal variations were observed in the general condition of a ⟨⟨standard⟩⟩ 50 mm shell length *A. opercularis:* in 1992 and 1993, gonad dry weight, body dry weight and stomach pigment content showed a sixteen-fold, three-fold and ten-fold variation, respectively. Sedimentation of the spring phytoplankton bloom as the main regulating factor for weight increases and development of reproductive tissue is questioned since variations of pigment uptake may not correlate with variations of pelagic trophic inputs to the benthos. Chlorophyll and phaeophytin concentrations in bottom-water were highest from April to June, while stomach pigment conent was lowest (maximum values in August and September). In contrast, sedimentation of summer phytoplankton blooms would be a main regulating factor for body weight increase.

WHEN IS IT TIME TO FEED THE SCALLOPS? G. H. Wikfors, B. C. Smith, J. H. Alix, and **M. S. Dixon,** NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford, CT 06460; **M. S. Dixon,** Marine Sciences and Technology Center, University of Connecticut, Groton, CT 06340.

As part of a research program to develop biochemically-based feeding standards for post-set bay scallops, *Argopecten irradians,* we conducted an experiment to determine how often to feed post-set scallops to achieve maximal growth and feed conversion efficiency. To accomplish this experiment, we designed and built a PC-controlled fluidics system for 12 molluscan rearing chambers that permits unattended control of dis-continuous feeding in each chamber. Experimental design compared growth of 5 mm scallops on a unialgal diet of *Tetraselmis chui,* strain PLY429, fed every 24, 12, 6, or 3 hours. Daily ration and cumulative feeding time were identical for all experimental feeding regimes.

Scallops fed every 6 hours grew more rapidly than those fed more or less often. The optimal 6-hour interval corresponds with the 6-hour tidal regime in the scallop's estuarine habitat, leading us to speculate that the scallop's digestive cycle is adapted to process food on this schedule. This finding suggests that knowledge of feeding behavior in nature may provide guidelines for farming bivalve mollusks.

MOLLUSCAN NUTRITION

FEEDING BEHAVIOUR AT HIGH AND VARIABLE SESTON LOADS. Brian Bayne* and **Tony Hawkins,** Plymouth Marine Laboratory, Prospect Place, Plymouth PL1 3DH, Devon, UK.

Suspension-feeding bivalves live in a diversity of food environments in which the suspended particulate matter may vary in concentration from low (<2 mg dry mass l^{-1}) to extremely high (>100 mg l^{-1}) values; the nutritional quality of this material may also differ, from diets high in living phytoplankton to those dominated by resuspended silt of low nutritious value. Both quantity and quality of food may vary over timescales from minutes, through tidal (hours and days) to seasonal scales. Such are some of the extrinsic (= supply) variables with which the animals must cope. In addition, there are intrinsic (= demand) variables, which result in individual and species variability and add further complexity to the analysis of feeding behaviour. Feeding may be considered as a linked sequence of traits which includes: filtration of material from suspension; sorting of this material into particles that are rejected and those ingested; digestion, absorption and assimilation of nutrients from the ingesta; egestion of non-absorbed matter. We need to understand the rates and efficiencies with which each of these processes is effected under different conditions of nutrient supply. An additional challenge is to understand the causes of intrinsic variability, both between individuals and between species.

In our paper we will review recent studies on various bivalve species whose objectives have been to analyse feeding behaviour under environmentally realistic conditions. We will seek generality for the processes of suspension-feeding itself, across a spectrum of food environments and of species. And we will briefly address questions of individual and species variability that pose particular challenges for the future.

OBLIGATE ENDOSYMBIOTIC ASSOCIATIONS BETWEEN CHEMOAUTOTROPHIC BACTERIA AND MARINE BIVALVES: NUTRITIONAL IMPLICATIONS TO THE EARLY LIFE STAGES. Craig S. Cary, College of Marine Studies, University of Delaware, Lewes, DE 19958.

The occurrence of obligate symbiotic associations between bacteria and invertebrate hosts appears widespread in marine organisms. These unique symbiotic associations are particularly abundant in marine mollusks where they are now know to occur in 5 families of bivalves and 2 other orders of mollusks. Whether the bacteria reside externally (episymbiosis) or within specialized cells of the host gill tissue (endosymbiosis) it is clear than in most circumstances host development and settlement are constrained by the initial acquisition and specific energy requirements of the symbiont. Biological communities associated with deep-sea hydrothermal vents and other reducing environments inhabit a highly variable and ephemeral environment characterized by large fluxes in abiotic and biotic conditions. Consequently, the reproductive, dispersal and nutritional strategies of the resident fauna must often accommodate relatively narrow windows of opportunity. In the bivalves, this appears to be achieved through continuous and broad range dispersal capabilities coupled with the vertical transmission

of the symbionts. These chemoautotrophic symbioses range from obligatory relationships in which the adult hosts completely lack digestive tracts to those which maintain fully functional particulate feeding in adult life. Still others are thought to have a functional feeding apparatus during the early life stages which is lost in the adult form. In all cases the distribution of the host is clearly related to factors directly associated with its symbiont's energy requirements (i.e. where both reduced sulfur or methane and molecular oxygen co-occur). It is therefore conceivable that the symbionts play an active role in habitat selection and the induction of metamorphosis and settlement of the host.

Although much of the early life history of the vent organisms and their shallower water analogs has remain unresolved molecular techniques are providing some clues as to the importance of the symbiont in the early life history of the host. Nucleic acid probing technologies provide the resolution necessary to identify symbionts even within the embryos providing key information of their role in early development.

LIPID NUTRITION AND FATTY ACID SYNTHESIS IN OYSTERS. Fu-Lin E. Chu, Virginia Institute of Marine Science, School of Marine Science, College of William and Mary, Gloucester Point, VA 23062.

Lipids play a unique role in reproduction of oysters. Egg storage lipids are important for early larval development. A fatty acid metabolism study revealed that *de novo* synthesis of saturated fatty acids (C16:0 & C18:0) occurred in adult oysters with reutilization of ^{14}C-acyl groups derived from β-oxidation. However, only limited elongation and no desaturation of ^{14}C-labeled palmitic, linoleic and linolenic acids (C16:0, C18:2-n6, C18:3-n3) was observed. Similarly, $<1.0\%$ of dietary ^{14}C-18 precursors were elongated to eicosapentaenoic (EPA, C20:5-n3) and docosahexaenoic (DHA, C22:6-n3) acids in spat. For reproductive success, EPA, DHA, C18:2-n6 and C18:3-n3 have to come mainly from a dietary source. Phytoplankton is the major food source of bivalve molluscs and is considered to be the main supplier of C18:2-n6, C18:3-n3, EPA and DHA in the marine food web. During phytoplankton blooms and whenever food is available, oysters store glycogen, rather than lipid as an energy reserve. They actively assimilate dietary fatty acids into their lipids prior to or during oogenesis. The seasonal variation of omega-3 PUFAs in the visceral mass corresponded to seasonal changes in the diet. There is evidence of active assimilation/incorporation of dietary fatty acids into the neutral lipids and phospholipids of oysters. During oogenesis, oysters probably synthesize lipids using recently ingested and stored fatty acids and at the expense of stored glycogen. Seasonal variation of lipids and omega-3 polyunsaturated fatty acids (PUFAs) in the visceral mass were related to the oyster's reproductive cycle. Total lipid and omega-3 PUFAs in the visceral mass were higher prior to the spawning season than after spawning.

CILIARY SUSPENSION-FEEDING AND PARTICLE SELECTION IN MOLLUSC LARVAE. S. M. Gallager, Woods Hole Oceanographic Institution, Woods Hole, MA 02543.

Most planktonic Mollusc larvae use cilia for feeding and locomotion. A brief review of the mechanisms available to Mollusc larvae for capturing particles and propelling fluid is presented together with appropriate morphological and functional constraints placed on each process. Mollusc larvae are used as models to illustrate how more than one capture mechanism may be functioning simultaneously depending on particle size and surface properties. For example, bivalve larvae capture flagellates at the base of the cilia using hydrodynamic retention, a mechanism for enhancing direct interception between cilia and non-sticky particles. Diatoms, however, are captured at the tips of the cilia by direct interception. Adhesion between cells and the tips of the cilia is enhanced by the cells' sticky surface which changes during cell growth. Particle encounter, capture, transport to the mouth, and selection for ingestion have distinct probabilities which must be observed and quantified independently and under a wide variety of environmental conditions to obtain accurate predictions of feeding success in suspension-feeders.

OMNIVORY BY THE MUSSEL, *GEUKENSIA DEMISSA*. Daniel A. Kreeger,* Patrick Center for Environmental Research, Academy of Natural Sciences, Philadelphia, PA 19103; **Roger I. E. Newell,** Horn Point Environmental Laboratory, University of Maryland, Cambridge, MD 21613.

Suspension-feeding bivalves must derive their nutrition from a complex suite of natural microparticulate material when phytoplankton are scarce. This is especially true for the ribbed mussel, *Geukensia demissa*, which is a prominent inhabitant of the high intertidal zone of eastern USA salt marshes. Ribbed mussels have numerous physiological adaptations for the acquisition and assimilation of alternative food particles, including vascular plant detritus, bacteria (free, aggregated and attached), benthic diatoms, and heterotrophic flagellates. As part of our ongoing program to determine how this mussel meets it's carbon and nitrogen requirements, we recently measured it's ability to ingest and digest cellulolytic bacteria and various identified species of heterotrophic flagellates and benthic diatoms. Ribbed mussels ingested radiolabeled carbon from bacteria, heterotrophic flagellates and benthic diatoms with efficiencies that were 59%, 88% and 78%, respectively, compared with a their carbon ingestion rates for a reference species of phytoplankton, *Isochrysis galbana* (clone T-ISO). Furthermore, carbon was assimilated from bacteria, heterotrophic flagellates and benthic diatoms with efficiencies (42, 44% and 87%, respectively) that were comparable to that from T-ISO (77%). We are currently measuring seasonal variation in the abundance of these alternative foodstuffs in situ to estimate their relative contributions to the mussel's carbon and nitrogen demand on an annual basis. Nevertheless, in comparison to

other suspension-feeding bivalves. *G. demissa* clearly has an exceptional capacity for omnivory, which could be a critical nutritional adaptation for life in a detritus-rich, phytoplankton-poor habitat.

HOW ARE BIVALVE BROODSTOCK AND LARVAE ADAPTED TO MEET THEIR NUTRITIONAL REQUIREMENTS FOR LIPIDS. **Philippe Soudant, Yanic Marty, Jean Rene Le Coz, Jeanne Moal,** and **Jean Francois Samain,*** Institut Francais pour l'Exploitation de la mer (IFREMER), Centre de Brest, BP 70, 29280, Plouzane, France.

Three microalgal diets, differing in composition in the levels of 20:4(n-6), 20:5(n-3) and 22:6(n-3) fatty acids and in sterol content, were used to study the nutritional lipid requirements for reproduction and larval development of the scallop *Pecten maximus*. A mixture of *Isochrysis galbana* (clone T. Iso) (T) and *Chaetoceros calcitrans* (C) plus *Pavlova lutheri* (P) and *Skeletonema costatum* (S) was fed to broodstock, and a mixed diet (PTS) was fed to larvae. Separation of the different polar lipids was performed using HPLC, then GC for their fatty acid composition. Phospatidyl choline (PC) and plasmalogens (PLSM) were the major classes identified, phosphatidyl inositol (PI), phosphatidyl serine (PS), phosphatidyl ethanolamine (PE), and a glycolipid (GLY) were minor classes.

Pecten maximus appears to regulate the composition of its polar lipid and sterol classes under different dietary conditions. In the PC class, 20:5(n-3) was not accumulated, but 22:6(n-3) and 20:4(n-6) were selectively accumulated compared to levels in the diet. The same fatty acids were selectively retained in plasmalogens when their concentration in the food was low, and levels of 22:6(n-3) and 20:5(n-3) differed in oocyte and sperm. Lastly, high selectivity was observed for 20:4(n-6) in PI, 22:6(n-3) in GLY, and 20:5(n-3) in PE, resulting in nearly constant levels that were almost independent of food composition. In the same way, cholesterol was preferentially concentrated, compared to other phytosterols, when the diet was deficient in this sterol. These differences in fatty acid, sterol or lipid class selectivities are discussed in reference to known or putative requirements for reproduction and larval development.

LOOKING INTO THE "BLACK BOX": FEEDING STRATEGIES AND LIMITATIONS OF SUSPENSION-FEEDING BIVALVES. **Evan J. Ward,*** Department of Biological Sciences, Salisbury State University, Salisbury, MD 21801; **Jeffrey Levinton,** Department of Ecology & Evolution, S.U.N.Y., Stony Brook, NY., 11794; **Sandra Shumway,** Natural Science Division, Southampton College, Southampton, NY 11968; **Terri Cucci,** Bigelow Laboratory for Ocean Sciences, Boothbay Harbor, ME 04575.

Benthic particle feeders are exposed to a food supply that varies in quality and quantity along both spatial and temporal scales. Previous studies have shown that bivalves deal with such fluctuating particle regimes in a variety of ways, including adjustments in pumping and ingestion rates, and rejection of non-nutritive particles as pseudofeces. Data such as these have led researchers to develop and test models of feeding compensation for changes in particle supply, but most models treat pallial organ processes like a "black box" with an input (capture/collection), one branch (pseudofeces) and an output (ingestion). There is evidence, however, that significant adjustments occur at a much finer scale.

To demonstrate some of these fine scale adjustments, we exposed the oysters *Crassostrea virginica* and *C. gigas* to a mixture of ground, aged *Spartina* sp. (3–10 μm) and similar sized phytoplankton (*Rhodomonas* sp. or *Tetraselmis* sp.) at three concentrations (10^3, 10^4, and 10^5 particles ml^{-1}). We then examined the gills and labial palps by means of endoscopy and sampled, *in vivo*, particulate material from various ciliated tracts. These samples were then analyzed with a flow cytometer. Preliminary results indicate that in oysters the gill is the main sorting organ, whereas the labial palps function more for controlling the volume of material to be ingested. Our study is the first to take a truly functional approach in modeling the way in which pallial structures respond to changing particle fields. Studies such as these will lead to a better understanding of pallial organ function, and help define the influence of bivalves on trophic dynamics of benthic ecosystems.

MOLLUSCAN REPRODUCTION

INDUCTION OF TRIPLOIDY IN UNCONDITIONED EASTERN OYSTERS, *CRASSOSTREA VIRGINICA*, USING NITROUS OXIDE UNDER INCREASED PRESSURE. **James W. Anderson*** and **Richard K. Wallace,** Auburn University Marine Extension and Research Center, 4170 Commander's Drive, Mobile, AL 36615.

Triploidy is commonly induced using cytochalasin B in Pacific oysters, *Crassostrea gigas*, to increase production. Use of cytochalasin B has raised safety concerns and there is interest in developing alternate methods for producing triploid oysters. Eastern oysters, *Crassostrea virginica*, were collected from Mobile Bay, Alabama and spawned by manually extracting the gametes. Eggs were treated with nitrous oxide at several pressures ranging from 0 to 19 atm. Initiation time and duration depended on development of the fertilized eggs. Triploidy was induced in up to 56.8% of the larvae, using a 15 min nitrous oxide treatment at 7 atm. When nitrous oxide was applied at ambient pressure, there was a maximum triploidy at 8.3%.

Effects of treating eggs for different durations were examined. Triploidy in the 20 min duration group (38.7%) was significantly different from the 10 min duration (16.0%) and control (6.0%) groups. Average survival rates to straight-hinge were 5, 22, and 52% for the 20 min duration, 10 min duration, and control groups, respectively. Nitrous oxide under pressure can induce triploidy in Eastern oysters when treatment conditions are ideal.

RECRUITMENT PATTERNS OF *MYA ARENARIA* L. FROM EASTERN AND SOUTHWESTERN MAINE: II. EFFECTS OF SITE, TIDAL HEIGHT, AND PREDATOR EXCLUSION. Brian F. Beal* and **K. W. Vencile,** University of Maine at Machias, 9 O'Brien Avenue, Machias, ME 04654; **Stephen R. Fegley,** Maine Maritime Academy, Castine, ME 04421.

Soft-shell clam landings in Main declined 67% from 450,000 bushels harvested in 1983 to less than 150,000 in 1994. The decline was spatially variable along the coast as landings in eastern Maine, where, historically 45–65% of all clams are harvested annually, dropped by 90% during this eleven-year period, but increased a modest 15% over the same time in southwestern portions of the coast. Reasons for the decline were not attributable to differences in harvesting pressure between the two coastal regions. We invoke three competing hypotheses to explain differences in relative clam abundances between the two regions: 1) differences in fecundity, 2) differences in larval survival, and 3) differences in post-settlement abundance and/or mortality. We employed a full-factorial, completely randomized block design at six intertidal sites in eastern Maine and six sites in southwestern Maine to examine the effects of substrate type (mud vs. sand), tidal height (low vs. high), and predator exclusion (protected vs. unprotected) on post-settlement survival success. If no apparent differences in post-settlement abundance/mortality exist between regions, two hypotheses remain to be tested.

Beginning in late March and continuing through April, 1995, we initiated a "long-term" experiment to examine the cumulative effects of settlement processes at each of twelve sites along the Maine coast. At each site, 120 plastic flower pots (11 cm diameter × 9.3 cm deep) filled with terrestrial "mason" sand (mean ϕ-diameter = 2) were pushed flush into the sediments near both high and low tide levels. At each tidal height, we employed 30 replicate blocks of four pots each in a 2 × 2 matrix. Two pots within each block were covered with a flexible, black plastic netting (aperture = 6.4 mm) to exclude large epibenthic predators. The remaining two pots within each block remained uncovered during the experimental period which extended to early November, 1995. Blocks were spaced at approximate 2-m intervals whereas pots within each block were spaced at approximate 1-m intervals.

Preliminary results indicate the importance of the block design in assessing spatial variability within each site. Although no apparent differences between protected and unprotected treatments were detected, significant tidal height effects occurred at most sites, but no consistent pattern was observed. Regional differences in settlement abundance may explain the disparity in landings between eastern and southwestern Maine. Regardless of tidal height or meshing treatment, fewer 0-year class individuals were found at easter Maine sites than those in the south-w

RECRUITMENT PATTERNS OF *MYA ARENARIA* L. FROM EASTERN AND SOUTHWESTERN MAINE: I. SHORT-TERM EFFECTS OF SITE, TIDAL HEIGHT, AND PREDATOR EXCLUSION. Stephen R. Fegley, Maine Maritime Academy, Castine, ME 04420; **Brian F. Beal** and **K. W. Vencile,** University of Maine at Machias, 9 O'Brien Ave., Machias ME 04654.

From 1970 through 1984 annual landings of soft-shell clams in eastern Maine ranged from 150,000 to 250,000 bushels. Over the same period harvests of soft-shell clams in southwestern Maine consistently fell below 40,000 bushels. Since 1985 landings in eastern Maine declined to less than 50,000 bushels per year (>67% decrease) while annual landings in southwestern Maine have expanded to over 50,000 bushels (~15% increase). Reasons for the changes in landings cannot be attributed to regional differences in either relative harvest pressure or to clamflat closures: lower abundances of soft-shell clams currently exist in eastern Maine where, historically 45–65% of all clams harvested in Maine were taken. Anecdotal and direct observations of intertidal flats indicated that eastern Maine soft-shell clam populations experienced repeated recruitment failures in the past decade while southwestern clam populations displayed fairly consistent annual recruitment. We questioned whether the absence of visible clam spat in eastern Maine occurred because of a failure of soft-shell clam larvae to reach the flats or because of high mortality of recently settled juveniles. To distinguish between these alternatives we employed a full-factorial, completely randomized block design that varied site (two sites in eastern Maine versus two sites in southwestern Maine), tidal height (mid versus low), and predator exclusion (protected versus unprotected).

In mid April we placed, at each tidal height at each site, 80 plastic flower pots (11 cm diameter × 9.3 cm high) filled with "mason" sand (mean ϕ diameter = 2) arrayed into 20 replicate blocks of four pots each in a 2 × 2 matrix. The pots were buried flush to the sediment surface and two pots within each block were covered with flexible, black plastic netting (aperature = 6.4 mm) to exclude large predators. At approximately two week intervals through August (and biweekly or monthly, depending on site, until early November) all pots at all sites were replaced with new pots filled with mason sand. The exposed pots were returned to the laboratory. The top 1 cm of sediments were scraped into plastic vials and fixed with formalin. The remaining sediment in each pot was sieved through a 1.3 mm mesh to retrieve all bivalves and bivalve predators. Bivalves were separated from the preserved sediment using a flotation technique utilizing dense sucrose solutions.

Peak settlement of *Mya* occurred from mid to late July in southwestern Maine and from late August to early September in eastern Maine. Average abundances were generally two orders of magnitude greater in southwestern Maine sites than in those in eastern Maine. Preliminary evidence provides no strong indication that predator exclusion or tidal height had consistent effects on initial

settlement and immediate (less than two week) *Mya* survival. Apparently the difference in abundance between southwestern and eastern Maine *Mya* populations begins prior to larval settlement from the plankton.

ESTIMATES OF RECRUITMENT AND ADULT ABUNDANCE IN THREE FLORIDA POPULATIONS OF BAY SCALLOPS (*ARGOPECTEN IRRADIANS*). Dan C. Marelli,* William S. Arnold, Catherine Bray, and **Melissa Harrison,** Florida Department of Environmental Protection, Florida Marine Research Institute, 100 8th Avenue SE, St. Petersburg, FL 33701.

Bay scallops occur in a series of disjunct populations along the Florida Gulf coast from Pensacola to Florida Bay in the Florida Keys. Each population is associated with a coastal basin, and these basins undoubtedly influence the larval-retention mechanisms responsible for the interannual maintenance of bay scallop populations. Recruitment in three Florida populations of bay scallops (*Argopecten irradians*) was examined using artificial spat collectors during 1994 and 1995. We have conducted surveys of adult densities and distributions in the same populations to examine the relationship between recruitment and adult population characteristics within and between basins. In 1994, we examined gonadal development to determine the relationship between spawning and recruitment in each of the populations. We have not seen a strong relationship between adult densities and levels of recruitment to artificial collectors in any of the three populations examined. Within basins, a stronger relationship appears to exist between recruitment and strength of the subsequent adult year-class. These data suggest that recruitment monitoring may allow us to make short-term predictions of adult bay scallop stock levels in Florida populations. Further, we speculate that this predictive ability could become important in making management decisions regarding recreational-harvest regulations.

VARIATIONS IN GAMETOGENESIS AND SEX RATIOS IN OYSTERS ALONG AN INTERTIDAL GRADIENT. Francis X. O'Beirn* and **Randal L. Walker,** Shellfish Aquaculture Laboratory, University of Georgia, Marine Extension Service, 20 Ocean Science Circle, Savannah, GA 31411–1011.

The spawning seasons of oysters in the southeastern U.S. extends from April through September. This study explored the possibility that different gametogenic maturation rates along an intertidal gradient was responsible for this prolonged season. Twenty oysters were taken on a biweekly basis from two tidal heights (high intertidal HI and low-intertidal LI) at each of two sites (House Creek HC and Skidaway River SK) in Wassaw Sound, Georgia, from June 1993 to September 1994. Gametogenic condition was evaluated by histological analysis of the gonads and image analysis. There was little or no retardation in gametogenic maturation and spawnings in the HI oysters. Also, the HI oysters tended to maintain higher gametogenic parameters throughout the year than the LI oysters. Multiple spawning events at each tidal height were also witnessed. Therefore, it is concluded that both prolonged maturation and multiple spawnings account for the extended spawning season. Sex ratios differed at the two tidal heights. Females were proportionally more abundant among oysters in high intertidal areas (3.45:1-HC, 3.12:1-SK vs 1.95:1-HC, 1.85:1-SK at HI and LI, respectively). The higher proportion of LI males was attributed to stresses on the oysters induced by a probable combination of predation, siltation, disease, and competition. These oysters, therefore, would have to allocate resources to repair and maintenance rather than gamete formation.

ANALYSES OF GONADAL CYCLING BY OYSTER BROODSTOCK, *CRASSOSTREA VIRGINICA* (GMELIN), IN LOUISIANA. John E. Supan,* Office of Sea Grant Development, L.S.U., Baton Rouge, LA 70803; **Charles A. Wilson,** Coastal Fisheries Institute, L.S.U., Baton Rouge, LA 70803.

Oysters held nearshore in Caminada Bay during the summer, typically exhibit hypertrophic gonads with prominent genital canals beneath translucent mantle tissue about four weeks post-hatchery spawning, indicating recycling. Broodstock ($N = 200$) were analyzed histologically over a two year period to document such gametogenesis, using Gonad/Body Ratios (GBR) and developmental stages. Ten oysters were randomly selected from a broodstock pool prior to each spawning attempt, and monthly during the winter-spring of 1992. As expected, the mean GBR before successful spawning attempts was significantly greater ($P \leq 0.05$) than the mean GBR before unsuccessful attempts. A dramatic drop in the percent occurrence of the advanced spawning and regression stage from May to June, a steady spawning stage occurrence, and fluctuations in the percent occurrence of early and later developmental stages during the summer months illustrates gonadal recycling during June–October.

THE EFFECT OF SALINITY CHANGE ON THE SYNCHRONY OF POLAR BODY DEVELOPMENT IN FERTILIZED OYSTER EGGS (*CRASSOSTREA VIRGINICA* [GMELIN]). John E. Supan, Office of Sea Grant Development, L.S.U., Baton Rouge, LA 70803; **Charles A. Wilson,*** Coastal Fisheries Institute, L.S.U., Baton Rouge, LA 70803; **Standish K. Allen, Jr.,** Haskin Shellfish Research Lab., Rutgers University, Port Norris, NJ 08349.

Broodstock, acclimated (1 week) to 13, 20, and 30%, were strip spawned at source salinity and the resultant eggs exposed to 3 different salinities (10, 20, and 30%) to evaluate the effect of rapid salinity change on egg development (synchrony = [the number of embryos observed at metaphase I] − [the number of embryos observed past metaphase I] ÷ [the total number of embryos counted]). Bonferroni pairwise comparison procedures were used to test for significant differences ($\alpha = 0.001$) between mean synchrony levels of treatment* broodstock interactions at mean de-

velopment time. A covariance model (synchrony = treatment salinity | broodstock salinity | development time) proved to be appropriate for defining the relationship between synchrony and changing salinity. High levels and rates of synchrony were achieved when the treatment salinity ≥ broodstock salinity, except when the broodstock salinity was 13‰.

THE EFFECT OF CYTOCHALASIN B (CB) DOSAGE IN THE SURVIVAL AND PLOIDY OF *CRASSOSTREA VIRGINICA* (GMELIN) LARVAE IN LOUISIANA. **John E. Supan,*** Office of Sea Grant Development, L.S.U., Baton Rouge, LA 70803; **Charles A. Wilson,** Coastal Fisheries Institute, L.S.U., Baton Rouge, LA 70803; **Standish K. Allen, Jr.,** Haskin Shellfish Research Lab., Rutgers University, Port Norris, NJ, 08349.

Survival and ploidy of D-stage oyster larvae were determined following the rearing of embryos exposed to CB dosages of 0.5 mg/l, 0.25 mg/l and 0.125 mg/l for 10–15 minutes, with 0.05% DMSO and ambient seawater as controls. Since timing did not permit true replication, the experiment was conducted three times on the same day with the same procedures and partially stripping the same male oysters; only different females were used. Treatments began when about 50% of the eggs reached PBI (24–31 min). Embryos were reared for 48-hrs at ambient temperature. Mean triploid percentages were 13% ± 6.7% (0.125 mgCB/l), 61.8% ± 6.2% (0.25 mgCB/l), and 68.2% ± 14.1% (0.5 mgCB/l). No significant difference ($P \leq 0.05$) in mean survival was found between the three CB treatments. Significant differences in mean survival between the three experiments (all dosages and controls combined) implies variability due to different sources of eggs.

A COMPARISON OF ARTIFICIAL SPAT COLLECTORS IN THE WESTPORT RIVER, MA. **Karin A. Tammi*** and **Michael A. Rice,** Department of Fisheries Animal and Veterinary Science, University of Rhode Island, Kingston, RI 02881; **Wayne H. Turner** and **Bethany A. Starr,** Water Works Group, P.O. Box 197 Westport Point, Ma. 02791.

Since 1993, the Bay Scallop Restoration Project has utilized 5,200 artificial spat collectors (>4 mm plastic mesh onion bags containing monofilament) for stock enhancement of the bay scallop, *Argopecten irradians* in the Westport River, Massachusetts. Researchers observed that poor scallop recruitment estimates may be attributed to crab predation and fouling inside the collectors. Research conducted in 1995 compared the performance of the original onion bag collector with that of a commercial fine (1.5 mm to 3.0 mm) mesh collector containing a poly-ethylene tube (40 cm × 80 cm) as the settlement substrate. Longlines consisting of 20 collectors, 10 of each bag type, were deployed at Coreys Island for a period of 4 weeks. After 1 month soaking time, longlines were harvested to assess scallop recruitment, crab abundance and fouling in each bag. The fine mesh collector displayed a significantly ($p < 0.05$) higher recruitment with a total of 887 scallops compared to 278 from the onion bags (50 collectors each). The fine mesh collector displayed a higher recruitment estimate averaging 18.1 scallops per collector, compared to 5.9 scallops per onion bag. Fine mesh bags had fewer mud crabs, *Panopeus* spp. < 1 mm in carapace length compared to the onion bag which had more crabs of larger size. Fouling was similar for both bag types, but the fine mesh collector appeared to have more siltation and algal fouling than the onion bag. This study indicates that the onion bag made from donated materials is a poorer spat collector design because it allows mud crab colonization, thus increasing the predation on newly settled scallops and the monofilament may settle when suspended in the water column, reducing the surface area available for larval settlement. Scallop recruitment estimates based on onion bag collectors may underestimate actual recruitment rates of scallops.

HISTOLOGICAL STUDY OF REPRODUCTION IN *ARGOPECTEN VENTRICOSUS*. **Janzel R. Villalaz,*** Departamento de Biologia, Acuática, Universidad de Panamá, Panamá.

A laboratory study was carried out in Delaware to observe changes in reproduction of *Argopecten ventricosus* by using histological techniques in gonads. During 66 days, combinations of monocultures (50:50) of C-1S0 and CH-1 were added daily to a tank with filtered and aerated seawater. Salinity and temperature of the water were measured with a salinity-temperature probe meter (YSI). Phytoplankton densities were recorded by direct count with a hematocytometer. This study is a contribution to the reproductive biology of *A. ventricosus* and fisheries management of the tropical scallop.

MARINE GENETICS

NUCLEAR DNA MARKERS FOR *CRASSOSTREA* SPECIES IDENTIFICATION. **Patrick M. Gaffney,*** College of Marine Studies, University of Delaware, Lewes, DE 11958; **Francis X. O'Beirn,** Department of Fisheries and Wildlife, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061–0321.

Unambiguous diagnostic methods for identification of cupped oyster (*Crassostrea*) species at all life stages are useful in a variety of purposes. These include genetic improvement programs involving hybridization or gene transfer, conservation of endangered broodstocks, and ecological monitoring of exotic species invasions. We present methods for identification of five *Crassostrea* species (*C. virginica*, *C. gigas*, *C. ariakensis* (*rivularis*), *C. sikamea* and *C. rhizophorae*) by restriction fragment length polymorphism (RFLP) analysis of both mitochondrial (16S rDNA, cytochrome oxidase I) and nuclear ribosomal genes (28S, ITS-1 and ITS-2) amplified by the polymerase chain reaction (PCR). We describe methods for analysis of individual eggs and larvae as well

as adult tissues, including hemolymph, which can be used for nondestructive identification of oysters. We illustrate the use of these methods to identify viable interspecific hybrids (*C. gigas* × *C. silkamea* and *C. gigas* × *C. ariakensis*) and to document unsuccessful attempts at hybridization of *C. virginica* with *C. gigas* and *C. ariakensis*.

GENETICS OF SEX DETERMINATION IN *CRASSOSTREA* OYSTERS: A SINGLE LOCUS MODEL. **Ximing Guo*** and **Standish K. Allen, Jr.**, Haskin Shellfish Research Laboratory, Institute of Marine of Coastal Sciences, Rutgers University, B-8, Port Norris, NJ 08349; **Dennis Hedgecock,** Bodega Marine Laboratory, University of California at Davis, Bodega Bay, CA 94923; **William K. Hershberger,** School of Fisheries, University of Washington, Seattle, WA 98105; **Kenneth Cooper,** DBI Consulting, 24888 Taree Drive NE, Kingston, WA 98346.

A unique feature of sex in the *Crassostrea* oysters at the coexistence of dioeciousism, sex change and functional hermaphroditism. To determine whether such a system is genetically controlled, we analyzed the sex ratio of over 100 factorial and nested families of the Pacific oyster, *Crassostrea gigas* Thunberg. The overall female percentages of one, two and three-year old oysters were 37%, 55% and 75%, respectively. The increasing female percentages with time suggest that there were a significant proportion of oysters that matured as males first and changed to females in later years. Detailed analysis of family sex ratio revealed significant family differences and parental effects, suggesting significant genetic control. Further, family sex ratios tended to distribute in four fragmented groups. Those and other data from the literature could be explained by a single locus model of sex determination. In this single locus model, there are two alleles: allele Y for maleness and X for femaleness, so that YY is true male, XX is true female, and XY matures as male first and can change sex later on. The three genotypes can produce four types of families under random mating. The sex change of XY oysters may be further influenced by other genetic and environmental factors, which often makes the four types of families less distinctive. Interestingly, this model of sex determination can account for the evolution of both XY and ZW types of sex determination, depending which allele gains dominance.

HYBRID VIGOR IS PERVASIVE IN CROSSES AMONG INBRED LINES OF PACIFIC OYSTERS. **Dennis Hedgecock,*** University of California, Davis, Bodega Marine Laboratory, Bodega Bay, CA 94923–0247.

The genetic and physiological bases of hybrid vigor (heterosis) are poorly understood even for major crops. Two alternative genetic explanations for heterosis have co-existed for nearly 80 years: dominance and overdominance. In the 1980s, this debate was renewed over numerous reports for bivalve molluscs that individual heterozygosity at allozyme-coding loci was positively correlated with fitness-related traits, primarily growth rate. Because this debate is not likely to be resolved without experiments, we have taken a classical approach to the study of heterosis in the Pacific oyster *Crassostrea gigas*, controlled crosses among inbred lines. In such mating experiments, heterosis (or potence, h_p) for one or more traits can be defined and quantified as $Q/L > 1.0$, where L is the difference between the trait values of the two parental inbred lines and Q is twice the deviation of the hybrid from the mid-parent value (Griffing 1990 *Genetics* 126:753).

Over 50 inbred lines of Pacific oyster have now been initiated by selfing of simultaneous hermaphrodites, self-fertilization with cryopreserved sperm after sex-reversal, or by brother-sister matings. Two sets of 2 × 2 crosses were made in both 1993 and 1994 and two sets of 3 × 3 crosses were made in 1995. Observations to date of larval survival and growth (or size-at-age) in larval, juvenile, and adult stages indicate that non-additive gene action and heterosis for these traits are pervasive. Potence values for growth or size-at-age are always significantly greater than zero and rarely less than 1.0; in the majority of cases h_p is significantly greater than 1.0, indicating heterosis. These observations alone do not discriminate between the dominance or overdominance hypotheses, although one case of negative heterosis suggests a third hypothesis, epistasis. The F_1 hybrids from these crosses are being reared in Tomales Bay, CA.

Mapping of quantitative trait loci (QTL) for heterosis will be done by following the segregation of protein and DNA markers and their associations with growth in the F_2 and backcross generations, as described in the abstract of McGOLDRICK and HEDGECOCK. Whether these QTL have dominant, overdominant or epistatic effects on growth can also be assessed, so this study should permit resolution of the causes of allozyme heterozygosity-fitness correlations. Ultimately, we want to know whether or not non-additive gene action will retard or prevent response to family selection and implicate crossbreeding as an important component of a genetic improvement program for farmed Pacific oysters.

MITOCHONDRIAL DNA VARIATION WITHIN AND AMONG LARVAL COHORTS OF PACIFIC OYSTER, *CRASSOSTREA GIGAS*, DETECTED BY PCR-SSCP ANALYSIS. **Gang Li*** and **Dennis Hedgecock,** Bodega Marine Laboratory, University of California at Davis, P.O. Box 247, Bodega Bay, CA 94923.

Detailed studies of genetic composition within and among cohorts of larvae produced by a semi-isolated population of Pacific oysters in Dabob Bay, Wa, are needed to test the hypothesis that large variance in reproductive success causes a previously reported 10^4-fold discrepancy between effective and actual population sizes.

We cloned and sequenced part of the mitochondrial genome of *Crassostrea gigas* and developed PCR primers to amplify, from individual larvae, four fragments totalling about 2.3 kb in length, or 13% of the genome. Each fragment was digested into

smaller pieces and screened for nucleotide-sequence variation by methods for detecting single-strand conformation polymorphism (SSCP).

Three temporal plankton samples from Quilcene Bay and two from north Dabob Bay (total N = 519) were surveyed by PCR-SSCP. A common haplotype is shared by 70–84% of all larvae sampled, so Monte-Carlo contingency chi-square methods are used to test the independence of haplotype frequency and sample. Quilcene Bay larval samples are homogeneous, but the north Dabob Bay larval samples are not. Haplotype frequencies in a cohort of larvae produced after mid-August in north Dabob Bay differ from those in a cohort that appeared in early August in both sites.

ATTEMPTED HYBRIDIZATION OF EASTERN AND PACIFIC OYSTERS USING BRIDGING CROSSES. Suifen Lyu,* Standish K. Allen, Jr., and **Gregory A. Debrosse,** Haskin Shellfish Research Laboratory, Rutgers University, Port Norris, NJ, 08349; **Patrick M. Gaffney,** College of Marine Studies, University of Delaware, Lewes, DE 19958.

Past attempts to cross the eastern oyster (*Crassostrea virginica*) with either of the Pacific oysters, *Crassostrea gigas* or *C. rivularis* have failed. In plants, where hybrids are not obtainable by direct means, hybridization can sometimes be carried out using new races or indirectly through bridging crosses: crosses within or between species meant to bridge the incongruity of species. The objective of this research was to test the use of bridging crosses in breaking the incompatibility between eastern and Pacific oysters. Initial experiments were done on the effect of sperm density on fertilization to determine appropriate sperm densities for hybrid crosses. About twice as much heterologous sperm is needed. For attempts with bridging crosses, 17 different varieties of oysters from three species were used. Moderate to high fertilization rates were common in most hybrid crosses. Straight-hinge larvae were obtained from all, but none survived in any hybrid combination. The effects of varying degrees of heterozygosity among the bridging crosses were tested for their effect on fertilization and survival. Highly heterozygous hybrid crosses were no more effective in breaking down hybrid barriers than simple crosses. Apparently the genetic distance, and consequently the incongruity, between eastern and Pacific oysters is too great.

MICROSATELLITE MARKER DEVELOPMENT IN THE PACIFIC OYSTER (*CRASSOSTREA GIGAS*): VARIABILITY TRANSMISSION, LINKAGE AND QTL MAPPING. Daniel J. McGoldrick* and **Dennis Hedgecock,** University of California, Davis-Bodega Marine Laboratory, P.O. Box 247 Bodega Bay, CA 94923.

The development of classes of highly variable, nuclear markers that can be assayed by the polymerase chain reaction (PCR) complements other areas of research including physiological measurement, population genetics, the inheritance of quantitative traits, and is integral to addressing a wide range of basic and applied biological questions e.g. "What is genetic basis of hybrid vigor in Pacific oysters?". To expand the usefulness of molecular approaches in addressing this question, I have cloned and sequenced several microsatellite inserts from a size-fractionated, oyster genomic DNA library. Of some 110 positive clones that I have identified, 50% have yielded PCR primer sets.

Testing has revealed that these microsatellites are indeed highly variable, derived from single loci, are co-dominant, and simply inherited, making them very suitable for linkage and mapping studies in *C. gigas*. I report the known linkage relationships among these microsatellites as well as 25 allozyme loci. Lastly, utilizing the best information from 24 backcross and intercross populations, I present developments in generating the quantitative trait locus (QTL) map for hybrid vigor in the Pacific oyster.

THE ROLE OF PHYLOGENETIC DISTANCE ON THE DISRUPTION OF DOUBLY UNIPARENTAL mtDNA INHERITANCE IN HYBRID MUSSEL (*MYTILUS*) POPULATIONS. P. D. Rawson and **T. J. Hilbish,** Dept. Biological Sciences, University of South Carolina, Columbia, SC 29208.

Blue mussels in the *Mytilus edulis* species complex have a doubly uniparental mode of mtDNA inheritance with separate maternal and paternal mtDNA lineages. Female mussels inherit their mtDNA solely from their mother while males inherit mtDNA from both parents. In the male gonad the paternal mtDNA is preferentially replicated so that only paternal mtDNA is transmitted from fathers to sons. Hybridization is common among differentiated blue mussel taxa; whenever it involves *M. trossulus* doubly uniparental mtDNA inheritance is disrupted. We have found high frequencies of males without and females with paternal mtDNA among hybrid mussels produced by interspecific matings between *M. galloprovincialis* and *M. trossulus*. In contrast, hybridization between *M. galloprovincialis* and *M. edulis* does not affect doubly uniparental inheritance, indicating a difference in the degree to which the mechanisms regulating mtDNA inheritance have diverged among the three blue mussel taxa. Our data indicate a high frequency of disrupted mtDNA transmission in F_1 hybrids and also suggest that two separate mechanisms, one regulating the transmission of paternal mtDNA to males and another inhibiting the establishment of paternal mtDNA in females, act to regulate doubly uniparental inheritance. We propose a model for the regulation of doubly uniparental inheritance which is consistent with these observations.

PERFORMANCE OF TRIPLOID OYSTERS, *CRASSOSTREA VIRGINICA*, GROWN BY PROJECT O.C.E.A.N. PARTICIPANTS. John Scarpa,* Leslie Sturmer, Everette Quesenberry, Ross Longley, and **David Vaughan,** Harbor Branch Oceanographic Inst., Ft. Pierce, FL 34946.

The use of triploid oysters to offset the poor quality of diploid oysters in the summer was tested in Cedar Key, Florida. One inch triploid oyster seed was distributed to 25 Project O.C.E.A.N.

participants in June 1994. Every three months 5–6 groups were sampled (n = 30 oysters/sample) and compared to either hatchery produced diploids or wild diploids. The triploid group was found to contain >90% triploids, therefore data of diploids was not removed from each data set. A condition index (CI) was calculated using a ratio of dry meat weight to dry shell weight multiplied by 100. In December, after six months of culture, the triploids outperformed the diploids in length (triploid/diploid: 78/68 mm), whole weight (56/40 g), dry meat weight (1.8/1.2 g) and dry shell weight (36.5/24.1 g), but not in CI (5.0/5.0) and prevalence of Dermo (28/7%). During the April sampling it was noted that mortality reached virtually 100% for one triploid group and the hatchery diploid group, therefore the June sample was compared to wild diploids of similar size and weight. In June the triploids had a higher dry meat weight (4.2/2.1 g) and CI (5.4/3.2) and lower prevalence of DERMO (65/96%). Although variability was evident among triploid groups, indicating local environmental differences as well as differences in culture practices between growers, the value of triploidy for producing highly quality oysters in the summer in Florida is evident.

DGGE REVEALS ADDITIONAL POPULATION STRUCTURE IN AMERICAN OYSTER (*CRASSOSTREA VIRGINICA*) POPULATIONS. Jeffrey R. Wakefield* and **Patrick M. Gaffney,** College of Marine Studies, University of Delaware, Lewes, DE 19958.

Sequence variation in the mitochondrial large subunit (16S) ribosomal gene of the American oyster (*Crassostrea virginica*) was investigated utilizing denaturing gradient gel electrophoresis (DGGE) and direct sequencing methods. The complete sequence of a 360 base pair fragment was characterized in 205 individuals from 21 populations. Three haplotypes (Gulf Coast, South Atlantic, and North Atlantic) accounted for 97% of oysters sampled from Maine to Mexico and displayed a high degree of geographic structuring. In contrast, a sample from Prince Edward Island (East River) Canada exhibited seven haplotypes with no single haplotype found in more than 5 of the 25 individuals assayed. We present a phylogeographic interpretation of these findings and discuss their implications for aquacultural operations.

GEOGRAPHIC VARIATION IN MORPHOLOGY OF THE BAY SCALLOP, *ARGOPECTEN IRRADIANS* (LAMARCK). Ami E. Wilbur* and **Patrick M. Gaffney,** College of Marine Studies, University of Delaware, Lewes, DE 19958.

The bay scallop, *Argopecten irradians*, exhibits extensive variation in morphology among geographically separated populations, resulting in the recognition of three major subspecies (*A. i. irradians, A. i. concentricus, A. i. amplicostatus*). The extent to which the morphological variation results from differing environmental conditions throughout the bay scallop's considerable geographic range is unknown. In this study scallops from Massachusetts, North Carolina, Florida and Texas were collected, spawned and the offspring reared in a common environment to determine if scallops raised under similar conditions exhibited the morphology expected given the geographic origin of their parents. Significant differences among populations were indicated by ANOVA in both wild-caught (13 of 14 morphological characters) and cultured (11 of 14 characters) scallops. Principal components analysis resulted in the clustering of individuals according to geographical origin, even when scallops were reared in a common environment. The morphological characters most influential in the clustering were plical width, plical number, interplical distance, and valve convexity. Geographical variation in morphology appears to have a strong genetic basis and reflects significant genetic differentiation among geographically separated populations of bay scallops.

OYSTER DISEASE RESEARCH PROGRAM

GLYCOSIDASES IN *PERKINSUS MARINUS:* PURIFICATION AND CHARACTERIZATION OF β-D-GLUCOSIDASE. Hafiz Ahmed* and **Gerardo R. Vasta,** Center of Marine Biotechnology, University of Maryland Biotechnology Institute, 701 E. Pratt St., Columbus Center, Suite 236, Baltimore, MD 21202.

The mass mortalities of oysters (*Crassostrea virginica*) in the Chesapeake Bay are due to infection by the apicomplexan parasite *Perkinsus marinus*. It remains unclear how the parasite gains entry into the host and avoids destruction by phagocytic cells, as well as the pathogenesis mechanisms. In prokaryotic and eukaryotic microorganisms, glycosidase activity has been associated with enhanced virulence. We have detected several cell surface glycosidases in *in vitro* propagated *P. marinus*, that may play a role in the host-parasite interactions. Of sixteen glycosidase activities tested in *P. marinus* cell extracts, activity was found for (in decreasing order) β-glucoside, β-xylosidase, N-acetyl β-glucosaminidase and N-acetyl β-galactosaminidase, whereas in the spend medium, the maximum activity was observed for N-acetyl β-glucosaminidase. The enzymatic activity was optimal at the following pH values: 4.0 for β-glucosidase, 5.0–7.0 for N-acetyl β-glucosaminidase, 4.0–7.5 for N-acetyl β-galactosaminidase. The temperature optimum was 70°C for all. The β-glucosidase was purified to homogeneity by anion-exchange chromatography followed by cation exchange chromatography using HQ/H, HS/M and SP/H perfusion columns. The purified enzyme exhibited a native mol. wt. of 66 kDa on HPLC and subunit mol. wt. of 70 kDa on SDS-PAGE suggesting its monomeric nature. The K_m and V_{max} values with p-nitrophenyl β-D-glucoside as the substrate at pH 5 and 37°C were 0.45 mM and 20.8 umol. $min^{-1} \cdot mg^{-1}$, respectively.

HETEROPLOID MOSAICS AND REVERSION AMONG TRIPLOID OYSTERS, *CRASSOSTREA GIGAS*. FACT OR ARTIFACT. **Standish K. Allen, Jr.*** and **Ximing Guo,** Haskin Shellfish Research Laboratory, Rutgers University, Port Norris, NJ, 08349; **Gene Burreson** and **Roger Mann,** Virginia Institute of Marine Science, Gloucester Point, VA 23062.

Triploids have been proposed for population control for introduction and testing of non-native species and for release of genetically modified organisms. This type of population control is especially important for marine systems where containment is nearly impossible. In the first field trial of its kind, certified triploid *Crassostrea gigas* were tested for disease resistance in Delaware and Chesapeake Bays. Certified triploids were obtained by biopsying about 1500 putative triploids, rejecting diploids, heteroploid mosaics, and those that were ambiguous. After a season of disease challenge in the field, about 15 and 20% of supposed triploids were, in fact, heteroploid mosaics as determined by flow cytometry. Mosaicism was expressed among all tissues within an individual, generally. Flow cytometric results were cross checked with karyology. This and other evidence suggests that heteroploid mosaics may have arisen as a consequence of reversion of triploids to a mosaic state. A tentative model for reversion of triploids invokes tripolar spindle formation amidst mitoses of triploids cells followed by differential cell division of the stem (diploid) cell relative to the triploids. Reversion may be a function of a single cell population, namely hemocytes.

GROWTH AND TIMING OF JUVENILE OYSTER DISEASE (JOD)-INDUCED MORTALITY OF *CRASSOSTREA VIRGINICA* IN THE DAMARISCOTTA RIVER, ME, USA. **Ryan B. Carnegie*** and **Bruce J. Barber,** Department of Animal, Veterinary, & Aquatic Sciences, University of Maine, Orono, ME 04469; **Christopher V. Davis,** Darling Marine Center and Department of Animal, Veterinary, & Aquatic Sciences, University of Maine, Walpole, ME 04573.

This study provides insight into growth and the timing and extent of JOD-induced mortality of juvenile *Crassostrea virginica* under conditions typical of commercial culture in Maine. Replicate groups of 500–1000 oysters 2–3 mm in size were deployed biweekly from May 23 through August 31, 1995, in floating trays at a commercial least site on the Damariscotta River. Growth and mortality were monitored weekly through September, and biweekly thereafter. Characteristic symptoms of JOD, a cupping of the left valve and chonchiolin deposits on inner valve surfaces, were observed in the final week of July; heavy mortality occurred three weeks later. Survival was highest (<20% cumulative mortality (CM)) in the group deployed on May 23, and survival was lowest in the groups deployed on July 20 and August 3 (>90% CM). Reduced mortality (<10% CM) has been seen in the group deployed on August 31. Oyster culturists in the Damariscotta River area will achieve acceptable survival of juvenile oysters by deploying them in May; more information on the survival of the late-August group over the winter is needed before late-summer deployment can be concluded to be a viable strategy for avoiding JOD-induced mortality.

INTRACELLULAR AND EXTRACELLULAR LYSOSOMAL ENZYME ACTIVITIES IN EASTERN OYSTERS (*CRASSOSTREA VIRGINICA*). **Fu-Lin E. Chu,*** **Aswani K. Volety,** and **Georgeta Constantin,** Virginia Institute of Marine Science, School of Marine Science, College of William & Mary, Gloucester Point, VA 23062.

It is generally believed that lysosomal enzymes play a role in host defense. The hemocyte, plasma and tissue lysosomal enzymes in oysters maintained at six temperatures (T) and salinity (S) conditions: 3 ppt at 10 and 25°C, 10 ppt at 10 and 25°C and 20 ppt at 10 and 25°C were assayed. The plasma and hemocyte lysosomal enzyme activities in oysters collected from high and low S habitats were also compared. Hemocyte lysozyme (L) concentration, and L-aminopeptidase (L-AP), and acid phosphatase (AP) activities were affected by both T and S. Hemocyte L and L-AP were higher at low T and S than at high T and S. However, highest AP activities were noted in oysters at 25°C and 10 ppt. The highest plasma L and L-AP were in oysters at 3 ppt. T did not affect the plasma L concentration, but L-AP activity was higher at 25°C than at 10°C. AP was not detected in the plasma. Neither T nor S affected the lysosomal enzymes levels or activities in oyster tissues. In both summer and winter months, the plasma L was significantly higher in oysters from low than from high S habitats. In a summer month, while AP was similar between these low and high S habitats, the L-AP was higher in low than high S habitats. In contrast, there was no difference in hemocyte L between low and high S habitats in either summer or winter months and the L-AP and AP were significantly higher in high S than low S habitats. Our results also reveal that plasma L tended to be lower in *Perkinsus marinus* infected oysters than in uninfected oysters.

COOPERATIVE REGIONAL OYSTER SELECTIVE BREEDING (CROSBREED) PROJECT. **Gregory A. Debrosse*** and **Standish K. Allen,** Institute of Marine and Coastal Sciences, Haskin Shellfish Research Lab, Rutgers the State University of New Jersey, Port Norris, NJ 08349.

Since 1962 the Haskin Shellfish Research Laboratory has been selectively breeding eastern oysters (*Crassostrea virginica*) for resistance to the parasite (*Haplosporidium nelsoni*) that causes MSX disease. MSX disease resistance was obtained relatively rapidly and pedigreed lines are still maintained. Since 1992, synthetic lines, developed from controlled matings of these same pedigreed lines, were begun in response to Dermo disease pressure. The synthetic lines have undergone 1 ½ generations of selection for Dermo resistance. Using the MSX resistance synthetic lines as a foundation and with collaboration of four mid Atlantic institutions (Haskin Shellfish Lab, Rutgers University; College of Marine Studies, University of Delaware; Horn Point

Environmental Laboratory, University of Maryland; Virginia Institute of Marine Science, College of William and Mary), the objective of this project is to institute a regional selective breeding program for developing oyster stocks resistant to both MSX and Dermo disease. Experimental groups were deployed to the participating institutions in August 1995. The experimental design, hatchery production, and progress of the project to date will be discussed.

RESISTANCE STUDIES FOR JUVENILE OYSTER DISEASE (JOD). **Austin C. Farley*** and **Earl J. Lewis**, National Marine Fisheries Service, Oxford, MD 21654; **David Relyea, Joseph Zahtila,** Frank M. Flower Co., Oyster Bay, NY 11771; **Gregg Rivara,** Cornell University, Cooperative Extension, Southold, NY 11971.

In a previous study, F_1 progeny from oyster brood stocks selected on the basis of survival of exposure to juvenile oyster disease (JOD) were exposed to the disease at two infective sites in 1994 along with seed oysters (FCT) from naive Connecticut brood stocks. Significant differences in survival warranted further studies.

Susceptible progeny, F_1 resistant progeny, and F_2 progeny from 1993 brood stocks from the F_1 generation were produced from June 1995 spawnings at the Frank M. Flower hatchery in Bayville, NY. Seed were placed in nursery raft trays on Aug. 4, 1995 and deployed at 7 sites in the Long Island area on Aug. 28. Evaluations for JOD (size, shell checks, conchiolin prevalence in live and dead oysters, and mortality) were made from weekly or biweekly samples between Aug. 28 and Nov. 6, 1995. No mortality was seen on Aug. 28 at the Flower site. Size culled FCT susceptible runts (16–20 mm) had mortalities of 13% on Sept. 12, 67% on Sept. 28, 74% on Oct. 17, and 75% on Oct. 30, 1995. Size culled resistant F_1 and F_2 runts had mortalities of 0 to 3% during this time. Unculled oysters had a maximum mortality of 15% in the FCT seed and <2% in the F_1 and F_2 seed over the same time period. At another Long Island Sound site, unculled FCT seed had mortalities of 30% on Sept. 18, 52% on Oct. 3, 62% on Oct. 16, and 72% on Oct. 30, 1995. The F_1 and F_2 seed had mortalities of 3%, 2%, 2%, 13%, and 3%, 4%, 3%, 15%, respectively, on the same dates.

At 5 Peconic Bay sites, mortalities of 43–72% were seen by Oct. 30 in the FCT seed, while the F_1 and F_2 seed mortality was between 1% and 15%. Conchiolin prevalences averaged 40% in FCT live seed, and 5% in the F_1 and F_2 live seed.

The results of this study demonstrate that seed oysters from JOD surviving brood stocks are 7 to 25 times better able to survive exposure to this disease than susceptible control populations. Use of these brood stocks on a commercial scale has brought production at the Frank M. Flower Co. back to pre-JOD levels for oysters.

INHIBITION OF *PERKINSUS MARINUS IN VITRO* PROLIFERATION BY HETEROLOGOUS PLASMA. **Julie D. Gauthier*** and **Gerardo R. Vasta,** Center of Marine Biotechnology, University of Maryland Biotechnology Institute, Columbus Center, Suite 236, 701 E. Pratt Street, Baltimore, MD 21202.

The endoparasitic protozoan *Perkinsus marinus* (Phylum Apicomplexa: Class Perkinsea) is considered the primary cause of mass mortalities of the eastern oyster *Crassostrea virginica*, and no natural resistance to the disease has been described. The *in vitro* culture of the pathogenic stage of *P. marinus* has provided a unique opportunity to examine its susceptibility to recognition and effector defense mechanisms operative in refractory bivalve species. We present the effect of medium supplementation with plasma from (1) uninfected to heavily infected oysters (*C. virginica*), (2) uninfected disease resistant west coast oysters (*C. gigas and C. rivularis*) and (3) other east coast bivalves (*Mytilus edulis*, *Mercenaria mercenaria* and *Anadara ovalis*) that are naturally exposed to the pathogen but show no signs of disease. Our results demonstrate a significant ($P < 0.05$, Fisher PLSD) decrease in growth of *P. marinus* in the presence of plasma from infected vs. uninfected *C. virginica*. Plasma (20%) from heavily infected oysters inhibited growth by 32% relative to control with no plasma supplementation. Plasma from other bivalves inhibited growth in a dose-dependent manner. Growth was significantly reduced ($P < 0.05$, Fisher PLSD) in media supplemented with *M. edulus* (5%), *A. ovalis* (10%) and *M. mercenaria* (20%) plasma. The highest inhibitory activity was found in *M. edulis;* only 5% plasma was needed to reduce growth by 35% compared with the control. Plasma from west coast oysters was not inhibitory; in fact growth was significantly enhanced ($P < 0.05$, Fisher PLSD) in media supplemented with *C. rivularis* (≥5%) or *C. gigas* (20%) plasma.

GENE TRANSFER THROUGH HYBRID PARTIAL GYNOGENESIS BETWEEN THE PACIFIC AND AMERICAN OYSTERS. **Ximing Guo,* Standish K. Allen, Jr.,** and **Patrick M. Gaffney,** Haskin Shellfish Research Laboratory, Rutgers University, B-8, Port Norris, NJ 08349; College of Marine Studies, University of Delaware, Lewes, DE 19958.

The transfer of disease resistance genes from the Pacific oyster to the American oyster is prohibited by post-gametic barriers to hybridization. It is possible that those barriers are caused by only a small number of incompatible genes and can be potentially bypassed with a partial genomic transfer via hybrid partial gynogenesis. Gynogenesis refers to the development of eggs promoted by genetically inactivated sperm. In partial gynogenesis, the sperm genome is partially inactivated, so that a fraction is incorporated in the gynogenetic development. In this study, we tested the feasibility of hybrid partial gynogenesis between the Pacific and American oysters. The goal is to use hybrid partial gynogenesis for the transfer of disease resistance between the two species.

Hybrid partial gynogenesis was induced by fertilizing American oyster eggs with ultraviolet (UV) irradiated sperm from the

Pacific oyster followed by blocking polar body II. UV irradiation was very effective in damaging sperm chromosomes. Partial destruction of sperm genome was produced at low UV dosages as evidenced by chromosomal fragments. Diploid American oyster embryos with Pacific oyster chromosomal fragments were produced at high efficiencies. However, chromosomal fragments created by UV irradiation greatly reduced the viability of oyster embryos. Only a small number of survivors were obtained from groups where sperm were irradiated at high dosages. Whether those survivors carry any genes from the Pacific oysters will be determined in future analysis.

JUVENILE OYSTER DISEASE—TRANSMISSION AND BACTERIOLOGICAL STUDIES. **Earl J. Lewis*** and **Austin C. Farley,** National Marine Fisheries Service, Oxford, MD 21654; **Ana Baya,** Maryland Department of Agriculture, College Park, MD 20740; **Eugene B. Small,** University of Maryland, College Park, MD 20742.

Juvenile oyster disease (JOD) has plagued the aquaculture industry in New York and New England since the late 1980s. This new disease causes devastating mortalities in oysters, *Crassostrea virginica*, typically less than 30 mm of size. While the identity of the disease agent is unknown, our studies have shown JOD to be a transmissible, temperature and salinity sensitive, waterborne, infectious disease with an incubation period of 3–7 weeks.

Experimental studies demonstrated the disease to be transmissible using JOD-infected oysters, or particulates filtered from ambient water at JOD-infected growing sites. Experimentally infected oysters consistently reveal the JOD syndrome as seen in naturally infected oysters. Transmission was evident at salinities of 18 ppt and above, but the disease agent was shown to survive in oysters held for 7 months in <5 ppt salinity water and to cause disease after salinity was raised to 26 ppt. Mortalities in JOD-infected oysters were also reduced by maintaining them in low salinity water.

Vibrio spp. have been isolated from JOD-infected oysters and water samples in bacteriological studies, but our data do not suggest a bacterial cause for this disease. Intracellular bodies, suggestive of a protistan parasite, have been observed consistently in histological sections of mantle from JOD-infected oysters. Protists isolated from ambient water at JOD-infected sites and aquaria used in transmission studies are being investigated as the possible causative agent(s).

ISOLATION AND CHARACTERIZATION OF MARKER GENES FOR *PERKINSUS MARINUS*. **Adam Marsh, Anita C. Wright,*** and **Gerardo R. Vasta,** Center of Marine Biotechnology, University of Maryland Biotechnology Institute, Suite 236, Columbus Center, 701 E. Pratt St., Baltimore, MD 21202.

Perkinsus marinus, an apicomplexan parasite of the Eastern oyster, *Crassostrea virginica*, is the causative agent of devastating lethal disease when the host is exposed to certain environmental conditions such as increased salinity and temperature *P. marinus* has been successfully cultured in host-free medium, greatly facilitating genetic studies of this organism, which were previously limited to sequence of ribosomal RNA. In this report, we present nucleic acid sequence with similarity to β actin. Messenger RNA was extracted from *P. marinus* growth in the presence of oyster serum and reverse transcribed to generate cDNA. Conserved sequence from the 5′ and 3′ ends of human actin nucleic acid sequence were used to design primers for PCR amplification of cDNA. Limited sequence of the cloned PCR product permitted elaboration of primers specific for *P. marinus* which were used for further amplification. PCR product was subcloned into pBluscript and sequenced.

While invertebrate and vertebrate species share 94–96% amino acid identity for actin, sequence of *P. marinus* revealed 83 to 84% identity to host (*C. virginica*) or mouse actin amino acid sequence, respectively. These findings are consistent with other apicomplexans, such as *Trypanosoma cruzi*, which have similar sequence diversity of 82 to 84% amino acid identity to vertebrate, invertebrate or *P. marinus* actin sequences.

RECONSTRUCTION OF A NATURAL OYSTER BAR IN THE CHOPTANK RIVER USING HATCHERY PRODUCED OYSTER SEED. **Donald Meritt,*** Horn Point Environmental Laboratory, University of Maryland, Cambridge, MD 21613; **Kennedy T. Paynter,** Department of Zoology, University of Maryland, College Park, MD 20742; **Robert Pfeiffer,** Oyster Recovery Partnership, Annapolis, MD 20676.

The Maryland Oyster Recovery Action plan calls for the use of disease-free hatchery seed in the reconstruction of oyster bars in specific zones of certain Maryland. These zones—designated A, B, & C—are areas in which specific restrictions are enforced. In Zone A, no oyster harvesting is allowed and no dermo or MSX infected seed may be planted. In Zone B, no infected seed may be planted but harvest can occur. Zone C areas have no particular restrictions at the present time. This plan will allow experimental projects to be performed to test how quickly oyster seed will become infected in these areas and how diseases affect oysters in relatively low salinity areas over a period of years.

Reconstruction of a natural oyster bar in the Choptank River was initiated in 1995 with funding from NOAA. The project was started with the deposition of 100,000 bushels of dredged fossil oyster shell by the Maryland Department of Natural Resources on a 10 acre portion of a natural bar in Zone A of the Choptank River. This produced a large, hard platform on which the hatchery-reared seed could be planted. Oyster larvae required for the project were produced at the Horn Point Environmental Lab hatchery in three large batches approximately 2 weeks apart from each other. Setting was conducted in two 10,000 liter tanks. Spat were held in these tanks 4 to 10 days after settlement then moved to nursery

sites in the Choptank River. After 4 to 6 weeks at the nursery sites where the spat grew to approximately 15 mm, they were planted on the prepared oyster bar. By October, 1995, the spat had grown to an average height of 28 mm. Surveys will continue through 1996 to determine growth, survival and infection rates of the oysters.

EVALUATING EASTERN OYSTER STOCKS FOR RESOURCE REHABILITATION. **Kennedy T. Paynter,*** Department of Zoology, University of Maryland, College Park, MD 20742; **Patrick M. Gaffney,** College of Marine Studies, University of Delaware, Lewes, DE 19958; **Donald Meritt,** Horn Point Environmental Laboratory, University of Maryland, Cambridge, MD 21613.

American oysters from several Atlantic and Gulf coast locations were used as broodstock to produce hatchery lines, which are now being grown out in low- and high-salinity Chesapeake Bay waters. They are being evaluated for growth rate and resistance to MSX and Dermo. In addition, both the hatchery lines and their respective progenitor broodstocks are being examined by denaturing gradient gel electrophoresis (DGGE) and direct DNA sequencing of mitochondrial genes, in order to determine genetic relationships among geographical populations and to obtain genetic markers for discriminating among them.

Genetic groups were deployed in September 1995, in the Chester and Choptank Rivers in Maryland and in Mobjack Bay, VA. Initial sizes of the groups were similar, ranging between 13 and 19 mm average shell height. By November, 1995, average shell heights ranged from 31 to 45 mm. In general the oysters were slightly larger at the Mobjack Bay site although some groups at the Maryland sites were larger than their counterparts at Mobjack Bay. *Haplosporidium nelsoni* was not detected in any groups in October, 1995, but 1 to 10 putative *Perkinsus marinus* cells were detected in a few individuals in a few groups. Initial genetic analysis indicates that the broodstocks can be divided into Gulf Coast, North Atlantic and South Atlantic groups, with mid-Atlantic waters containing both Atlantic types.

Information obtained will be used to 1) determine the extent to which geographical populations are genetically distinct stocks; 2) determine which of these stocks would be best for replenishing native populations in disease-ravaged areas; 3) provide genetic markers useful for determining the geographic origin of oysters, for use in management, enforcement and breeding programs.

ASSESSMENT OF GEOGRAPHIC VARIABILITY IN *PERKINSUS MARINUS*. **José Antonio F. Robledo,* Adam G. Marsh, Anita C. Wright,** and **Gerardo R. Vasta,** Center of Marine Biotechnology, University of Maryland Biotechnology Institute, 701 E. Pratt St., Columbus Center, Suite 236, Baltimore, MD 21202.

Perkinsus marinus is the major cause of mortality of the eastern oyster, *Crassostrea virginica*, in the Chesapeake Bay. This situation has resulted in a reduction of the oyster production, and better understanding of this organism at the molecular level is needed. PCR primers derived from a non-transcribed spacer (NTS) domain between the 5S and 17S small subunit of rRNA sequence, previously shown to be specific for *P. marinus*, were used to compare *P. marinus* sequence from infected Chesapeake Bay and Gulf Coast oysters. Total DNA (1 μg) was extracted from oysters (n = 8) obtained from either Maryland or Louisiana. PCR products of the amplified 307 bp target region were cloned into pBlueScript, and several clones of each isolate carrying the inserts were sequenced. *P. marinus* sequence of the NTS domains from all Maryland oysters were identical. The nucleotide sequence of the NTS from Louisiana isolates showed greater variability. Most of the sequence dissimilarity (four positions) was concentrated in a region of nine nucleotides. Two of the Louisiana samples were identical to sequence of *P. marinus* from Maryland; however, within the nine-base region, the other six sequences exhibited only 55.5% nucleotide identity to Maryland sequence but were identical to each other. Differences between NTS domain rRNA sequences may represent genetic diversity within *P. marinus* populations in various geographic areas and might provide a tool to better understand the relationship between *P. marinus* strains isolated from oysters at different locations.

ACID PHOSPHATASE: A VIRULENCE FACTOR OF THE PROTISTAN PARASITE, *PERKINSUS MARINUS* AGAINST HOST, OYSTER'S DEFENSE? **Aswani K. Volety*** and **Fu-Lin E. Chu,** Virginia Institute of Marine Science, School of Marine Science, College of William & Mary, Gloucester Point, VA 23062.

Acid phosphatase (AP) in parasites, has been postulated to play a role in evasion of the host defense by dephosphorylation of host phosphoproteins and/or suppression of the oxygen intermediates released by the host phagocytes. The effect of temperature (4, 12, 20 and 28°C) and osmolality (400, 570 and 840 mOsm/kg) on extracellular AP secretion by the oyster parasite, *Perkinsus marinus* was investigated *in vitro*. In addition, ultrastructural localization of acid phosphatase activity in *P. marinus*, was also examined. Temperature significantly affected AP secretion by *P. marinus* ($p < 0.0001$). AP activity in *P. marinus* increased with the increase of temperature ($p < 0.05$). The extracellular AP secretion was *P. marinus* cell density-dependent ($p < 0.001$). Increasing temperatures resulted in increased proliferation of *P. marinus* cells. Similarly, osmolality significantly affected extracellular AP secretion by *P. marinus* ($p < 0.0001$). AP secretion was higher in *P. marinus* cells cultured at 400 and 570 mOsm/kg than those cultured at 870 mOsm/kg media. In the ultrastructure study, intense AP activity was found in the nucleus of the parasite. Based on the activity and distribution of AP in the nucleus, AP may aid the parasite in avoiding host defense and maybe involved in cell cycle regulation.

SHELLFISH NEOPLASIA

GONADAL NEOPLASIA IN HARD CLAMS (*MERCENARIA* SPP.) FROM THE INDIAN RIVER LAGOON, FLORIDA. W. S. Arnold* and **T. M. Bert,** Florida Department of Environmental Protection, Florida Marine Research Institute, 100 Eighth Avenue S.E., St. Petersburg, FL 33701; **D. M. Hesselman,** United States Food and Drug Administration, P.O. Box 21077, Charleston, SC 29413; **N. J. Blake,** Department of Marine Science, University of South Florida, 140 Seventh Avenue S., St. Petersburg, FL 33701.

Within the past 20 years, numerous cases of neoplastic disease in bivalve molluscs have been reported. These neoplastic diseases have typically been of hemic origin, but until recently only a very few cases of gonadal neoplasia in bivalve molluscs had been reported. Of those cases in which the neoplasm was of gonadal origin, only one occurrence had been documented for the hard clam *Mercenaria*. However, during a recent three-year study, we documented a relatively high incidence of gonadal neoplasia in hard clams from the Indian River lagoon, Florida.

The Indian River hard clam population is composed of approximately 68% *M. mercenaria*, 4% *M. campechiensis*, and 28% hybrid genotypes. The incidence of gonadal neoplasia is substantially higher in hybrids than in either of the two pure species. Although there is a locational component to the incidences of gonadal neoplasia, the pattern is attributable to the proportion of hybrids at that location rather than to any locational characteristics *per se*. In fact, water quality in the lagoon is generally good, and the fact that we have not found any localized concentrations of neoplastic clams indicates that water-borne carcinogens are not present. The frequency of occurrence of gonadal neoplasia was different for males and females; we observed an overall lower frequency of the disease in males than in females, and the disease was most common in males of intermediate age but in females of very old age. However, hybridity rather than environmental or other biological factors appears to determine susceptibility, implicating a genetic mechanism in the etiology of the disease.

Genetically mediated gonadal neoplasia may provide an endogenous mechanism that operates in conjunction with exogenously mediated, genotype-specific growth differences to balance selection on hard clam genotype classes in the Indian River lagoon. This balancing selection acts to maintain the Indian River hard clam hybrid zone in spite of a preponderance of *M. mercenaria* alleles within the population.

GONADAL NEOPLASMS IN *MYA ARENARIA*: WHAT DO WE KNOW? Bruce J. Barber,* Department of Animal, Veterinary & Aquatic Sciences, University of Maine, Orono, ME 04469.

1) Gonadal neoplasms have only been found in *Mya arenaria* from Maine. Prevalence of gonadal neoplasms in adult clams from Washington County, ME, since September 1993 has ranged from 10% to 43%. Intensity of neoplasms varies from a few, small foci of undifferentiated germ cells (Stage 1), to 50–100% of gonadal follicles being involved (Stage 2), to invasion and metastasis with loss of tissue architecture (Stage 3), indicating that the disease is progressive and lethal. Clams of both sexes are affected, but females are significantly more likely ($P \leq 0.025$) to have neoplasms than males. **2) Neoplasms negatively impact gameotogenesis.** Female clams with neoplasms produce 66% fewer oocytes than healthy clams ($P \leq 0.001$) as the result of direct displacement by tumor cells. In addition, female clams with neoplasms have a significantly lower mean oocyte diameter before spawning and a significantly greater mean oocyte diameter after spawning ($P \leq 0.001$) than healthy clams, as the result of a general inhibition of normal oogenesis and spawning. **3) Absolute diagnosis of gonadal neoplasms requires histological examination.** Clams with advanced neoplasms can also be identified visually by the presence of a mottled gonad; clams without discernable mottling, however, may have Stage 1 neoplasms. Blood from clams with gonadal neoplasms has levels of glucose and total protein similar to levels in healthy clams.

GONADAL NEOPLASIA IN NORTHERN AND SOUTHERN QUAHOGS AND THEIR HYBRIDS IN SOUTH CAROLINA. Arnold G. Eversole,* Department of Aquaculture, Fisheries and Wildlife, Clemson University, Clemson, SC 29634-0362; **Peter B. Heffernan,** Marine Institute, 80 Harcourt Street, Dublin 2, Ireland.

Histopathological examination of northern and southern quahogs and their hybrids revealed gonadal neoplasia in 47% of the samples ($n = 440$). Severity of neoplasia was assigned scores from very low (1) to a high severity condition (5). Average severity value was 1.89, and the low severity category was the most frequently (44%) encountered condition. Highest severity values were observed May–July and September–October and lowest values were in March and August. Shell lengths (SL) of samples were 19.9–61.4 mm and those with neoplasia were 20.1–51.2 mm. Average SL of quahogs with neoplasia were $\geq$ than the SL of specimens without neoplasia. The SL of hybrids with more advanced stages of neoplasia were $>$ than SL of hybrids without neoplasia. Sex composition of the samples were 39% male, 40% female and 21% undifferentiated. Neoplasia prevalence (59%) and severity (2.22) were highest in the undifferentiated sex category. Average monthly prevalence and severity of neoplasia was $>$ than in either parental species. Gonadal neoplasia was diagnosed in all the hybrid monthly samples and 58% of the parental species monthly samples. Maximum monthly prevalence was 100% in the hybrids and 75% in the parental species. The frequency of gonadal neoplasia in the hybrids increased between 1988 and 1992; average prevalence was 95–100% and severity was 3.41–4.21 in 1992. Invasive stages of neoplastic cells were observed in the hepatopancreas. Examination of gonads from more recent collections

will be compared with these samples to determine progression of the condition and degree of invasion of other tissues.

AN UPDATE ON SOFTSHELL CLAM (*MYA ARENARIA*) SARCOMA IN THE CHESAPEAKE BAY AND THE DECLINING FISHERY. **Shawn M. McLaughlin*** and **Austin C. Farley,** National marine Fisheries Service, Southeast Fisheries Science Center, 904 S. Morris St., Oxford, MD 21654; **Christopher C. Judy,** Maryland Department of Natural Resources, 580 Taylor Ave., Annapolis, MD 21401.

Presumptive hematopoietic neoplasms (sarcomas) of softshell clams, *Mya arenaria,* were rarely observed in Chesapeake Bay populations prior to the first documented epizootic in 1984. Softshell clam sarcoma epizootics were subsequently reported in six Chesapeake Bay sites, including Swan Point in the Chester River, during 1984–1988. Subsequent monitoring of the Swan Point site showed sarcoma prevalences of 78% in December of 1990, 0% during summer of 1991, and 20% in January of 1992. Softshell clam sarcomas at Swan Point have continued at low prevalences (0–3%) since 1992 with small peaks at 12% each in March 1994 and July 1995. The apparent seasonal and cyclic nature of softshell clam sarcoma epizootics suggests another peak in sarcoma prevalences may be imminent at Swan Point. However, actual sarcoma prevalences may be obscured by a reduced sampling frequency due, in part, to decreased commercial clamming activity. Softshell clam harvests in the Maryland portion of the Chesapeake Bay have declined from a high of 680,358 bushels in 1964 to a low of 29,616 bushels in 1992 and only reached 37,386 bushels in 1994. The relationship between softshell clam sarcomas and the declining commercial harvest in the Chesapeake Bay is complicated by environmental and biological factors. High prevalences and intensities of softshell clam *Perkinsus* sp. and a chlamydia-like organism have also been observed in Chesapeake Bay softshell clams. In addition, extreme summer temperatures have been associated with high softshell clam mortalities in the Chesapeake Bay.

THE RATE OCCURRENCE OF NEOPLASIA IN CRUSTACEA: MYTH OR SAMPLING ARTIFACT. **J. Frank Morado*** and **Donald V. Lightner,** National Oceanic & Atmospheric Administration, National Marine Fisheries Service, Alaska Fisheries Science Center, 7600 Sand Point Way NE, Seattle, WA 98115; Environmental Research Laboratory, University of Arizona, 2601 East Airport Drive, Tucson, AR 85706.

The Class Crustacea is both an ecologically and economically important taxon that contains roughly 30000 species. It is a highly diverse and successful taxon possessing easily recognizable adult stages such as crabs, lobsters and shrimp to the highly aberrant Rhizocephala and parasitic isopods and copepods. The majority of the Crustacea are aquatic, but many are terrestrial. Survival is dependent upon the production of thousands to millions of progeny that must pass through several larval stages during which large increments in growth occur before they acquire their adult characteristics.

Two basic tenets for the induction of cancer were presented by Cotran, Kumar and Robbins (*Pathologic Basis of Disease*, 1989). The first simply stated that risk was associated with survival. The second identified cell replication because it is involved in cancerous transformation—"regenerative, hyperplastic and dysplastic proliferations are fertile soil for the origin of a malignant neoplasm." Crustaceans possess these two criteria, but why is neoplasia rare in this class of animals, especially when embryos, larvae and adults in aquatic coastal environments are exposed to the same chemical promoters of neoplasia as fish?

To date, only a handful of published reports present realistic accounts of neoplastic disorders in crustaceans. Two may be classified as benign neoplasms while the three most recently described cases may be classified as carcinomas. Descriptions of these proliferative anomalies as well as their prevalences and distributions will be presented. Probable reasons for the rarity of neoplastic lesions in crustaceans, regardless of their life history stage will be discussed.

EFFECTS OF HEMATOPOIETIC NEOPLASIA ON REPRODUCTION AND POPULATION SIZE DISTRIBUTION IN THE SOFT-SHELL CLAM. **Mary-Susan Potts,*** Biology Dept. Northeastern University, Boston, MA 02115.

The soft-shell clam, *Mya arenaria*, is susceptible to Hematopoietic neoplasia (Hn), a disease in which increasing numbers of atypical cells invade the hemolymph and connective tissue of the clam's circulatory, digestive, reproductive and excretory systems. In this study disease effects on the morphology of the clam's reproductive organs were examined. In addition, Hn prevalence was evaluated with respect to sex and as a function of size in the total population and in a cohort of clams. Methods utilized included field sampling, routine histology and computer image analysis. Comparisons of mean gonadal follicle size and number of follicles were made between normal and Hn clams.

Qualitatively, Hn effects on the gonads of both male and female clams were increasingly apparent as abnormal cells filled the clam's connective tissue. As Hn advanced, the gonadal follicles became significantly smaller in size but were not reduced in number. While Hn prevalence was unrelated to the sex of the clam, the data supported a relationship between Hn prevalence and size. Clams 40–70 mm had the highest prevalence of Hn, and a cohort of clams showed increasing Hn prevalence as they grew into this disease-susceptible size range. In general the data suggest that Hn reduces the reproductive capabilities of diseased individuals and may directly alter the size distribution of soft-shell clam populations by removing particular size classes through mortality.

NEOPLASIA AND OTHER POLLUTION ASSOCIATED LESIONS IN *MYA ARENARIA* FROM BOSTON HARBOR.
Roxanna Smolowitz,* Laboratory for Marine Animal Health, U. Of Pennsylvania, Marine Biological Laboratory, Woods Hole, MA 02543; **Dale Leavitt,** WHOI, Woods Hole, MA 02543.

Northeast coastal populations of *Mya arenaria,* the soft shell clam, are affected by Hematopoietic Neoplasia. High prevalences of this disease has been related to highly polluted waters by some researchers. Therefore *Mya* was chosen to investigate as a possible biological monitor of pollution in Boston Harbor. Twenty animals were collected from 5 sites in Boston Harbor and 2 control sites in Cape Cod Bay. The occurrence of Hematopoietic Neoplasia in these samples was determined using a neoplasia cell specific antibody in a modified immunocytochemical staining method. In addition, paraffin embedded tissues were examined from each animal for other possible pathologies. Multivariant statistical methods were used to determine what pathologies were highly correlated with animals collected from polluted sites. Out of 33 possible pathologies, the occurrence of the following in *Mya* were highly correlated with pollution: gonadal inflammation, inflammation of the mantle, gill inflammation, gill hyperplasia/papilloma, kidney hyperplasia, brown cell accumulation in the renal epithelium, protozoal infection in the kidney and pericardial gland changes. Hematopoietic neoplasia was not positively corrected with site pollution. Surprisingly, 5/20 animals from only one polluted site showed a neoplasia not identified previously in *Mya.* This neoplasia appeared to be branchial in origin and had numerous metastatic nodules in other tissues. Whether this neoplasia was caused by pollution, an infectious agent or a combination of causes, is not known.

INVESTIGATION OF MOLECULAR MECHANISMS OF TUMORIGENESIS IN BIVALVE GONADAL TUMORS.
R. J. Van Beneden and **L. R. Rhodes,** University of Maine, Orono, ME; **D. J. Brown,** Duke University, Durham, NC; and **G. R. Gardner,** EPA, Narragansett, RI.

Epidemiological investigations of germ cell tumors of Maine soft shell clams (*Mya arenaria*) and hard shell clams (*Mercenaria spp.*) from Florida demonstrate the prevalence of histogenically similar gonadal cancers as high as 40% and 60%, respectively. Human mortality rates due to ovarian cancer from the same areas are significantly greater than the national average and show a rise over the last two decades of the survey. This correlates with the increasing use of herbicides in these areas and in the appearance of significant numbers of tumors of the analogous reproductive system in the clams. We have investigated the molecular mechanism of tumor induction in the clams. Results of photoaffinity binding studies using the TCDD photoaffinity analog [^{125}-I]-2-azido-3-iodo-7,8-dibromodibenzo-*p*-dioxin to detect two cytosolic proteins (28 and 39 kDa) in *Mercenaria mercenaria* and one (35 kDa) in *M. arenaria* which specifically bound this ligand. Their role in dioxin toxicity and their relationship to the vertebrate Ah receptor is under investigation. Differential display PCR analysis has revealed the presence of differentially-expressed messages in tumors from *M. arenaria* which have potential roles in signal transduction pathways.

EXPRESSION OF THE TUMOR SUPPRESSOR GENE, P53 IN NORMAL AND LEUKEMIC CLAM BLOOD CELLS *IN VIVO* AND *IN VITRO*.
Charles W. Walker, Sharon A. Key, Joseph E. Mulkern, Shalini Verma, and **Jocelin A. Jacobs,** Department of Zoology, University of New Hampshire, Durham, NH 03824.

Mya arenaria, the soft shelled clam, can develop a diffuse blood tumor or leukemia. The disease is fatal and may deplete commercial clam populations. The only externally obvious signs of the disease are slow decline and death of affected individuals. Thus, the degree of destruction of local clam beds may not be obvious to local fishermen and has not been carefully documented anywhere in the Gulf of Maine. Until now it has been impossible to culture these rapidly dividing cells for use in molecular or other studies. We have developed a method for mass culture and cryopreservation that should permit more widespread study of these tumor cells. Using our chemically defined medium (modified from Sible et al., 1991) in spinner culture flasks (8–10°C), leukemic cells harvested directly from the clam heart double in number in 40–50 hours and can easily be subcultured. We can also maintain leukemic cells in biofreezers (−196°C) for extended periods of time (≥3 months) and can subculture recovered cells. In both cases, we have confirmed their identity with the clam leukemia specific antibody 1E10 (Miosky et al., 1989) and cytology. We have partially cloned the tumor suppressor gene p53 from normal clam blood and gonadal tissues. In the available sequence, 3 of the 5 vertebrate DNA binding domains exist and are highly conserved as are the nuclear translocation and dimerization and tetramerization domains. Based on structural conservation, the p53 gene product should function similarly in down-regulating cell division in clams and vertebrates. When compared with normal clam blood cells from New Bedford Harbor, Massachusetts, our data from *in situ* hybridizations and cytochemistry demonstrate that expression of clam p53 is depressed in fully developed leukemia cells. This expression pattern is consistent with that seen in vertebrate leukemias. Because of the high degree of similarity between p53 and many of the genes involved in mitogenic signal transduction cascades between mammals and clams, our studies may have substantial implications for understanding leukemia in mammals. Also, molecular data that we gather about leukemia in clams should point out mechanisms for the diagnosis, treatment and prevention of clam leukemia and should lead to an understanding of how the disease is transmitted and promoted in clam populations at risk for this fatal disease. Supported by Hatch Grant #353 to C. W. Walker; McNair, to S. A. Key, SURF to J. E. Mulkern and UROP to S. Verma.

STOCK ASSESSMENT

USING REAL TIME DATA WITH A PC-BASED GIS FOR SHELLFISH MANAGEMENT. James G. Boyd,* Duke University School of the Environment, Beaufort, NC 28516; **William D. Anderson** and **Guy M. Yianopoulos,** South Carolina Department of Natural Resources, Charleston, SC 29422.

Estuarine environments are inherently dynamic systems. Consequently, management of estuarine resources is facilitated by tools that allow for rapid evaluation and decision making. Using a pc-based geographic information system (GIS/ArcView2) to display shellfish resources in a portion of the James Island USGS quadrangle, Charleston, SC provides a demonstration of the utility that near real time data can have for managing shellfish resources.

An intertidal oyster resource assessment from the mid 1980's was compared to the most recent oyster survey completed in the summer of 1995. In addition, an historical intertidal oyster survey completed in the winter of 1890–91 was included in the comparison. Qualitative observations made up the bulk of the historical survey, which included a limited but useful map of the resource. A GIS comparison of the three surveys illustrated the dynamic nature of the resource over an extended period. Field data included physical descriptions of the oyster beds, water temperature and salinity, tidal stage, locations of private and commercial docks, as well as erosional banks. The pc-based GIS allows data to be presented in single or multiple map layers so that specific effects may be examined exclusively as well as inclusively.

Comparative analysis over time allows managers to discern trends or abrupt changes in resource status. Policy measures can be adopted and implemented more accurately based on data and management tools that contrast historic and contemporary information. The benefits of this system extend to commercial and recreational users as well as resource managers.

RELATIVE EFFECTS OF HARVEST AND DISEASE MORTALITY ON EASTERN OYSTER POPULATIONS IN DELAWARE BAY. Stephen R. Fegley, Maine Maritime Academy, Castine, ME 04420; **Susan E. Ford, John N. Kraeuter,** and **Harold H. Haskin,** Haskin Shellfish Research Laboratory, Rutgers University, Port Norris, NJ 08349; **David R. Jones,** Grice Marine Biology Lab, College of Charleston, Charleston, SC 29412.

The decline in size of many Eastern oyster (*Crassostrea virginica*) populations and their failure to recover to historical abundances are frequently attributed to excessive harvests and the presence of oyster diseases. Few attempts have been made to estimate the relative importance of these mortality sources to each other and to other factors affecting oyster abundance. We used a 40-year data set that included hydrography, abundances of oyster life-history stages, stage-specific oyster mortality, harvest intensity, and MSX disease prevalence and intensity to determine which of these factors most influenced adult oyster abundance on natural oyster beds in Delaware Bay.

For all oyster beds combined, the prevalence of MSX disease was significantly and negatively correlated to oyster abundance; harvest volume was not related to subsequent oyster abundance. A highly significant, multivariable regression model containing only three factors (1-yearling oyster abundance, 2-mean annual Delaware River flow, and 3-mean proportion of oysters infected with MSX disease in the spring) explained almost two-thirds of the variance in adult oyster abundances on oyster beds. The absence of a general, negative effect of harvesting on Delaware Bay oysters probably results from the conservative management program existing in the New Jersey oyster fishery since the 1950's.

EASTERN OYSTER STOCK ASSESSMENT IN MARYLAND. Mark L. Homer, Mitchell Tarnowski, and **Lisa Baylis,** Maryland Department of Natural Resources, Piney Point Aquaculture Center, PO Box 150, Piney Point, MD 20674.

During the last 13 years Maryland's public oyster fishery has declined from a 50 year average of 2.5 million bushels per season to less than 0.2 million bushels. Although this decline was and is concurrent with severe and widespread epizootics of *Dermo* and *MSX* other causal factors such as overharvesting and habitat loss have been suggested. The debate that arose from attempts to attribute causality to the declining fishery led to the development of an oyster stock assessment program by the Maryland Department of Natural Resources in 1989. The initial goal of this effort was to produce a statistically sound sampling procedure that would give unbiased estimates of oyster abundance, population structure, and habitat volume. This was accomplished in 1990 through the development of a randomly initiated, systematic sampling scheme using hydraulic patent tongs covering 1 m^2 of substrate. Since then over 86,000 acres of charted oyster bars have been surveyed, including over 15,000 acres originally surveyed in 1975) when harvests were still high) using a similar and compatible methodology.

The results from the patent tong-based surveys indicate that there has been moderate to substantial habitat loss in some low to moderate salinity areas, and that these areas have been overharvested in recent years. In most of the moderate to high salinity areas, however, neither of these two factors can account for the severe harvest declines. Many areas will support oyster populations as large (numerically) as those recorded in 1975 although severe truncation of size class structures and substantial decreases in the live to dead ratio of oysters have occurred. In addition, many of the moderate to high salinity areas have not been commercially exploited during the last 5 to 9 years and habitat loss in higher salinity zones has been minimal since 1975. Interestingly, the period from 1990 to the present has seen remarkable fluctuations in parasite severity, oyster recruitment, and freshwater dis-

charge into the Chesapeake Bay. These fluctuations combined with the stock assessment data suggest that the primary factor in the decline of Maryland's oyster harvest has been parasite-related mortality.

MORPHOLOGICAL DIFFERENTIATION OF THE FRINGING AND PATCH OYSTER REEF TYPES IN CHESAPEAKE BAY: A COMPARATIVE EVALUATION. **G. F. Smith** and **K. N. Greenhawk,** Cooperative Oxford Laboratory, Maryland Department of Natural Resources, Oxford, MD.

Morphological and bathymetric examination of oyster reefs in Maryland Chesapeake Bay waters indicates that reefs may be segregated into two principal classifications: fringing reefs and patch reefs. Although gradation between the two reef types is common, morphological differences between these reef types can help explain patterns of variation in historic cultch loss on charged oyster bars. Three dimensional analysis utilizing GIS technology has allowed for a general comparison of habitat loss from the turn of the century until the mid 1970's. A three dimensional integration of bathymetry, historic charged oyster bar boundaries, and recent bottom composition surveys has identified principal causes of habitat loss for selected sites. Principal causes of habitat loss due to sedimentation may be inferred to result from local processes rather than baywide general siltation.

MOLLUSCAN INVENTORY OF MARYLAND'S COASTAL BAYS. **Mitchell L. Tarnowski,* Mark L. Homer, Lisa Baylis,** and **Robert Bussell,** Maryland Department of Natural Resources, 580 Taylor Ave., C-2, Annapolis, MD 21401.

MDNR's Shellfish Program is in the third year of a comprehensive effort to inventory the molluscan fauna of Maryland's coastal bays. Intended to establish baseline values for future management needs, both commercially important molluscs and ecologically valuable species have been targeted. To date approximately 1700 stations have been sampled using five different collection methods. Nearly 50,000 individuals comprising 52 mollusc species have been collected. Two of these species represent range extensions and another 12 had not been reported in previously published accounts of the coastal bays.

The first phase of the inventory was conducted in Chincoteague Bay, the largest and least environmentally impacted of the old coastal bays. Hard clam populations are 25% of the estimates made a quarter century ago, when hydraulic dredges were first introduced to the fishery. A juvenile hard clam survey showed recruitment to be substantially lower than in other regions. Severe predation, particularly by blue crabs, aggravated by the disappearance of oyster shell as a protective cover, may be a primary factor in limiting recruitment. The once highly prized Chincoteague oyster no longer inhabits the subtidal bars of the bay, having succumbed to diseases, intense predation and competition for substrate. To a large extent the bars themselves have been buried by sediment or smothered by fouling organisms, greatly reducing this ecologically important habitat. Relic populations of oysters still exist intertidally, although the ribbed mussel *Geukensia demissa* is the dominant species in this zone. The inventory of ecologically valuable molluscs generated information on population and community parameters such as species composition, distribution, abundance, size structure, and habitat characterization. Among the findings was the elucidation of ecological communities and functions of Chincoteague molluscs, the positive effect of detritus derived from salt marshes and seagrass meadows on molluscan abundance, and the importance of shell cover to species diversity. Also in Chincoteague Bay, an experiment is examining the growth and survivorship of hatchery reared, disease free oysters suspended in the water column.

The inventory has been expanded into the other coastal bays, affording a synoptic view of the entire lagoonal system and allowing comparisons between relatively unimpacted and more degraded ecosystems. Another round of sampling in Chincoteague Bay will give some idea of the temporal variability of population and community parameters.

WATER QUALITY AND GOVERNMENT REGULATION

CONTROL OF VIBRIO VULNIFICUS GROWTH TO REDUCE RISK IN SHELLFISH CONSUMPTION. **Paul G. Comar,*** National Marine Fisheries Service, Charleston Laboratory, Charleston, SC 29412.

Vibrio vulnificus is a naturally-occurring bacterium present at elevated levels in estuarine waters and bivalve shellfish during warmer months. From 1992 through 1995, there has been an average of 22 illnesses resulting in 10 deaths caused by *V. vulnificus* each year in the United States in raw shellfish consumers with several known pre-existing medical conditions. Nearly all of these shellfish illnesses have been linked to consumption of raw oysters from the Gulf of Mexico.

Education of groups at risk for the disease and product advisory labeling at retail are risk reduction measures under development and implementation by regulatory agencies and the shellfish industry through the coordination of the Interstate Shellfish Sanitation Conference (ISSC). In 1996, a new approach will be implemented to limit the increase in *V. vulnificus* levels in shellfish post-harvest and thereby reduce the risk of illness. It will require more rapid refrigeration of shellfish during warm weather months in states whose shellfish have been implicated in two or more *V. vulnificus* illnesses.

This report describes the design of a cooperative analytical investigation to be conducted in 1996 to assess the control in *V. vulnificus* levels afforded by the new refrigeration requirements and how these data will be used with other information in determining the effectiveness of controls.

ASSESSING WATER QUALITY: NEW DIRECTIONS. **Elizabeth Fellows,*** EPA Office of Water, 4503F, 401 M Street, Washington, DC 20460.

Two major new activities will help the public and water managers understand water quality and set management priorities.

The first is implementation of a nationwide strategy to improve water quality monitoring. The strategy was developed by the Intergovernmental Task Force on Monitoring Water Quality (ITFM), a Federal/State consortium with an advisory committee of local and private experts. The strategy addresses nationwide monitoring design and collaboration, watershed and ecosystem components, environmental indicators, comparable monitoring methods, quality assurance and control, assessment and reporting, and specific monitoring tools. The other activity is the first national water environmental indicators report that characterizes the nation's waters and how well we are meeting the goals of the Clean Water Act. The indicators measure how well the nation is doing to achieve goals of public and ecosystem health, attainment of water uses such as fishing and swimming, improvement of ambient conditions, and prevention or reduction of pollutant loadings and other stressors. Two of the indicators concerns shellfish consumption and the condition of shellfish beds. The indicators will depend upon and employ a wide range of data providers and users such as the shellfish management industry.

TARGETING STRATEGIES FOR SHELLFISH RESTORATION IN THE GULF OF MEXICO: RESULTS OF A REGIONAL STRATEGIC ASSESSMENT PROCESS. **Paul Orlando, John Klein, Daniel Farrow, Anthony Pait, Dorothy Leonard,** and **Jamison Higgins,** Office of Ocean Resources Conservation and Assessment (N/ORCA1), National Oceanic and Atmospheric Administration, 1305 East-West Hwy., Silver Spring, MD 20910.

NOAA's Strategic Environmental Assessments Division (SEA), in partnership with EPA's Gulf of Mexico Program (GMP), has been conducting a strategic assessment to identify and geographically-target management strategies to meet the GMP's Shellfish Challenge, which is to "increase Gulf shellfish beds available for safe harvesting by 10 percent". The assessment process brings together key data sets characterizing shellfish bed closure problems with state and regional experts to produce an integrated plan to meet the Shellfish Challenge. Since July 1994, there have been two regional workshops, numerous state visits, completion of the 1995 National Shellfish Register, and the compilation of an extensive data base and mapping system that describes the classified shellfish harvest areas, pollution sources, estuarine salinity, and cultch planting activities. Nearly 30 strategies have been developed to reduce fecal coliform bacteria loadings, enhance shellfish habitat enhancement, safeguard public health, and increase resource abundance. These strategies were prioritized and geographically-targeted to the 50 estuarine watersheds in the Gulf region.

One of the highest-rated strategies was an expansion of cultch planting activities. This presentation will discuss how, through the strategic assessment process, areas suitable for shell planting were identified based on salinity requirements and resource productivity estimates in approved harvest areas (i.e., acceptable water quality). The next step is to evaluate the feasibility of implementing several restoration strategies in one or more "good candidate" estuarine watersheds before proceeding with full-scale restoration.

THE EFFECTS OF URBANIZATION ON THE AMERICAN OYSTER, *CRASSOSTREA VIRGINICA* (GMELIN). **G. I. Scott,* M. H. Fulton, E. D. Strozier, P. B. Key,** and **J. W. Daugomah,** US National Marine Fisheries Service, Southeast Fisheries Science Center, Charleston Laboratory, Charleston, SC; **D. Porter,** Marine Science Program University of South Carolina, Columbia, SC; **S. Strozier,** School of Public Health, University of South Carolina, Columbia, SC.

Rapid development of coastal areas of the southeastern US has resulted in significant alterations of upland terrestrial habitats adjacent to sensitive estuarine salt marsh ecosystems in the southeastern US. Most remaining coastal development in the southeastern US will be residential and tourism/service related industries rather than industrial development and will occur around the >300 small high salinity tidal creeks and estuaries found in the region. These alterations may result in potential impacts to living resources within estuaries, including molluscan shellfish such as the American oyster, *Crassostrea virginica.*

The Urbanization in Southeastern Estuarine Systems (USES) study has addressed impacts of coastal development on adjacent small, high salinity estuaries of the southeastern US by comparing Murrells Inlet (MI), a highly urbanized estuary with pristine North Inlet (NI). A total of 60 monitoring stations were sampled in both estuaries. Surface waters, sediments and oysters (*Crassostrea virginica*) were monitored for fecal coliform bacteria densities; serotyped to individual bacterial species; analyzed for trace metals, polycyclic aromatic hydrocarbons (PAHs), pesticides and polychlorinated biphenyls (PCBs) to characterize chemical contaminant inputs; and adult oysters were monitored for survival, condition index, gonadal index and juvenile spat settlement. Geographical Information Processing (GIP) was conducted on multiple data layers to indicate geographic regions where multiple contaminant interactions had occurred.

One of the more significant effects from urbanization study was the increased closure of shellfish harvesting waters due to increased inputs of fecal coliform bacteria. More than 67% of the sampling sites in MI exceedede the SA water classification fecal coliform standard versus 35% in NI. Fecal coliform monitoring of shellfish meats indicated that >50% of stations in each estuary exceeded the Interstate Shellfish Sanitation Conference Depuration Meat Standard. Mortality rates among adult and juvenile oysters was much higher in MI than NI, and the pattern of spat settlement was different. GIP aqnalysis indicated areas where mul-

tiple contaminant interaction occurred and where coastal ecosystem health was adversely affected.

SYNOPSIS OF FDA RESEARCH RELATED TO WATER QUALITY. **William D. Watkins,*** HFS-417, Office of Seafood, U.S. FDA, 200 'C' Street, S.W., Washington, D.C. 20204.

FDA research involving water quality focuses primarily on issues involving molluscan bivalve shellfish and the National Shellfish Sanitation Program. The goal of this research is to enable the prevention of various health hazards sometimes associated with the consumption of raw shellfish, and to provide sound scientific data for regulatory and compliance decisions.

Research involving traditional and alternative indicators of fecal contamination demonstrate the utility and significance of using an array of microbial indicators. This array variously includes total and fecal coliforms, *Escherichia coli,* enterococci, *Clostridium perfringens,* and male-specific bacteriophage. Studies have provided valuable information on shellfish depuration, wastewater disinfection, microbial survival, assessments of sanitation in marine environments, and shellfish-forne illness outbreaks. Other studies on densities of the naturally occurring, opportunistic pathogen, *Vibrio vulnificus,* show the importance of storage temperatures (and times) and elucidate the effects of applying various intervening measures. Water quality research related to shellfish toxins suggest that phytoplankton monitoring may provide reliable indications of impending toxic algal blooms.

Several important issues related to molluscan shellfish remain unresolved. Future water quality research will need to address the development of new criteria for effective closure zones around point sources, re-opening criteria for offshore areas, and a more human-specific indicator of fecal contamination.

POSTER SESSION

INCIDENCE OF FOULING AT TWO MARICULTURE SITES IN BON SECOUR BAY, ALABAMA. **Susan B. Athanas*** and **David B. Rouse,** Department of Fisheries and Allied Aquacultures, Auburn University, Auburn, AL 36849.

Private oyster producers in Bon Secour Bay, Alabama have recently adopted off bottom aquaculture practices. Barnacle fouling has increased tremendously, increasing labor cost for market preparation. A survey was conducted from July 1994 to June 1995 to determine seasonality of barnacle spawning in order to discover possible prevention techniques. Location within the bay and within the water column were also examined. *Balanus eberneus* was determined to be the only barnacle species fouling oysters. Most barnacle sets occurred from January to May with peak fouling observed in March. Little differences were observed in location or depth. Possible management strategies might be to stock young oysters in the bay after June, allowing the accumulation of s[illegible] and bryazoans which will discourage later barnacle fouling.

CAN A SPECIES BE "FOULED" INTO EXTINCTION? ZEBRA MUSSELS VS NATIVE BIVALVES IN THE UPPER MISSISSIPPI RIVER. **P. Baker*** and **D. J. Hornbach,** Macalester College, St. Paul, MN 55105.

Zebra mussels (*Dreissena*) are known to locally eradicate native clam (Unionacea) populations, by heavily fouling the exposed shells. Native bivalves thus far impacted have been protected from extinction by their large ranges, which include waters unsuitable for *Dreissena*. As *Dreissena* spreads, however, it has encountered unionaceans with restricted ranges. Our research examined the threat posed to one such species, the endangered *Lampsilis higginsi*.

All but two of the reproducing populations of *L. higginsi* are in the mainstem Mississippi River. The remaining populations are in the lacustrine downstream region of the St. Croix River (MN & WI). *Dreissena* have reached high densities on all Mississippi habitats, and are in the process of invading the lower St. Croix. Water chemistry and the long water residence time in the lower St. Croix are favorable for *Dreissena* reproduction. If *Dreissena* invades as predicted, and affects unionaceans as it has elsewhere, uncommon species such as *L. higginsi* will probably be reduced to densities below the minimum required for successful spawning. Extinction could follow in the time it takes for the remaining *L. higginsi* to die of old age. Transplanting or culturing *L. higginsi* may preserve this species.

PADDLES OR SIEVES: TESTING THE MECHANISM OF PARTICLE RETENTION IN BIVALVES. **Kerri M. Bentkowski*** and **J. Evan Ward,** Department of Biological Sciences, Salisbury State University, Salisbury, MD 21801; **Roger I. E. Newell,** Horn Point Environmental Laboratory, University of Maryland, Cambridge, MD 21613.

The mechanism of particle capture in suspension-feeding bivalves is controversial and poorly understood. Despite the traditional view that the gill and its associative rows of laterofrontal cilia or cirri physically trap particles in a manner similar to a filter, recent insights into the physical forces that interact at small size scales show an inconsistency in the paradigm. For example, viscous forces of the water tend to over-ride inertial forces at the size scales of the cilia.

In order to test hypotheses concerning the forces that mediate particle capture, we manipulated the kinematic viscosity of the fluid by changing water temperature. Retention efficiency was measured for particles 1 μm and 10 μm in diameter with a Coulter Multisizer. Previous workers have found that retention efficiency of 1 μm particles in some planktonic suspension-feeders is mediated by fluid viscosity. In contrast, our preliminary data with *M. edulis* indicate that retention efficiency of 1 μm particles is independent of kinematic viscosity even when viscosity increases by 81%. Our results support the hypothesis that laterofrontal cirri function more like paddles than like sieves. Implications of our results to feeding mechanisms in bivalves will be discussed.

SEASONAL CYCLE OF *HAPLOSPORIDIUM NELSONI* (MSX) IN INTERTIDAL OYSTERS, *CRASSOSTREA VIRGINICA*, IN SOUTH CAROLINA. **M. Yvonne Bobo*** and **Donnia Richardson,** South Carolina Department of Natural Resources (SCDNR), Marine Resources Research Institute, Charleston, SC 29412; **Thomas C. Cheng,** Shellfish Research Institute, Charleston, SC 29412; **Elizabeth McGovern** and **Loren Coen,** SCDNR, Marine Resources Research Institute, Charleston, SC 29412.

Little is known about the seasonal cycle of the oyster pathogen *Haplosporidium nelsoni* (MSX) in the southeastern United States. South Carolina oysters are predominantly intertidal (>95%) and occupy a very different habitat from their northern counterparts which are predominantly subtidal. MSX disease was first reported in SC in 1993 in oysters collected from Charleston Harbor and subsequently observed in oysters from other SC coastal sites. During summer, 1994, an extensive survey was conducted to determine the prevalence of MSX over a larger geographic area. Twenty-one stations were sampled (n = 925 oysters examined). MSX was present in oysters from 10 of the 21 stations surveyed (48%), with prevalences as high as 28%. In June 1994, monthly sampling of a Charleston Harbor site was initiated. In September 1994, as part of a long-term intertidal oyster ecosystem study, two additional sites were added, one at a degraded marina and the other in a pristine tidal creek system surrounded by marsh and mudflats. Monthly sampling (n = 25) from each of these three sites has continued since June 1994, and has revealed that the highest prevalence and intensity occurred during the Fall. MSX was present at all three sites, with maximum prevalence ranging from 28 to 42% and intensities ranging from rare to heavy. All diagnoses were by examination of representative histological sections. Concurrent physical environmental data (DO, temperature, salinity) were collected at the sites. Mortalities due to MSX were not apparent.

EFFECTS OF *PERKINSUS MARINUS* ON CULTURED MOBILE BAY OYSTERS. **Guy W. Brunt** and **Yolanda J. Brady,** Department of Fisheries and Allied Aquacultures, Auburn University, Auburn, AL 36849–5419.

A protozoan parasite, *Perkinsus marinus*, is both common and widespread in the Gulf of Mexico and has been responsible for severe infection and mortality of eastern oysters, *Crassostrea virginica*. This study investigated the effects of *P. marinus* on oysters being cultured in a suspended bag system in the Bon Secour area of eastern Mobile Bay. Alabama. Determinations of infection prevalence and intensity as well as related regulating factors were made, as were determinations of the effects of *P. marinus* infection on oyster digestive diverticula and vesicular connective tissue. Also, differences between first and second year age class oysters and differences between oysters cultured near the top and bottom of the water column were assessed.

P. marinus infection prevalences were high (usually 85% or above) throughout the study period. Infection intensities were generally light, however, and were not ususally at levels associated with severe oyster mortality. Higher infection prevalences and intensities were associated with the warmer months, though salinity was concluded to be the primary regulating factor. Condition of oyster digestive diverticula and vesicular connective tissue decreased as infection intensity increased, although other factors probably exerted some influence on oyster condition as well. No consistent differences were found between oyster groups (first and second year, high and low in water column), with the exception that second year oysters had more atrophic digestive diverticula than first year oysters.

PREDATOR- AND PREY-DIFFERENTIATED REPAIR FREQUENCIES IN THE MOON SNAILS *EUSPIRA HEROS* AND *NEVERITA DUPLICATA* VERSUS THE WHELKS *BUSYCON CARICA* AND *BUSYCON CANALICULATUM* FROM CAPE MAY COUNTY, NEW JERSEY. **Gregory P. Dietl,** Dept. of Geological and Marine Sciences, Rider University, Lawrenceville, New Jersey 08648.

More than 1300 specimens combined from the moon snails *Euspira heros* and *Neverita duplicata,* and the whelks *Busycon carica* and *Busycon canaliculatum*, were collected from Hereford Inlet and Great Egg Harbor in Cape May County, New Jersey. Body whorl diameter and apertural lip thickness were measured, and the number of sublethal scars per final whorl counted, for each specimen. Although the mean number of repairs per specimen was different among the four species (ANOVA, p = .0001), the average was comparable for the two moon snails, namely 1.1 and 0.9 for *N. duplicata* and *E. heros*, and identical (6.3) for *B. carica* and *B. canaliculatum*. Repair frequencies per specimen ranged from 0 to 13 for both whelk species, 0 to 12 for *N. duplicata,* and 0 to 7 for *E. heros*. Only 4% and 3% of *B. canaliculatum* and *B. carica*, respectively, lack repairs, whereas 48% and 57% of *E. heros* and *N.. duplicata*, respectively, lack repairs. Repair frequencies are strongly correlated with both body whorl diameter and apertural lip thickness for both whelks and *N. duplicata*, but not for *E. heros* from the Hereford Inlet locality.

The greater mean frequency of repairs in *B. canaliculatum* and *B. carica* relative to the two moon snails is attributed to shell breakage from predation by crabs on whelks combined with apertural lip fracture during attempts by whelks to wedge apart the valves of their bivalve prey. In contrast, manipulation of shelled prey by moon snails in preparation for drilling doesn't induce apertural fracture. Consequently, less than 5% of whelk shells show no damage in contrast to the more than 50% of moon snails lacking repairs. Surprisingly, the thinner whelk *B. canaliculatum* and moon snail *E. heros* do not have number of repairs-frequency distributions different from their thicker lipped relatives *B. carica* and *N. duplicata*, respectively. (Kolmogorov-Smirnov test; p = .20 between whelks; p = .28 between moon snails). Repaired fractures on the apertural lips of the largest whelks and moon snails indicate a lack of a "size refuge" from sublethal predation.

SPATIAL PATTERNS OF INTERTIDAL OYSTER REEFS IN THE CANAVERAL NATIONAL SEASHORE, FLORIDA. **Ray Grizzle,*** Randall Environmental Studies Center, Taylor University, Upland, IN 46989; **Mike Castagna,** Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062.

The Canaveral National Seashore includes much of the northern Mosquito Lagoon in northeastern Florida, and some of the few remaining large intertidal oyster (*Crassostrea virginica*) reefs along the Atlantic coast. We characterized intra-reef patterns at a scale of 1 m by sampling quadrats quarterly (July 1994–March 1995) along fixed transect lines on ten different reefs, and lagoon-wide (inter-reef) distribution patterns using low-altitude aerial photography (1:6,000 imagery, color IR film) in January 1995.

Intra-reef patterns included a strong "edge effect" with substantially greater spat settlement and oyster density (but no differences in mean shell height) in a 2 to 3-m fringe around most reefs. Inter-reef patterns showed two strong trends: a lagoon-wide S to N increase in areal coverages by the reefs correlated with increasing tidal ranges; and multiple-reef patterns that suggested a relationship to tidal flow patterns. Tide range was also positively correlated with oyster and spat densities, but not oyster size. Though a complete analysis has not been done, reefs showed obvious complex spatial patterns relative to tidal channels. The largest reefs and many small reefs were oriented parallel to and/or along the edges of major tidal channels. In contrast, there were several clusters of moderate-sized reefs arranged in dendritic patterns associated with multiple tidal channels. Both intra- and inter-reef patterns can be explained by existing hydrodynamical models concerned with water flow and food fluxes.

SERIAL DEPLETION IN MARINE INVERTEBRATE DIVING FISHERIES. **Peter L. Haaker,*** California Department of Fish and Game, Long Beach, CA 90802; **Gary E. Davis,** National Biological Service, Ventura, CA 93001; **Ian K. Taniguchi,** California Department of Fish and Game, Long Beach, CA 90802.

California's diving fisheries relied primarily on five abalones, one sea urchin, and a few other invertebrates in the 20th century. Improvements in technology, e.g., fast boats, electronic navigational and survey equipment, SCUBA, and thermal protection for divers, increased diver efficiency and provided access to remote territories and deep habitats. As populations of the most accessible and valuable species declined, divers switched to less accessible and lower valued species. Red abalone dominated landings prior to World War II, followed by pink abalone in the 1950s and 1960s. Green and white abalones contributed briefly in the mid-1970s, before harvest shifted to intertidal black abalone and red sea urchins. Current abalone landings are ~10% of historic levels. When urchin landings in southern California began to wane in the early 1980s, fishing effort moved to new territories in northern California. The shift from abalones to urchins required an order of magnitude increase in harvested biomass to sustain total fleet income, e.g., 2,000 tonnes of abalone yielded about the same income as 20,000 tonnes of sea urchin. This frontier approach to sustained fisheries continues today. The dive fishery is currently exploring more species, e.g., small purple sea urchin, wavy top turban snail, purple coral, and sea cucumber.

As species were depleted, their harvest was not curtailed to allow recovery, consequently those populations remain at dangerously low levels. When new fisheries were developed no entry restrictions were imposed, leading to overcapitalization and over fishing. As technological improvements increased the efficiency of harvest, management paradigms failed, and important marine resource stocks declined to the point of collapse. Even though serial depletion of marine resources may have been advantageous to the fishing industry in the 20th century, it is not sustainable. Restoration of public benefits derived from the productivity of coastal waters will now require expensive restoration of depleted populations and a loss of benefits for many decades while restoration is affected.

FEDERALLY ENDANGERED FRESHWATER MUSSELS IN THE ST. CROIX RIVER: MICROHABITAT AND MUSSEL COMMUNITY ASSOCIATIONS. **D. J. Hornbach,* T. Deneka,** and **P. Baker,** Dept. Biol., Macalester Coll., St. Paul, MN 55105.

Recent studies indicate that macrohabitat rather than microhabitat factors are better predictors of freshwater mussel community structure. However, microhabitat factors can be important in describing local abundance and distribution of mussels. Unfortunately, for many rare mussels, factors responsible for controlling their abundance and distribution have not been studied. Our hypothesis was that endangered mussels are found in high quality mussel habitat rather than in peculiar niches and must be found in localized populations if they have been successful in maintain a viably reproducing population.

We conducted this study in a Mississippi River tributary, the St. Croix River, which contains at least 38 mussel species including two federally endangered species: *Lampsilis higginsi* and *Quadrula fragosa*. Using SCUBA, 494 0.25 m^2 quadrats were examined to characterize the mussel community and microhabitat (sediment size, water depth and flow). We also conducted searches specifically for *Q. fragosa* and *L. higginsi* quantitatively sampling areas where specimens were found.

The most dense and rich mussel communities were associated with specific substrate types in conjunction with particular water depth and flow regimes. *Q. fragosa* and *L. higginsi* were found in areas of rich and diverse mussel assemblages. Consequently these endangered species did not have peculiar niches, indicating that a community assessment technique may be helpful in endangered mussel management.

THE EFFECTS OF LARVAL STOCKING DENSITY ON GROWTH AND SURVIVAL OF LABORATORY REARED *SPISULA SOLIDISSIMA SIMILIS*. Dorset H. Hurley* and **Randal L. Walker,** Shellfish Aquaculture Laboratory, University of Georgia, Marine Extension Service, 20 Ocean Science Circle, Savannah, GA 31411–1011.

Growth and survival of *Spisula solidissima similis* (Say, 1822) larvae stocked at 10, 20, 30 and 50 larvae per ml were determined in a laboratory growth study to define the optimum stocking density for culture of this subspecies. Twenty-four hour old larvae were stocked at the above densities within 500 ml flasks containing filtered seawater at 25 ppt and 20°C in a constant temperature-controlled room. All treatments received a daily food ration of 100,000 cells per ml of Tahitian strain *Isochrysis* sp. with a complete water exchange per flask every two days. Three replicate flasks per treatment were subsampled ($N = 5$), on days 1, 5, 9, 15, and 27. No significant differences ($p = 0.3539$) in survival occurred between stocking densities at day 27 with percent survival ranging from 61% for the 10 larvae per ml to 32% for the 50 larvae per ml stocking treatments. Larval size (μm) was significantly different for all treatments on day 9 ($p < 0.0001$: 50, $\bar{x} = 88.4 < 20$, $\bar{x} = 95.0 < 30$, $\bar{x} = 101.8 < 10$, $\bar{x} = 117.0$) and day 27 ($p < 0.0001$; 50, $\bar{x} = 145.8 < 30$, $\bar{x} = 175.6 < 20$, $\bar{x} = 195.7 < 10$, $\bar{x} = 263.0$). A higher percentage of animals had undergone complete metamorphosis at day 27 in the lower stocking density treatment of 10 (87%) than in the higher stocking density treatments of 20, 30, and 50 larvae per ml (13%, 3.6%, and 0%, respectively).

SALINITY EFFECTS ON INTRODUCED DREISSENID MUSSELS. V. S. Kennedy,* M. Aspleen, and **T. Hall,** University of Maryland System, Horn Point Environmental Laboratory, Cambridge, MD 21613.

We tested the effects of salinity on movement, byssal attachment, and mortality of smaller (<1.5 cm) and larger (>2 cm) zebra (*Dreissena polymorpha*) and quagga (*D. bugensis*) mussels acclimated to freshwater, and to 3 and 5 ppt (quagga mussels; QM) or to 4 and 6 ppt (zebra mussels; ZM). Freshwater-acclimated mussels demonstrated a decrease in movement after 48 h in salinities of 7+ ppt (ZM) and 4+ ppt (QM), a decline in byssal attachment at 5+ ppt (ZM) and 3+ ppt (QM), and increased mortality at 6 ppt (ZM) and 4+ ppt (QM). Acclimation to higher salinities at a rate of 1 ppt every 3 d raised the salinities at which movement and attachment were inhibited after 48 h for both mussel species. Zebra mussels survived exposure to 10 ppt with limited mortality (>30%); quagga mussels tolerated up to 8 ppt. Larger zebra and quagga mussels were less likely to move, attach, or survive than were smaller mussels in similar salinities. Additional experiments on the native dreissenid in Chesapeake Bay, *Mytilopsis leucophaeata*, found that movement, attachment, and mortality were minimally affected by salinities down to 2 ppt for individuals acclimated to 12 ppt, with limited mortality even in fresh water.

Some zebra and quagga mussels spawned spontaneously in the experimental bowls during the salinity tolerance experiments. Embryos formed in salinities above about 4 ppt did not survive past the early stages of cell division, even if derived from mussels acclimated to higher salinities. Eggs spawned into fresh water by mussels acclimated to 3 to 6 ppt went unfertilized, ultimately enlarging, then disappearing. We conclude that zebra and quagga mussels can adjust to oligohaline conditions, that they could overlap with *M. leucophaeata*, but that spawning in the upper estuary may not produce viable embryos.

GENETICS AND SYSTEMATICS OF FRESHWATER MUSSEL SPECIES: A TISSUE REPOSITORY. Tim L. King,* Mary E. Smith, Rita F. Villella, Priscilla I. Washington, and **David A. Weller,** Department of the Interior, National Biological Service—Leetown Science Center, Aquatic Ecology Laboratory, 1700 Leetown Road, Kearneysville, WV 25430.

Recognizing a lack of population genetics and phylogenetics information with respect to freshwater mussel conservation efforts, the National Biological Service—Leetown Science Center's Aquatic Ecology Laboratory has established a repository and associated database to coordinate tissue samples collected for genetics and systematics research. The repository provides a centralized location for researchers to obtain properly catalogued and preserved adductor muscle, mantle, foot, gill (including glochidia), and digestive gland tissue samples. All data generated for the repository are maintained in the PARADOX for Windows relational database package. Collection information compiled for each specimen includes data, site name, site description, and habitat characteristics. Database content reports are generated and provided to interested researchers. Currently the database contains in excess of 260 individuals representing 46 species inhabiting Atlantic slope and Interior Basin drainages. All researchers utilizing the repository are required to accommodate a standard numbering scheme to allow comparisons of the same individuals among diverse studies and methodologies. Potentially, the repository would reduce the number of animals sacrificed and sampling time while providing comprehensive data to multiple researchers. A single collection of mussels can provide ecophenotypic, protein, DNA, and immunological information for species and population structure delineation. This poster describes the development of the repository, provides instructions for tissue contribution and retrieval, and presents data collection protocols, preservation methods, database structure, and a current report of the database contents.

DISCONTINUITY IN THE GENETIC POPULATION STRUCTURE OF THE GREEN FLOATER *LASMIGONA SUBVIRIDIS*. Tim L. King,* Rita F. Villella, Mary E. Smith, and **Michael S. Eackles,** Department of the Interior, National Biological Service—Leetown Science Center, Aquatic Ecology Laboratory, 1700 Leetown Road, Kearneysville, WV 25430.

Modern molecular genetic techniques have revealed population genetic structure and phylogenetic associations within and among many rare taxonomic groups. However, little genetic-based pop-

ulation or phylogenetic information is available for most freshwater mussel species. To address the need for mussel genetics research, we have evaluated techniques to identify and assess genetic variability in selected ribosomal and mitochondrial (mtDNA) DNAs among geographic populations of the green floater *Lasmigona subviridis*. A total of 43 *L. subviridis* were sampled from nine geographic populations representing five rivers draining the Atlantic slope (Susquehanna, 2 sites; Potomac, 2 sites; Rappahannock; James; and Neuse Rivers) and the New River (2 sites) an Interior Basin drainage. Each mussel was subjected to polymerase chain reaction amplification and restrictionase digests of the first internal transcribed spacer region (ITS-1) of nuclear ribosomal DNA and the cytochrome oxidase I (COI) region of mitochondrial DNA. Diagnostic genetic differentiation was observed in both ribosomal and mitochondrial DNA among geographic populations of *L. subviridis*. The 570 base-pair (bp) ITS-1 fragment, digested with the enzyme *Dde* I, produced a diagnostic restriction site polymorphism between the northern populations (Susquehanna and Potomac Rivers) and the remaining populations. The northern populations also exhibited a diagnostic restriction site polymorphism in the 710 bp COI region of mtDNA when digested with the enzyme *Cfo* I. Preliminary results suggest the presence of a latitudinal discontinuity in the population structure of *L. subviridis*, and possibly the absence of gene exchange. No differentiation among the Atlantic slope and Interior Basin populations has been observed.

EVALUATION OF TAG TYPES AND ADHESIVES FOR MARKING FRESHWATER MUSSELS. **David P. Lemarié, David R. Smith, Rita F. Villella,** and **David A. Weller,** National Biological Service—Leetown Science Center, Aquatic Ecology Laboratory, 1700 Leetown Road, Kearneysville, WV 25430.

External identification of individual mussels is highly desirable for following passive and active movements, population studies, and labeling for studies of growth, reproduction, genetics, and physiology. Ideally, tags must be easy to apply, inexpensive, and provide excellent long term legibility and retention.

In this study we evaluated three varieties of tags (Northwest Marine Technology Visual Implant Tag, Floy Fingerling Tag, and Hallprint Shellfish Tag), two types of adhesives (3M two-part epoxy and Krazy Glue cyanoacrylate), and four bonding times before immersion in water (2, 5, 10, and 15 min). Tags were applied to shells of dead animals. Tag/glue combinations showing good initial legibility after complete curing of the adhesive were further tested under natural conditions in a shallow stream and in a standard gem tumbler containing coarse metal shavings.

This poster provides an illustrated summary of the advantages and disadvantages of each of the tag types and adhesives tested. Preliminary results suggest that the best combination is a flexible polyethylene shellfish tag bonded to the shell with cyanoacrylate. Cyanoacrylate can be immersed in water in as little as two minutes after application.

SPECIES SPECIFICITY AND EFFECT OF PH ON THE RESPONSE OF FRESHWATER MUSSEL JUVENILES TO ACUTE COPPER TOXICITY. **A. D. McKinney,** Tennessee Wildlife Resources Agency, Nashville, TN; **R. G. Hudson,** Presbyterian College, Clinton, S.C.; **Margaret L. Barfield,** Arkansas State University, Jonesboro, AK.

Utterbackia imbecilis is the only freshwater mussel species whose juveniles have routinely been used for toxicity testing. This species is somewhat ubiquitous, occurring in a variety of habitats. A review of literature showing that pH affects the toxicity of certain metal ions in nonmolluscan species is given. To determine if this is true also with mussel juveniles, a range of copper ion concentrations was tested on *U. imbecilis* juveniles at two different pH levels. Furthermore, to show whether this species is representative of a flowing water species, *Ellipitio angustata* juveniles were also tested at the more neutral pH range. Tests were made with four repetitions of 10 juveniles/repetition in each test group using moderately hard water with 800 mg/l silt and the addition of bloomed plankton, imitating their natural environment as much as possible. Copper concentrations ranged from 1–4 ppm, with a control. Results show that the lower pH values caused the sensitivity of the juveniles to more than double, with a LC_{50} value of 1.28 ppm in the lower pH group and 2.75 ppm in the higher pH group. Comparison of the higher pH group with juveniles of *E. angustata* revealed that the two had identical LC_{50} values. The immediate significance of this preliminary study is to emphasize the consideration of pH when conducting and comparing toxicity studies involving juvenile mussels. Furthermore, the increased stress on populations in acidified water is obvious, and standards regulating known pollutants should be qualified by a statement of acceptable pH range. Finally, juveniles of *U. imbecilis* were representative of this other stream dwelling mussel as far as copper sensitivity is concerned.

FATE OF POTENTIAL BACTERIAL CONTAMINANTS AS A FUNCTION OF CONTACT SURFACE IN SHELLFISH WET HOLDING TANKS. **Carter R. Newell,*** Great Eastern Mussel Farms, Inc., Tenants Harbor, ME 0486; **Bohdan M. Slabyj,** Department of Food Science, University of Maine, Orono, ME 04469.

Perceived risks associated with the use of masonry surfaces in shellfish holding tanks and associated reservoirs have led to the enforcement of "food contact surface" requirements in those systems. In preparation for the 1995 ISSC meeting, a study was performed using both porous (cement) and non-porous (fiberglass) mussel (*Mytilus edulis*) miniature wet holding tanks (microcosms). The incoming water was seeded with *Escherchia coli, Enterococcus faecium, Listeria innocua* and a non-pathogenic *Vibrio* isolate. Influent and effluent water, tank sediments and mussels were sampled from 0–72 hours. Mussel lots were changed daily, and tanks were steamed cleaned between lots mirroring normal production conditions. In both types of tanks (concrete and fiberglass) all

seeded bacteria disappeared with time and were essentially removed with routine cleaning. The experiments should be repeated for confirmation. Nonetheless, they indicate the beneficial role of the natural seawater flora in outcompeting potential pathogens in seawater from approved shellfish growing areas.

RFLP ANALYSIS OF GENETIC DIVERSITY IN A SIBERIAN POPULATION OF THE JAPANESE SCALLOP (*PATINOPECTEN YESSOENSIS*). **Elizabeth A. Orbacz,* Ami E. Wilbur, Jeffrey R. Wakefield,** and **Patrick M. Gaffney,** College of Marine Studies, University of Delaware, Lewes, DE 19958.

Restriction fragment length polymorphism (RFLP) analysis was used to compare the genetic diversity of a Siberian population of the Japanese scallop (*Patinopecten yessoensis*) with populations previously examined by Boulding et al. (1993, Can. J. Fish. Aquat. Sci. 50: 1147–1157) including a small hatchery population in British Columbia and two wild populations from Mutsu Bay (Aomori) and Uchiura Bay (Hokkaido) in Japan. The polymerase chain reaction (PCR) was used to selectively amplify three coding regions of the mitochondrial genome in 20 individuals from Peter the Great Bay (Primorye Region, Russia). These regions included (1) most of ATP synthetase subunit 6 and cytochrome *c* oxidase subunit 3 (1.5 kb), (2) tRNA for threonine (1.1 kb), and (3) part of cytochrome *b* apoenzyme (1.4 kb). Digestion of the PCR products with 11 restriction enzymes revealed 22 polymorphic sites and 19 distinct composite haplotypes. Haplotype diversity and within-population nucleotide diversity were high in the Siberian population (0.98 and 0.015 respectively). Both of these estimates of genetic diversity are much greater than those calculated by Boulding et al. (1993) for the hatchery (haplotype diversity = 0.53, nucleotide diversity = 0.0012) and Japanese populations (mean haplotype diversity = 0.72, mean nucleotide diversity = 0.0017).

MANAGEMENT STRATEGIES FOR FOULING CONTROL IN ALABAMA OYSTER CULTURE. **F. Scott Rikard** and **Richard K. Wallace,** Auburn University Marine Extension and Research Center, Mobile, AL; **Christopher L. Nelson,** Bon Secour Fisheries, Inc., Bon Secour, AL.

Fouling by marine organisms is a major impediment to the development of inshore mariculture. Fouling control methods for off bottom oyster culture were analyzed experimentally over a two year period for effects on fouling, oyster growth, oyster condition and oyster survival. Oysters were held in plastic mesh bags attached to a belt system suspended in the water column. The first year study focused on pressure washing treatments at 2, 4, and 8 week intervals, and biological control treatments using blue crabs, hermit crabs and stone crabs, and a control receiving no washing or animals. Frequentlyt washed oysters (2 and 4 week intervals) had significantly less fouling than the 8 week wash interval or the unwashed control but were significantly smaller and suffered greater mortality. Stone crabs showed the most potential for biological fouling control but also appeared to prey significantly on the oysters. Based on the first years results, methods were refined and a second experiment designed. Treatments analyzed were a 6 week was interval, a bag change treatment, biological treatments using larger blue crabs, and a control. There were no significant differences in fouling, mortality and condition among all treatments at the time of harvest. Some significant differences in the fouling index between 6 week wash interval and other treatments were seen during peak fouling times. There was a significant difference in growth between the bag change treatment and the control at the time of harvest. Current management suggestions are to pressure wash bags at a 6 week or greater interval and also target washing to coicide with peak settlement of fouling organisms.

SENSORY PHYSIOLOGY OF GLOCHIDIA LARVAE OF THE FRESHWATER MUSSELS *UTTERBACKIA IMBECILLIS* AND *MEGALONAIS NERVOSA*. **Melanie K. Shadoan*** and **Ronald V. Dimock, Jr.,** Department of Biology, Wake Forest University, Winston-Salem, NC.

The stimuli involved in the attachment of larvae of freshwater mussels to fish hosts are almost completely unknown, with a few observations suggesting fish mucus or body fluids as an important cue. The responses of glochidia of *Utterbackia imbecillis* and *Megalonais nervosa* to chemical and mechanical stimulation were monitored. Larvae with hooked shells (*U. imbecillis*) typically attach to fins and opercula, while hookless larvae (*M. nervosa*) attach to gills. The larvae were exposed to fish epithelial mucus (blue gill, bass, goldfish) and to mucus that was fractioned by ultrafiltration (fractions: >10KD, <10 >3KD, <3KD). Putative components of fish mucus including sialic acid, galactose, mannose, and free amino acids (pH 6.5) also were tested. In addition, larvae were mechanically stimulated (stroked until tonic closure) with a 30 μm glass micropipet in a micromanipulator and also mechanically stimulated in the presence of selected amino acids to determine if a synergistic effect between chemical and mechanical stimulation occurred. When exposed to fish epithelial mucus, all larvae of both species of mussels experienced tonic closure; however, the duration of closure was longer for *U. imbecillis*. Glochidia responded only to the <3KD fraction of fish mucus. Of the non-amino acids tested, only sialic acid induced rhythmic adductions, with *M. nervosa* showing a greater response. *M. nervosa* was also more sensitive than *U. imbecillis* to all tested amino acids at 10^{-2}M, responding with more rhythmic adductions and a higher percentage of larvae undergoing tonic closure. Mechanical stimulation induced tonic closure in both species, but *M. nervosa* required more stimuli before it closed. Synergism between mechanical and chemical stimuli was evident from a significant increase in the duration of tonic closure by larvae of both species.

THE CULTURE VARIABILITY OF *MONOCHRYSIS LUTHERI* AS AN ADVANTAGE FOR SHELLFISH CULTURE. **Lioudmila V. Spektorova,** Harbor Branch Oceanographic Institution, 5600 Old Dixie Highway, Fort Pierce, FL 34946.

During different developmental stages, mollusks need algae of various chemical compositions. Aquaculturists require better knowledge of conditions that ensure a biomass yield rich in protein, lipids or with a high HUFA level. From this point of view, *Monochrysis lutheri* is attractive, because it has a flexible metabolism. *M. lutheri* contains high level of lipids and both essential fatty acid 20:5w3 and 22:6w3, in similar proportions to oyster tissue.

Microalgae experiments were made outdoors in a closed tubular photobioreactor of 160 l volume capacity on the Black Sea Experimental Station between May and October. The daily radiant energy averaged 132 W m^{-2} in May, 171 W m^{-2} in July and 80 W m^{-2} in October. Nutrient concentrations were maintained at 230–250 mg N l^{-1}, 50–70 mg P l^{-1} at a pH 7.0–7.7. The optimal temperature for this strain was 28°. We tested the role of three factors in influencing the chemical composition of *M. lutheri:* season, amount of nitrogen, source of nitrogen. Most favourable conditions were in July, when the culture reached a density of 400–600 10^6 cells/ml. Cells contain 44–46% of protein and 16–18% of lipid. The highest percent of 20:5w3 was recorded in July (19% of total fatty acids). In the autumn, the culture density was only 200 10^6 cells/ml, the protein content decreases to 33%, lipids increased to 32% and amount of 20:5w3 dropped to 3,5%. The decline of nitrogen level in the medium from 250 to 120 mg N^{-1} lowered the protein level by 6,5%. The highest amount of protein was found in cells which were grown using the combination of ammoniacal and urea nitrogen. The amount of lipids can reach 40–45% under unfavourable conditions (e.g. decreasing temperature, low level of both irradiance and nitrogen).

EFFECTS OF THE TOXIC DINOFLAGELLATE, *PFIESTERIA PISCICIDA*, ON JUVENILE BAY SCALLOPS (*ARGOPECTEN IRRADIANS*, LAMARCK). **Jeffrey J. Springer*** and **JoAnn Burkholder,** Department of Botany, North Carolina State University, Raleigh, NC 27695; **Sandra E. Shumway,** Division of Natural Sciences, Southampton College of Long Island University, Southampton, NY 11968.

The recently discovered toxic dinoflagellate, *Pfiesteria piscicida*, has been implicated as the causative agent in at least 50% of the major finfish kills (>1,000 fish) since 1991 along the North Carolina coast. Preliminary data indicate that a neurotoxin released by this dinoflagellate adversely affects certain species of shellfish as well.

Zoospores of *P. piscicida* release a yet-to-be characterized neurotoxin that may render a fish helpless in minutes. Sloughed scales and tissue from the dying fish are then fed upon by the zoospores. In the period between a fish kill, *Pfiesteria piscicida* may encyst and remain in the sediments until the next bloom is triggered. It can also survive in a multitude of forms including amoebae as well as non-toxic zoospores (NZs), subsisting heterotrophically on diets of flagellated algal prey. However, those forms which do not encyst can transform into toxic zoospores (TZs) within minutes after being introduced to fish.

Release of the toxin(s) associated with *P. piscicida* is known to be lethal to finfish both in the field and laboratory when dinoflagellate cells are present in sufficient densities (>300 cells/ml). This toxin has also been documented to cause adverse neurological and immunological effects in humans.

While a major fish kill event involving *P. piscicida* is occurring, floating fish carcasses are highly visible and tend to draw public interest. However, what is not known is the effects of the dinoflagellate's zoospores on shellfish located in the general vicinity of the kill. The goal of this research was to determine short-term effects of zoospores on shellfish populations in estuarine ecosystems. A grazing study was conducted to assess the potential impact on commercially important shellfish as indicated by the bay scallop, *Argopecten irradians*. A significant decrease in clearance rate was noted along with a "narcotizing" effect on exposed scallops. Some scallops ceased feeding after 15 minutes of continuous exposure to zoospores. The effects of long-term exposure to zoospores must be studied in further research to determine adverse impacts on shellfish populations in areas repeatedly affected by toxic outbreaks of *P. piscicida*.

DEVELOPMENTAL SHIFTS IN THE FEEDING BIODYNAMICS OF JUVENILE *UTTERBACKIA IMBECILIS* (MOLLUSCA: BIVALVIA). **R. A. Tankersley,*** Department of Biological Sciences, University of Maryland, Baltimore County, MD; **J. J. Hart** and **M. G. Wieber,** Biology Department, Gonzaga University, Spokane, WA.

Ontogenetic shifts in the feeding mechanisms utilized by juvenile mussels (*Utterbackia imbecilis*) immediately following transformation were determined and associated with morphological changes in pallial feeding structures. Video recordings of feeding activities indicated juvenile *U. imbecilis* utilize a combination of interstitial suspension and deposit feeding to capture and ingest particles. Cilia located on the foot, gills, and anterior edge of the mantle produce anterior inhalant currents that draw suspended particles into the mantle cavity for ingestion. Deposited particles were collected and drawn toward the pedal gape using both pedal-sweep and pedal locomotory feeding. The relative contribution of each feeding mode to the ingestion rate of 8, 14 and 24 day old juveniles was determined by examining the gut contents of mussels fed fluorecently labeled latex beads. Dominant feeding mode varied with age, with younger juveniles relying more heavily upon deposit feeding mechanisms than older mussels. The rate of deposit feeding was enhanced by the presence of fine silt (<202 μm), suggesting that particles too large to ingest may serve as important substrata for deposit feeding. Ontogenetic shifts in the mode of particle acquisition were accompanied by changes in the functional

morphology of suspension feeding structures, including the size and number of ctenidial filaments and ciliary tracts.

WHAT A YEAR TO BE A MUD CRAB! THREE YEARS ON THE BAY SCALLOP RESTORATION PROJECT, WESTPORT RIVER ESTUARY, MASSACHUSETTS. **Wayne H. Turner,* Karin A. Tammi,** and **Bethany A. Starr.** The Water Works Group, Inc., Post Office Box 197, Westport Point, MA 02791.

The Bay Scallop Restoration Project (BSRP) was launched in 1993 with the goal of generating the interest, involvement, and enthusiasm required to restore and enhance the renewable economic resources of traditional fishing and farming communities. 83,000 hours of volunteer work enthusiastically invested by teams of people have been channeled into this effort. These people: *students, teachers, parents, graduate students*—have left a major impact, not only on the bay scallop, *Argopecten irradians*, but on the way in which communities participate and positively affect the direction of their economic future and environmental quality.

With three years of research on the BSRP, community volunteers, led by graduate students have uncovered several significant clues about bay scallop propagation in the Westport River. In 1993, when the BSRP first began, mud crabs, *Panopeus* spp., went largely unnoticed as very few were found on the river bottom or in the propagation equipment. Green crabs, *Carcinus maenas*, on the other hand, were plentiful and practically every spat bag (propagation equipment used to catch juvenile scallops) had at least one if not two green crabs associated with it.

Strangely enough, in the summer of 1994, green crabs took a dive and mud crabs surged. Researchers began counting thousands of mud crabs as they poured from nearly every spat bag. Because of the recent prevalence of mud crabs, an experiment using floating rafts was set up in the Westport River; each raft housing a different combination of four ingredients: mud crabs, green crabs, spat size bay scallops, and yearling tautog, *Tautoga onitis* (approximately two inches in length).

The conclusions drawn from this study are intriguing: 1) mud crabs ate the bay scallops; 2) green crabs did not eat the bay scallops and instead ate the mud crabs; 3) yearling tautog could not seem to handle a green crab (probably due to size differences), but cleverly enough, researchers observed that mud crabs in a raft with yearling tautog lost one leg per day. By the fourth day of the experiment, the mud crab could no longer move and the tautog ate it. 1995, therefore appears to be a good year to be a mud crab for several reasons. First, green crabs have been down in numbers for the past two years. Secondly, yearling tautog, commonly found in spat bags in 1993 were virtually absent during 1994 and 1995. Finally, supporting this abundant supply of mud crabs is the propagation activities of the BSRP which is increasing bay scallop spat production, a preferable food source for mud crabs.

FECUNDITY ESTIMATES OF THE SOUTHERN SURFCLAM *SPISULA SOLIDISSIMA SIMILIS*. **Randal L. Walker,* Dorset H. Hurley,** and **Michelle L. Jansen,** Shellfish Aquaculture Laboratory, University of Georgia Marine Extension Service, 20 Ocean Science Circle, Savannah, GA 31411–1011.

Fecundity estimates for two stocks of *Spisula solidissima similis* (Say, 1822) were determined in laboratory spawning trials. One stock was dredged from St. Catherines Sound, but transplanted to field grow-out cages planted in a sand flat at the mouth of House Creek, Little Tybee Island, Wassaw Sound, for six months prior to the spawning trial. A second stock was dredged from St. Catherines Sound prior to the spawning trial. One-year-old clams were injected weekly with 0.2 ml serotonin in the posterior adductor muscle to induce spawning from March 29, 1995 until the end of June 1995. For clams ranging in shell length from 26 to 50 mm, egg production per female ranged from 0.14 to 13 million eggs. The House Creek stock produced a greater mean number of eggs per female (4.8×10^6 versus 1.7×10^6) and spawned 2.8 times more than St. Catherine's Sound stock. No relationship of number of eggs per female to shell length occurred for either stock. Overall, eggs from the House Creek stock ($\bar{x} = 59.1$ μm) were significantly larger ($p < 0.0001$) than eggs ($\bar{x} = 57.4$ μm) from the St. Catherine's Sound stock. Although small in size, one-year-old southern surfclams can produce sufficient numbers of eggs per female to be utilized as brood stock for the development of an aquaculture fishery for this subspecies in the southeastern U.S.

PREPARED FOOD COUPLED WITH MANIPULATION OF PHOTOPERIOD YIELD AN OUT-OF-SEASON CROP FOR THE NORTHEASTERN SEA URCHIN. **Charles W. Walker** and **Michael P. Lesser,** Department of Zoology, University of New Hampshire, Durham, NH 03824.

The fishery for the green sea urchin (*Strongylocentrotus droebachiensis*) has rapidly grown to become the second largest in the Northeastern United States behind lobsters. Overfishing has drastically depleted once abundant natural populations. Two other problems naturally plague the industry. One of these is poor roe quality in a large percentage of the urchins harvested, leading to a lower than maximum price. Another is the short period when roe quality is high. There is a window of time from September until February when urchins have firm, ripe gonads. If urchins in a land based aquaculture facility could be fed a prepared food and be induced to ripen again after February, then the period of availability of highest quality roe could be expanded, greatly increasing the market potential for Gulf of Maine urchin roe. We have coupled: 1) enhancement of gonadal growth of poorly fed urchins utilizing prepared food with 2) photoperiodic manipulation of the gametogenic cycle to produce an out-of-season crop which could be used to exploit a lucrative end of summer market now supplied by Chile. Rather spawned urchins (March, 1995; ≤6% gonad index) were held under artificial illumination, using astronomic clocks set to simulate June photoperiod and were feed 3 g prepared food/

animal/week. This resulted in a significant increase in gonad size compaired with field populations (≥25%) without a corresponding increase in test size. Histological examination of monthly samples of gonads indicates that this growth is a result of increase in size of nutritive phagocytes (which are intragonadal nutrient storage cells) yielding significantly higher gonadal indices than those simultaneously observed in field populations. After 3 months on this feeding regime, urchins were then exposed to September photoperiod which is known to naturally stimulate gametogenesis for urchins in the field. Stereological analysis of histological sections, indicate that spermatogonia in such animals undergo rapid proliferation and normal spermatogenesis three months early. Oogonia also proliferate early, but resulting oocytes undergo minimal vitellogenesis. Testes and ovaries both increase in size to gonad indices of 28–30% which is based on accumulation of normal spermatozoa in males and continued growth of nutritive phagocytes in females. Supported by Sea Grant Development Funds, New Hampshire and Hatch Grant #353 to C. W. Walker and M. P. Lesser.

MYELOPEROXIDASE ACTIVITY FROM BLOOD CELLS OF THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA*. **Jennifer Wojcik*** and **Kennedy T. Paynter**, Department of Zoology, University of Maryland, College Park, MD 20742.

Hemocytes of most bivalve molluscs are amoeboid, phagocytic cells which comprise an important part of the bivalve immune system. Similar to vertebrate macrophages, hemocytes engulf invading or other non-host entities and attack them within the phagosome through a series of biochemical reactions including lysosome and protease activation, and reactive oxygen intermediate (ROI) production. There are four types of ROIs typically produced: superoxide anions ($O2^-$), hydrogen peroxide (H2O2), hydroxyl radicals (OH), and hypochlorous acid (HOCl). In oysters, oxidative killing is thought to be one of the primary forms of immune response.

In most phagocytic cells, myeloperoxidase (MPO) catalyzes the production of hypochlorous acid from hydrogen peroxide and chloride. Hemocytes from a number of molluscan species have been shown to produce reactive oxygen metabolites, which could serve as a substrate for myeloperoxidase. When cells engulf the foreign particles, enzymes are brought into the phagosome and are then activated as the pH decreases. The enzymes catalyze the production of ROIs which have damaging biochemical effects on the foreign cells. ROI production is quantified by using a chemiluminescent assay. Taurine, a scavenger of hypochlorous acid, completely quenched chemiluminescence in oyster hemocytes, indicating that the cells produce hypochlorous acid during and shortly after phagocytosis.

We measured myeloperoxidase activity in extracts of oyster hemocytes using a variety of techniques. Tetramethylbenzidine (TMB) was peroxidized readily by small amounts of hemocyte extract, indicating a significant amount of MPO may be present. TMB peroxidation had a pH optimum of approximately 5.5. Attempts to differentiate between halide-independent and halide-dependent activities using diethanolamine and a taurochloramine assay yielded ambiguous results. Other techniques are currently being used to assess putative MPO activity and its importance in the oysters cytotoxic response.

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COVER PHOTO: Thurlow Nelson on board the *Julius Nelson* ca. 1948–1949. Photo courtesy of Haskin Shellfish Research Laboratory.

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Robert R. L. Guillard
Honored Life Member
National Shellfisheries Association

As we honor Dr. Robert R. L. Guillard—Bob—with lifetime membership in the National Shellfisheries Association, we should remind ourselves of two important aspects of Bob's relationship with shellfish. The first is that the rearing of shellfish in captivity, both for experimental research and aquaculture production, would not be possible without Bob's pioneering work in phytoplankton culture. The second important point is that Bob's contributions to the world of shellfish, although not exactly inadvertent, are no more than fortuitous offshoots of his research focus on the physiological ecology of phytoplankton. We should all hope that our sidelines are so successful!

Bob began his professional life as an electrical engineer at the Navy Yard in New York City; perhaps mussels set on the hulls, but otherwise, this seems a long way from shellfish. Graduate studies in microbial ecology at Yale, leading to a Ph.D. in 1954, brought Bob to Connecticut, where he became acquainted with Victor Loosanoff. Dr. Loosanoff then was Director of the U.S. Fish and Wildlife Service's Milford Marine Biological Laboratory and a fixture at Yale marine science seminars, having completed his own Ph.D. there. Apparently, Loosanoff would preface all questions of seminar speakers in his strong Russian accent, "As you know, I am interested from oysters . . ." Efforts to grow oyster larvae at the Milford Laboratory on fertilized, bloomed seawater had met with limited success, and communication with the Plymouth Laboratory suggested advantages of feeding selected phytoplankton to larval shellfish. Loosanoff must have seen in one student, Bob Guillard, the expertise needed to produce baby food for his oysters; a position funded by the Oyster Institute of North America was secured for Bob to spend several years at Milford.

During his time at Milford, Bob isolated a number of the phytoplankton cultures used widely to this day in marine research and shellfish culture, including 3H *Thalassiosira pseudonana*, and *Synechococcus bacillaris* (a cyanobacterium that would revolutionize biological oceanography 20 years later). Bob Guillard's first full research report was an article published in 1957, not coincidentally in the *Proceedings of the National Shellfisheries Association* (Vol. 48, pp. 134–142), titled, "Some Factors in the Use of Nannoplankton Cultures as Food for Larval and Juvenile Bivalves." Between this article and the 1958 *USFWS Fish. Bull.* 136 (Vol. 58), "Relative Value of Ten Genera of Microorganisms as Foods for Oyster and Clam Larvae," by Harry Davis and Bob Guillard, most of the practical information we use to this day in deciding what phytoplankton to feed molluscan larvae was established. Countless studies of basic shellfish biology, not to mention the establishment of hatchery-based shellfish aquaculture, were made possible by Bob Guillard's identification of practical algal diets.

If Bob Guillard did nothing more to benefit the shellfish community, his place among the legendary figures of shellfish biology would be assured. Then, he invented f/2. In July 1958, Bob had accepted a research position at Woods Hole Oceanographic Institution. While working to establish a collection of marine phytoplankton cultures for studies of plankton ecology, Bob faced the challenge of developing a nutrient enrichment for seawater that would support survival and growth of the widest possible range of microalgal taxa—no mean feat, considering the physiological diversity represented. Achievement of the "right recipe" was coincident with completion of a study,

published with John Ryther in 1962, having the seemingly arcane title, "Studies of Marine Planktonic Diatoms. I. *Cyclotella nana* Hustedt and *Detonula confervaceae* (Cleve) Gran." in the *Canadian Journal of Microbiology* (Vol. 8, 229–239). This article would become one of the most-cited in marine science, not because of extreme interest in the two diatoms that dominate the title, but because the seawater enrichment detailed in this report—designated f/2—turned out to be the most successful algal-culture medium ever developed. "f/2" has trademark recognition in marine science that would be the envy of most breakfast cereals, and a number of aquaculture-supply companies market premixed products of this composition. For this contribution, Bob Guillard does not deserve to be merely famous (which he is anyway), but he deserves to be very wealthy!

A move to the Bigelow Laboratory for Ocean Sciences in West Boothbay Harbor, ME, in 1982 led to the establishment of the Provasoli-Guillard Center for the Culture of Marine Phytoplankton (CCMP) there in 1985. Bob was director of this institution from its inception until his "retirement" in 1989. CCMP brought together the two great, privately held U.S. collections of marine phytoplankton, Bob's Woods Hole Collection and that of the late Dr. Luigi Provasoli of the famed Haskins Laboratories. The Center also established a framework and organization to ensure that phytoplankton strains of known origin and identity are available to the research community far into the future. Again, we have Bob Guillard to thank for building an algal supermarket where we can shop for shellfish munchies and know we are getting what we ask for.

As a publication record of well over 100 articles and many book chapters, notes, abstracts, etc. attests, Bob Guillard continues to contribute mightily to the field of phytoplankton ecology. In his "retirement," Bob has only increased the pace of his scientific activities, having shaken loose the chains of bureaucracy that inevitably accompany the title, "Director." Bob continues, as he has for many years, to teach. There are the legendary short courses. There are the endless telephone calls for help to which we subject him; there is no known instance of anyone whose call for help was ignored or given short shrift. There are the endless telephone calls *he* makes to students and professionals alike to suggest research directions and ideas—these seem to pop into his head much faster than even he can follow up on. There is the boundless curiosity and enthusiasm that continue to inspire.

A sign of maturity is the ability to articulate ideas in direct, simple language. Bob expresses the question driving his work with phytoplankton as, "Why do they live where they do?" This seemingly simple question weaves physical, chemical, and biological threads into the fabric of the invisible ecosystem—that of organisms too small for us to see, catch, dissect, and catalog with our unaided senses. To cultivate, using the limited resolution of the microscope and a dizzying array of indirect methods of measurement, a garden in which the smallest flowers will thrive requires a rare combination of knowledge, insight, and intuition. Bob Guillard has brought these talents to bear on the challenges of culturing phytoplankton . . . relentlessly. As we honor him at our annual meeting, Bob is back in his laboratory nursing along another new "bug," one that may reveal more secrets of his invisible ecosystem. We wish him luck with it and, for our own sakes, hope that it is the perfect food for larval oysters.

Bob has shared with some of us the irony of his interactions with shellfish farmers. He describes the typical telephone troubleshooting scenario as a series of phone calls in which the hatchery operator relates a problem and Bob suggests a response. The hatchery operator calls back to say that the suggested action did not fix the problem. Bob suggests the next step. The process is repeated. "Eventually," says Bob, "they stop calling. That's how I know what finally worked." Bob Guillard, as we thank you for over 40 years of help fixing our most difficult problems, we want you to know, "IT WORKED!"

Gary H. Wikfors
Milford, CT

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EFFECTS OF DIFFERENT SUBSTRATA AND PROTECTIVE MESH BAGS ON COLLECTION OF SPAT OF THE PEARL OYSTERS, *PINCTADA MARGARITIFERA* (LINNAEUS, 1758) AND *PINCTADA MACULATA* (GOULD, 1850)[1]

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ABSTRACT Refining techniques for the collection of spat is important to the culture of blacklip pearl oysters, *Pinctada margaritifera*, especially where the collection of spat is marginally effective. We deployed 40 spat collectors at 15 sites within the open reef complexes of Solomon Islands to test the effects of different collectors (constructed of shademesh and plastic sheeting) and protective mesh bags on the abundance of spat. After 6 mo, we recorded abundances of *P. margaritifera*, and another pearl oyster, *P. maculata*, together with the numbers of predators associated with the collectors. Significantly more *P. margaritifera* were found on the shademesh, whereas live *P. maculata* were more abundant on the plastic sheeting. Collectors inside protective mesh bags did not yield more pearl oysters than those left unprotected. Mesh bags trapped predators such as *Cymatium* spp. gastropods and portunid crabs settling to the collectors from the plankton. The bags also fouled easily, impeding waterflow to the collector. We conclude that experiments should be conducted to identify optimal materials for collecting the target species of pearl oyster and that collectors should not be placed in protective mesh bags in environments similar to those of Solomon Islands.

KEY WORDS: Aquaculture, pearl oysters, *Pinctada*, spat, settlement, substrates [1]ICLARM contribution no. 1240.

INTRODUCTION

In the past 20 y, there has been rapid expansion in the farming of blacklip pearl oysters, *Pinctada margaritifera*, for the culture of black pearls (Intes and Coeroli 1985). The expansion of this industry has been particularly pronounced in French Polynesia and Cook Islands (Rowntree 1993), where it rivals tourism as the major source of foreign exchange. The culture of blacklip pearl oysters in French Polynesia and Cook Islands was based initially on the use of wild shell from the lagoons of selected atolls: collection of spat provided only a minor proportion of the farmed shell (Coeroli et al. 1984). In the last 1980s and early 1990s, however, legislation was introduced to parts of French Polynesia and Cook Islands banning the use of wild shells. Consequently, the industry became more dependent on the collection of spat to provide the oysters needed for pearl culture.

The spat of the blacklip pearl oysters are collected on subsurface longlines, using a variety of settlement materials, ranging from branches of selected trees (Coeroli et al., 1984, Victor 1987, Passfield 1989) to a variety of plastic sheets, ropes, and meshes (Coeroli et al. 1984, Cabral et al. 1985). The use of plastic substrata is now widespread because of the ease of use and durability (N. Sims, pers. comm.). Spat collectors are hung at depths of 2–4 m, where settlement is greatest (Shirai 1970, Cabral et al. 1985, Sims 1993). Collectors are buoyed clear of the substrate to isolate them from benthic predators (Swift 1985), and in some cases, mesh bags are used to protect spat on the collectors from predators (Coeroli et al. 1984, Gervis and Sims 1992).

In the course of a large-scale sampling program to identify spatial variation in an abundance of spat *P. margaritifera* in Solomon Islands, we designed experiments to answer two questions aimed at refining methods for the collection of pearl oyster spat. These questions were: (1) Do mesh coverings ("spat bags") increase the number of spat harvested from collectors? (2) Is there a difference in the number of spat harvested from collectors made of plastic sheeting and those made from shademesh?

We found that the use of spat bags did not increase the number of *P. margaritifera* spat on collectors and that more spat were collected from shademesh than from plastic sheeting. During the experiments, large numbers of another pearl oyster, *Pinctada maculata*, also settled on the collectors. This species, which produces baroque pearls of smaller size and value than those found in *P. margaritifera* (Sims 1988), also provided a useful test for the effect of spat bags. At two of the three sites where this species settled in abundance, there were significantly fewer spat on collectors within the bags.

METHODS

Sampling Sites

We deployed spat collectors at three sites in each of the five regions (i.e., a total of 15 sites) in the Solomon Islands (Fig. 1). These sites encompassed the range of habitats thought to be suitable for the settlement of *P. margaritifera*. Sites were selected on the basis of maps and aerial photographs, on-site inspections, and information on past harvests of *P. margaritifera* supplied by local communities.

Within sites, spat collectors were positioned where larvae were likely to be entrained by nearby channel flows or in areas that were semienclosed. Where possible, we placed collectors on the lee side of bays, where prevailing winds were likely to concentrate spat in the surface waters (Sims 1989).

Longlines

At each site, spat collectors were suspended from a single longline consisting of a 100-m headline (12-mm polypropylene rope) supported every 20 m by a 30-cm-diameter buoy. Using SCUBA, we submerged the longline to a depth of 3 m by pulling down on the attached buoys with a dropper line (10-mm polypropylene rope). The dropper lines were secured to large colonies of coral at depths of 8–19 m.

Longlines were positioned across shallow reef areas (<20 m deep), and the ends of the headline were attached to coral heads in

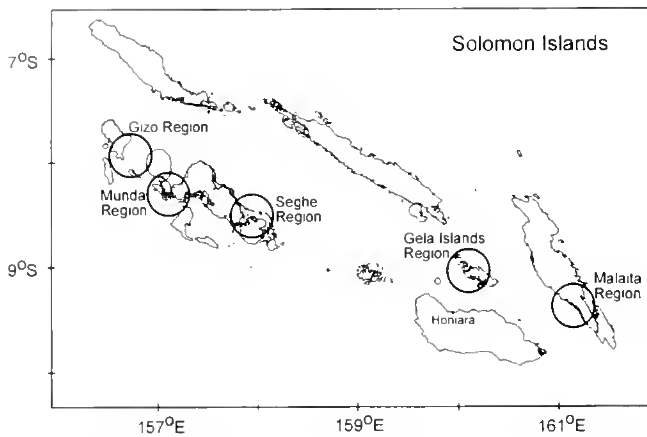

Figure 1. Map of the Solomon Islands showing the regions where spat were collected (circles).

shallow water (<5 m deep). This allowed the headline to be tensioned easily to that all collectors could be maintained at the required depth (2–4 m).

Spat Collectors

We selected materials for the construction of spat collectors on the basis of the previous experience of Cabral et al. (1985) in French Polynesia and Sims (1989) in Cook Islands. These materials were black plastic polyethylene sheeting (50 μm thick) and black plastic shademesh (55% shade). One type of collector was constructed of each material.

We constructed the plastic sheeting collector from 16 strips, measuring 10 × 100 cm. Each strip was threaded loosely, four or five times, onto a 110-cm length of 3-mm polypropylene line. The ends of several strips were passed through the weave of the 3-mm line to prevent the strips from "bunching" at the base of the collector once it was fouled. We made the shademesh collector from a folded single sheet of shademesh threaded four or five times onto a 110-cm length of 3-mm polypropylene line. Both types of collectors had the same surface area (1.6 m^2).

Experimental Design

Twenty replicates of each type of collector were suspended from each longline. Protective mesh bags measuring 80 × 40 cm, with a mesh size of 2 × 5mm, were used to enclose 10 replicates of each type of collector. Thus, we had 10 replicates of four treatments for each longline: 10 × plastic sheeting open, 10 × plastic sheeting bagged, 10 × shademesh open, and 10 × shademesh bagged. These were randomly allocated to attachment points ever 2 m along each longline.

Collectors were soaked for 6 mo (between January and July 1994), after which they were removed from the water and checked for spat of pearl oysters. In addition to recording data for *P. margaritifera*, we counted the numbers of the closely related pearl oyster, *P. maculata*. Data recorded for each collector were: the number of live and dead spat for *P. margaritifera* and *P. maculata*, the dorsoventral measurement (DVM) (Nicholls 1931) of each individual *P. margaritifera*, and the DVM of up to 10 live individuals of *P. maculata*. All measurements were made to the nearest 1 mm. Additionally, we recorded the numbers of several of the predators of juvenile pearl oysters identified by Sims (1989), Dharmaraj et al. (1987), and Govan (1994), especially *Cymatium* spp. gastropods and crabs. Govan (1994) recognized four species of *Cymatium* as predators of juvenile bivalves in Solomon Islands. We pooled counts for these species because it was difficult to distinguish among the juveniles.

Some of the predators concealed within the collectors were highly mobile, e.g., fish from the family *Balistidae*. To ensure that we sampled them effectively, we used a fine-mesh harvesting bag measuring 80 × 130 cm to surround the collector before it was removed from the longline.

We used data from the four types of spat collectors to test the following null hypotheses for both *P. margaritifera* and *P. maculata:*

(1) There was no difference in the abundance of spat recorded on the collectors made of plastic sheeting and shademesh, and
(2) There was no difference in the abundance of spat collected from open and bagged collectors.

Analysis of Data

To test the null hypotheses, we used data only from sites that had relatively high numbers of spat (Table 1). For *P. margaritifera*, we used five sites to analyze variation in the following measures: total (live and dead) abundance of spat, ratio of live to total number of spat, and DVM. We also analyzed variation in the abundance and size of predators from these five sites. In general, *P. maculata* settled in far greater abundance than *P. margaritifera*. However, three sites received the majority of *P. maculata* spat (Table 1), and so, we restricted the analysis of data for this species to those sites.

We analyzed variation in the total abundance of spat due to the effect of site (random factor), collecting material (fixed factor), and bag protection (fixed factor) for both species of pearl oysters in a balanced three-way analysis of variance (ANOVA). We also analyzed the total abundance of *Cymatium* spp. in the same manner. To investigate variation in the DVM of live spat of *P. margaritifera* and *P. maculata* among collectors, we used data pooled across sites in a two-way ANOVA (materials × protection). Before all analyses, we tested for homogeneity of variance using Cochrans C test and transformed data to $\log_{10}(\times + 1)$ when variances were nonhomogeneous.

In the case of data for the abundance of live *P. maculata*, transformation did not result in homogeneity of variances. For this variable, data were pooled for each type of collector across the three sites and were presented graphically to describe variability. Where ANOVA indicated that there were significant differences among means, the Student Neuman-Keuls (SNK) test or Tukey HSD test for unequal sample sizes was used to identify the nature of these differences.

RESULTS

Variation in Collections of **P. margaritifera** *Among Sites and Collectors*

A total of 154 *P. margaritifera* spat were collected at the 15 sites (Table 1), but distribution was highly patchy. There was a significant difference in the total abundance of spat among the five sites where the greatest number of *P. margaritifera* were collected

TABLE 1.

Total abundance of *P. margaritifera* and *P. maculata* spat at each of three sites in five regions.[a]

Region	Site	*P. margaritifera* Total	*P. margaritifera* Alive	*P. margaritifera* % Alive	*P. maculata* Total	*P. maculata* Alive	*P. maculata* % Alive	Site Description
Gela Islands	1	17[a]	9	52.9	88	32	36.4	Embayed reef
	2	6	5	83.3	133	60	45.1	Open reef system
	3	14[a]	10	71.4	1,257[a]	924	73.5	Reef system beside channel
Seghe	4	0	0	0	10	6	60.0	Lagoonal reef
	5	0	0	0	3	2	66.7	Lagoonal reef
	6	2	0	0	82	13	15.8	Inside edge of lagoonal reef beside a channel
Munda	7	0	0	0	18	11	61.1	Channel side embayment
	8	1	1	100	21	10	47.6	Channel side embayment
	9	1	1	100	60	26	43.3	Lagoonal reef
Gizo	10	27[a]	11	40.7	698[a]	301	43.1	Open reef beside a channel
	11	2	0	0	104	19	18.3	Embayed reef
	12	41[a]	15	36.6	1,147[a]	81	7.1	Embayed reef
South Malaita	13	37[a]	14	37.8	104	32	30.8	Inside edge of lagoon beside a channel
	14	6	4	66.7	17	4	23.5	Lagoonal reef
	15	0	0	0	1	0	0	Bay affected by large mangrove system
Total		154	70		3,743	1,521		

[a] Indicates those sites where data were used in ANOVA

(Table 2; p = 0.006), but the SNK test could not differentiate among the means. Collections at the best site (Site 12) averaged just over one spat per collector (Table 1).

There was a significant difference in the total abundance of *P. margaritifera* on collectors made of shademesh and plastic sheeting (Table 2; p = 0.01). A mean of 0.98 (±0.13 SE) *P. margaritifera* was found on collectors made from shademesh, whereas a mean of 0.38 (± 0.06 SE) spat was collected from those made of plastic sheeting. There was no significant difference in the total abundance of spat harvested from the open ($\bar{X}$ = 0.75 ± 0.11 SE) and bagged ($\bar{X}$ = 0.61 ± 0.10 SE) collectors (Table 2). When only live spat were considered, there were totals of 24 and 19 spat on open and bagged shademesh, respectively, and 8 spat on both bagged and open collectors made of sheeting.

Variation in Collections of **P. maculata** *Among Sites and Collectors*

More than 1,000 *P. maculata* were collected at two sites, and >100 individuals were collected at another four sites (Table 1). There was a significant positive correlation (Pearson's r = 0.62, df = 13, p < 0.05) between the abundance of *P. margaritifera* and *P. maculata* across all 15 sites. Thus, sites with high abundances of *P. maculata* were relatively good sites for the settlement of *P. margaritifera*.

There was a significant interaction between the effects of site and protection on the abundance of *P. maculata* (Table 2; p = 0.02). Open collectors had significantly more spat than bagged collectors at two of the three sites (Table 3). There was no signif-

TABLE 2.

Results of three-way ANOVA for the effects of site (S), material (M), and protection (P) on the total abundance of *P. margaritifera* and *P. maculata*.[a]

Source	*P. margaritifera* df	*P. margaritifera* F	*P. margaritifera* p	*P. maculata* df	*P. maculata* F	*P. maculata* p
Sites	4	3.7298	0.0061[b]	2	4.9544	0.0087[b]
Material	1	19.0641	0.0120[c]	1	6.6906	0.1226
Protection	1	0.6652	0.4605	1	2.4311	0.2593
S × M	4	0.9499	0.4365	2	1.1269	0.3278
S × P	4	1.4740	0.2119	2	3.8839	0.0235[c]
M × P	1	2.2250	0.2101	1	12.5877	0.0710
S × M × P	4	0.4528	0.7702	2	0.2550	0.7754
Residual	180					

[a] Data for both species were transformed to $\log_{10}$ (x + 1).
[b] Significance: p < 0.01.
[c] Significance: p < 0.05.

TABLE 3.

Mean abundance of *P. maculata* harvested from open and bagged collectors at three sites and mean abundance of *Cymatium* spp. harvested from open and bagged collectors at five sites.

Species	Site	Protection[a] Open	Protection[a] Bagged
P. maculata	3	43.5	19.4
	10	26.0	8.9
	12	20.3	37.5
Cymatium	3	0.80	0.95
	10	0.45	2.30
	12	0.95	1.45
	1	0.60	1.55
	13	1.20	1.30

[a] Underlined means do not differ significantly by SNK test. Real means are displayed, but SNK tests were performed on data transformed to $\log_{10}$ (x + 1).

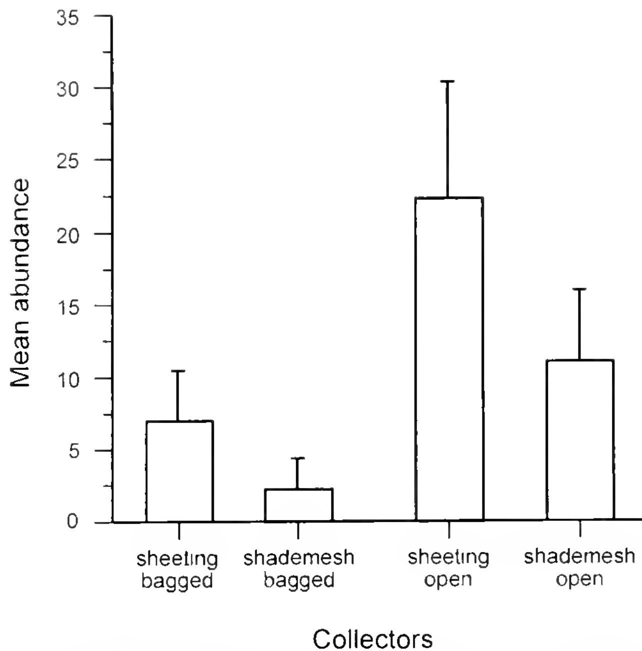

Figure 2. Mean abundance of *P. maculata* found on each type of collector. Data were pooled across the three sites of greatest settlement. Error bars are standard errors.

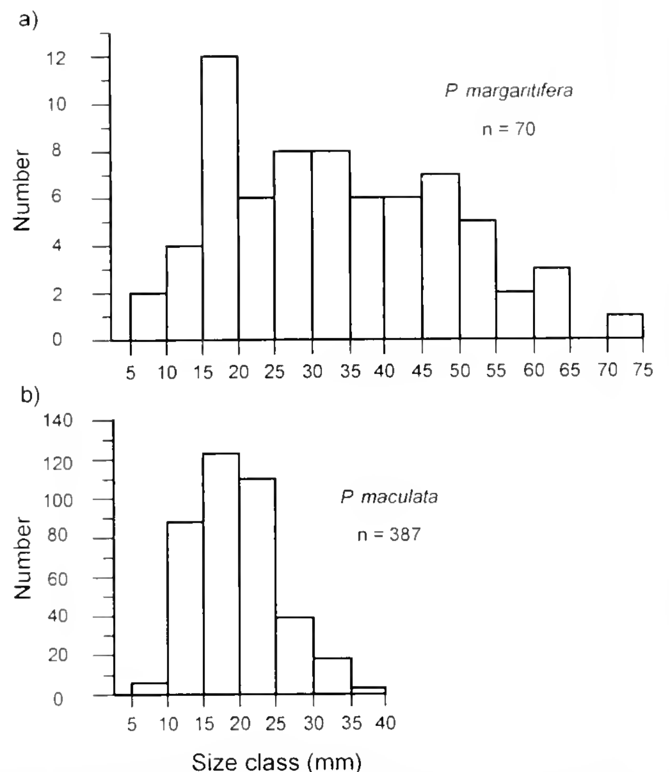

Figure 3. Size frequency distributions of live spat of *P. margaritifera* and *P. maculata* removed from collectors after 6 mo. Data for *P. maculata* are a subsample of the 1,521 individuals.

icant difference in the number of *P. maculata* collected from the two types of material (Table 2; p = 0.1): a mean of 20.8 ± 5.54 SE spat was collected from shademesh, a mean of 30.9 ± 6.94 SE was harvested from plastic sheeting.

Live spat of *P. maculata* had a higher mean abundance on the open and plastic collectors than on bagged or shademesh collectors (Fig. 2). There was, however, great variation in the number of live spat for each type of collector (Fig. 2). In the case of open collectors, for example, 20% of collectors had large numbers of live spat with a mean of 79.6 ± 45.9 SE, whereas the other 80% only averaged 0.9 ± 0.2 SE.

Variation in Size of Spat

The spat of *P. margaritifera* alive at harvest ranged from 8 to 71 mm DVM (Fig. 3a). The mean DVM of spat was 32.4 mm (±1.7 SE). All types of collectors had a wide size range of live *P. margaritifera*, except for the open sheeting collectors, which held none smaller than 30 mm (Fig. 4).

The modal size of *P. maculata* was smaller than that of *P. margaritifera* (Fig. 3b). There were significant differences (df = 3, F = 17.45, p = 0.001) in the size of *P. maculata* among collectors. *P. maculata* were significantly larger on open sheeting collectors ($\bar{X}$ = 22.1 ± 0.59 mm SE) than on open shademesh ($\bar{X}$ = 17.9 ± 0.82 mm SE), bagged sheeting ($\bar{X}$ = 16.9 ± 0.55 mm SE), and bagged shademesh ($\bar{X}$ = 16.3 ± 0.8 mm SE).

Ratio of Live Spat to Total Number of Spat

There were few differences in the ratios of live to total number of spat of *P. margaritifera* among the different collectors. The ratios ranged from 40 to 46.3%. The ratios of live to total numbers of spat of *P. maculata* were higher on open collectors, (68.9% for sheeting and 40.1% for shademesh) than on the bagged collectors (23.8% for sheeting and 22.9% for shademesh).

Variation in Abundance of Cymatium *sp. Among Sites and Collectors*

We found a total of 185 *Cymatium* spp. on collectors at the five sites that held the greatest abundance of *P. margaritifera*. There was a significant interaction between the effects of site and "protection" on the abundance of these gastropods (Table 4; p = 0.047): bagged collectors had significantly more *Cymatium* spp. than did open collectors at one of the sites (Table 3). At the other four sites, there was no significant difference in the numbers of *Cymatium* spp. between open and bagged collectors (Table 3).

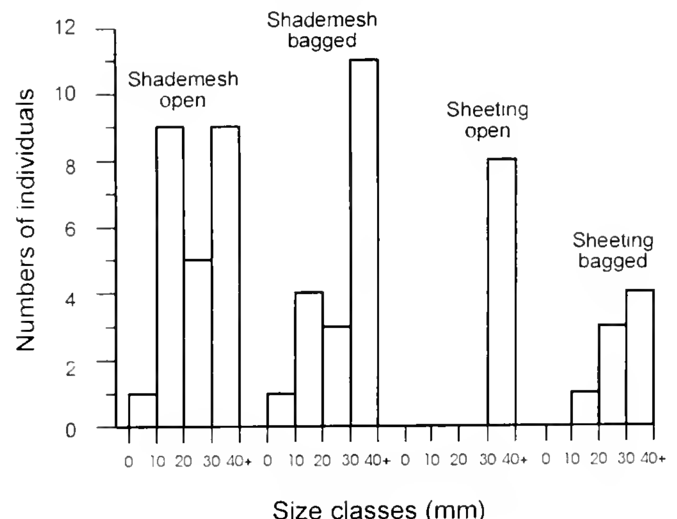

Figure 4. Size frequency distributions (DVM) of live *P. margaritifera* on the four types of collectors.

TABLE 4.

Results of a three-way ANOVA for the effects of site (S), material (M), and protection (P) on *Cymatium* spp. abundance among collectors.[a]

Source	*Cymatium*		
	df	F	p
Site	4	0.492	0.745
Material	1	1.328	0.313
Protection	1	4.674	0.097
S × M	4	1.007	0.405
S × P	4	2.465	0.047[b]
M × P	1	0.963	0.382
S × M × P	4	1.628	0.169
Residual	180		

[a] Data were transformed to $\log_{10}(x + 1)$.
[b] Significant ($p < 0.05$).

The sizes of *Cymatium* spp. ranged from 6 to 67 mm ($\overline{X}$ = 24.5 ± 0.72 mm SE). There were no significant differences in the mean shell sizes of *Cymatium* spp. among the four types of collectors.

Other Predators

Large numbers of predatory portunid crabs, *Thalamita quadridens* (A. Milne Edwards 1869), were found on the collectors. Smaller numbers of xanthid crabs, e.g., *Gaillardiellus orientalis* (Odhner, 1925), were also found. These crabs are predator/scavengers and are adapted to scrape off sessile invertebrates (P. Davie, pers. comm.). The majority of individuals found had a carapace width <20 mm; however, larger crabs with carapace widths ≥40 mm were found. The majority of crabs with a carapace width >20 mm (76% of all crabs) were associated with collectors "protected" in mesh bags.

Sixteen trigger fish (Balistidae) and three octopus were also collected from the five sites. All but one of these individuals were found on open collectors.

DISCUSSION

Our study showed conclusively that there were differences in the abundance of both *P. margaritifera* and *P. maculata* with respect to type of collector. These differences appeared to be related to the structure of the collectors, the texture of the materials used in their construction, and the survival rates of spat.

Despite the low numbers, *P. margaritifera* were more than twice as abundant on shademesh than on plastic sheeting, being found alive in the pockets and folds of the shademesh collectors at harvest. *P. margaritifera* prefer dark surfaces for settlement (Sims 1989), with black or dark blue spat collectors producing the best yields (Coeroli 1983). Both of the substrate we used were black and had the same surface area, but shademesh presented a multistranded surface that differed from the smooth surfaces of the plastic sheeting. Dayton et al. (1989) postulated that oyster larvae are affected by microscale transport elements and behaviors, such as boundary layers, chemical cues, microtopography, and predation from established individuals. We do not know which characteristics of shademesh were favored over plastic sheeting, but the multistranded surface evidently provided suitable points for settlement.

In contrast to *P. margaritifera*, abundances of live *P. maculata* were far greater on collectors made of plastic sheeting than on shademesh (in the absence of bags). The strips of flat plastic had a looser structure, allowing spat that had settled to maintain good water exchange. The "habitat" of the open plastic collectors apparently suited *P. maculata:* individuals found on this type of substratum were significantly larger at harvest than those on the shademesh and closed collectors. Interestingly, the spat of *P. maculata* associated with shademesh were found usually on the edges of the sheets, where they had improved access to water flow.

Comparisons of the two materials used to collect spat cannot be made without consideration of the collection environment. Our observations were that, within the high island/open reef environments of Solomon Islands, elevated levels of particulate matter in the water after heavy rainfall, as well as high fouling rates, accentuated differences in the "habitats" created by the two substrata. These "habitats" influenced the settlement and survival rates of the two pearl oyster species. For example, shademesh was made less suitable for *P. maculata* because particulates accumulated within "dead spaces." This was not such a problem on collectors made of plastic strips. However, if either type of collector became fouled too heavily, or covered in particulates, both species were mostly dead at harvest. Similar problems have been encountered elsewhere. Collections of *P. margaritifera* in the Philippines were "unsuccessful" because of the high productivity of the water and heavy fouling of collectors (Gervis and Sims 1992).

Predation plays an important role in the dynamics of pearl oyster stocks (Hornell 1914a & b). The presence of predators on the collectors and the high incidence of dead spat indicate that predation also influenced the number of live spat on our collectors. Our observations indicate, however, that shademesh provided better protection from predators than the plastic sheeting: the spat of *P. margaritifera* were able to live on the inside of folds in the "permeable" shademesh. In this position, spat were presumably afforded greater refuge from predators than on the flat surfaces of the collectors made of plastic sheeting. Judging from the size of the live shells found, *P. margaritifera* continued to settle and grow on the shademesh collectors throughout the 6-mo period. In contrast, small (<30 mm) *P. margaritifera* shells were not seen on the open plastic collectors. Ambrose et al. (1992), studying recruitment of scallops, proposed that collectors with greater structural complexity may inhibit the activities of predators.

For reasons already suggested, *P. maculata* were seldom found on the inside folds of shademesh collectors, but they were common on open plastic collectors. In this vulnerable position, how did they persist? The answer may lie in the large number that settled (20% of collectors held over 79 live individuals): predation pressures were insufficient to markedly affect abundances.

Although shademesh was two to five times more effective at catching the spat of *P. margaritifera* than plastic sheeting, it was four times more expensive. However, this difference in costs is small in proportion to the total farm costs and is outweighed by the fact that shademesh sheets are more easy to handle (thread and shred) than the plastic strip collectors, therefore saving on labor costs. In addition, the shademesh material can be used many times, whereas the plastic sheeting is less easy to recycle.

A major finding of our experiment was that the abundance of neither *P. margaritifera* nor *P. maculata* was significantly greater

on spat collectors placed inside protective bags. In the case of *P. maculata*, abundances were actually significantly greater on open collectors than on collectors in bags at two of the three sites analyzed in detail. This result can be attributed to two main factors. First, deterioration in the quality of the "habitat" within the bag over the 6-mo period, due to reduced scope for water exchange. This may have been caused in part by the baffling effect of the mesh itself, but was also due to the accumulation of particulates and the heavy fouling of the bags. After 6 mo, the bags supported a complex community of algae, sponges, and ascidians. These organisms blocked much of the mesh. These conditions were likely to lower the settlement success of spat (Sumpton et al. 1990) and reduce their growth (Coeroli et al. 1984). If the bags became fouled heavily or filled with particulates, both species were mostly dead at harvest.

Second, the bags were not effective at protecting spat from all types of predation. On the contrary, spat bags included rather than excluded *Cymatium* spp. and portunid crabs, which are effective predators of juvenile bivalves (Appukuttan 1987, Govan 1994). These animals evidently recruited to the spat collectors as larvae and were trapped within the 2 × 5 mm mesh of the protective bags as they grew. Dayton et al. (1989) also recognized "extremely strong" predation pressures by invertebrates, e.g., gastropods, crabs, flatworms, and octopus, on oysters protected from fish predation during a study of oyster settlement on the Great Barrier Reef.

In some cases, the "protective" bags were torn, indicating that oysters did not receive comprehensive protection from predatory fish. Chellam et al. (1987) found that they needed to put secondary fish nets (mesh, 10 mm) around spat already protected by fine meshes to stop fish predation during growout.

We do not know the relative rates of predation by fish, crabs, and *Cymatium* spp. and so cannot assess the relative advantage of excluding one type of predator at the expense of retaining another. It is clear, however, that bags do not exclude two important types of predators, *Cymatium* spp. and crabs, and that, in some cases, the use of bags increases their abundance.

CONCLUSIONS

The choice of substrate used to construct collectors had a significant influence on the abundance of spat: *P. margaritifera* preferred shademesh, and *P. maculata* preferred plastic sheeting. This implies that further experiments are needed to select the best materials for collecting the spat of *P. margaritifera*, and that farmers may be able to design collectors that target particular species over potentially competitive species. Such experimentation is critical where the collection of spat is marginally effective.

Predators such as *Cymatium* spp. gastropods and portunid crabs settle to spat collectors from the plankton. Bags placed around spat collectors to exclude predators such as fish can enclose *Cymatium* spp. and crabs as they grow, resulting in increased predation by these invertebrates. "Protective" bags also become heavily fouled. In severe cases, this fouling may render the "habitat" within the bag unsuitable for the growth and survival of pearl oyster spat. Because the number of spat on collectors held in protective mesh bags was significantly lower at some sites and because the installation of bags adds considerably to the cost of spat collectors, we do not recommend the use of spat bags for the collection of pearl oysters within environments similar to those in Solomon Islands.

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PHYSIOLOGIC VARIABILITY OF EASTERN OYSTERS FROM APALACHICOLA BAY, FLORIDA

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ABSTRACT Eastern oysters, *Crassostrea virginica*, were collected monthly during a 1-y period from two study sites in Apalachicola Bay, FL, and several measurements were made of their physiologic condition. Continuous and intermittent temperature measurements at both sites shows highly coincident ambient temperature regimens. Salinity measurements, however, were erratic and varied dramatically between sites. Oyster gonad size and gametogenic condition were highly synchronous at both sites, supporting the concept of temperature-driven reproductive cycles. Other measurements, including condition index, wet:dry tissue weight ratio, digestive tubule condition, and vesicular connective tissue condition, showed significant variability as the result of sampling month, but also differed because of site and/or interaction between date and site, indicating that local effects influenced oyster physiology. Temperature control over condition index and wet:dry tissue weight seems apparent, but it is not known whether the changes resulted directly from temperature or from temperature-driven reproductive and metabolic cycles. A significant difference between site means at specific dates was observed for digestive tubule condition and may relate to short-term salinity differences. Other physiologic variations could not be attributed to any of the physical conditions monitored (temperature, salinity, pH, and dissolved oxygen). The variability of oyster physiologic measurements inherent at different sites and seasons must be well understood to properly interpret them in the context of biologic indicators of environmental condition.

KEY WORDS: Eastern oysters, *Crassostrea virginica*, bivalve physiology

INTRODUCTION

The interpretation of physiologic measurements of bivalve molluscs as indicators of their health or of environmental condition is complicated by the wide ranges and multiple sources of variability. A primary source of variability is the reproductive cycle, which is driven by temperature and causes annual metabolic changes associated with gonadal development and spawning (Galtsoff 1964, Quick 1971). Other environmental factors in oyster habitats, such as salinity, dissolved oxygen, nutrients, toxicants, parasites, and disease, typically show intermittent and short-term fluctuations, and their effects can be either obscured or magnified by the seasonal reproductive effects. The proper interpretation of physiologic measurements, whether used as indicators of health (Engle 1951; Bayne et al. 1976) or as biologic indicators of environmental condition (e.g., International Mussel Watch [IMW] 1980), requires a concrete understanding of both seasonal and intermittent variations in the natural environment.

This study describes the range of variability in certain physiologic measurements of eastern oysters, *Crassostrea virginica*, in a Gulf of Mexico estuary and attempts to distinguish seasonal reproductive effects from those caused by local environmental fluctuations. Two oyster beds were selected in Apalachicola Bay (Franklin County, FL) to study monthly changes in selected physiologic and immunologic characteristics. The sites lie within 15 km of each other in a bay system protected by a chain of barrier islands (Gorsline 1963). Their proximity assures a reasonably similar temperature regimen, whereas currents, tides, and river inflow create smaller-scale and intermittent environmental diversity, which can cause physiologic differences between inhabitants of the two sites.

The Apalachicola Bay system (Fig. 1) is a wide (210 square miles), shallow, and relatively unpolluted body of water (Thompson et al. 1990) fed primarily by the Apalachicola River. The system is a highly productive lagoon/barrier island complex that typically yields $12–$16 million in dockside seafood landings annually (Florida Department of Natural Resources 1986). Since the early 1900s, commercial fishing has been the most important economic activity within the bay and currently supports 60–85% of the local community (Rockwood and Leitman 1977). Historically, revenue from this industry has accounted for nearly half of Franklin County's income (Whitfield and Beaumariage 1977).

The eastern oyster is the most important commercial invertebrate in Apalachicola Bay, which supplies nearly 90% of the oysters harvested in Florida. Production on commercial oyster bars has been estimated at between 400 and 1,200 bushels/acre per year (Ednoff 1984, Berrigan pers. comm.). Because of relatively mild temperatures in the area, rapid oyster growth is sustained throughout the year. Harvestable oysters, those larger than 3 inches (76 mm), have been grown from spat in as little as 39 wk. The spawning season is one of the longest in the United States (Ingle and Dawson 1952).

Although oysters from Apalachicola Bay have frequently been evaluated from a commercial standpoint, they have not been extensively investigated in terms of their physiology, immunology, and disease. The monthly measurement of several physiologic

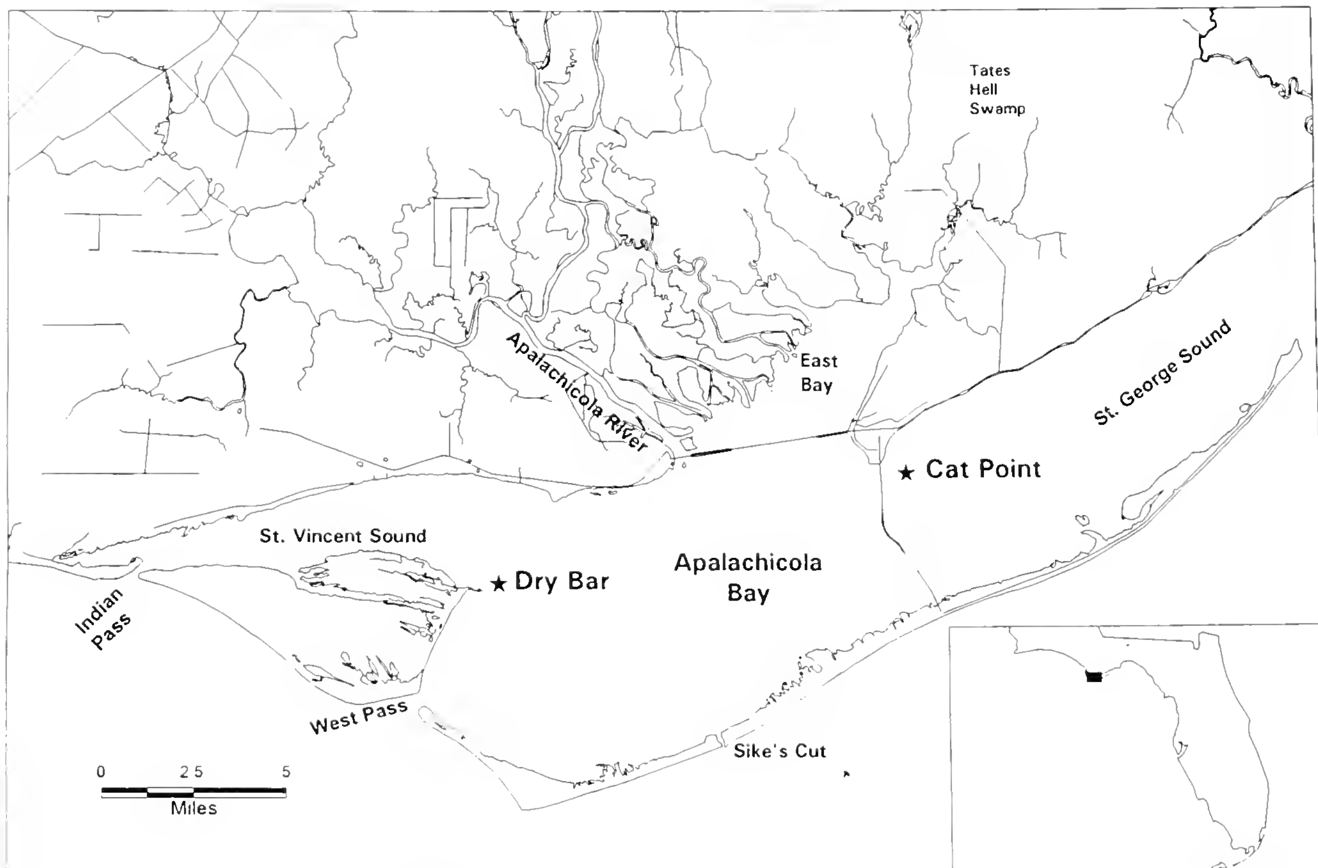


Figure 1. The Apalachicola River and Bay system with the two oyster collection sites, Cat Point Bar and Dry Bar (also known as St. Vincent's Bar), noted with stars. The sites are in northwest Florida (inset).

characteristics presented here provides a baseline of information on comparatively vigorous and stable oyster populations in a relatively unpolluted environment.

MATERIALS AND METHODS

Collection Sites

Oysters were collected from two of the most commercially productive oyster bars in Apalachicola Bay—Cat Point and Dry Bar (also named Drybar and St. Vincent's Bar). The Apalachicola estuarine system is divisible into four sections based on natural bathymetry and man-made structural alterations: East Bay, St. Vincent Sound, Apalachicola Bay, and St. George Sound (Fig. 1). The average depth in these bays ranges from 1 m in East Bay to 3 m in Apalachicola Bay, with maximum depths of 7 m occurring near the barrier islands (Dawson 1955, Gorsline 1963). The depths of the two oyster bars ranged from 1 to 2m.

Oyster bars cover over 10,600 acres, or ~10% of the submerged bottom within the boundaries of the Apalachicola National Estuarine Research Reserve (NERR). The two oyster collection sites are within NERR and were selected for their relatively large, easily harvested oysters, their locations on either side of the Apalachicola River (Fig. 1), and the availability of historical water quality data from the Florida Department of Environmental Protection (Thompson et al. 1990). The Dry Bar site is off St. Vincent's Island on the western edge of Apalachicola Bay, ~8 km WSW from the mouth of the Apalachicola River (latitude [Lat] 29:40.25, longitude [Lon] 85:03.33). The Cat Point site is located on the western edge of St. George Sound, just east of the St. George Island bridge, ~6 km ESE of the mouth of the Apalachicola River (Lat 29:43.05, Lon 84:52.93). The two sites are less than 15 km apart. Both sites lie within the chain of protective barrier islands, but the physical and chemical characteristics of the water are influenced by wind, tide, and river currents, as noted above.

Water Quality Measurements

Water salinity and temperature were recorded from both sites on each oyster collection date with a refractometer and digital thermometer. All "continuous" water quality characteristics were recorded every 30 min from June 1992 to December 1993, except for short maintenance periods. They were measured with Hydrolab[1] Datasonde 3 dataloggers that were programmed to measure temperature (±0.15 C), pH (±0.2 units), dissolved oxygen (±0.2 mg/L), salinity (±0.2 ppt), and tide height (±0.09 m) (Hydrolab Corporation 1991). The dataloggers were equipped with an external submersible battery pack and 70K extended memory, which allowed in situ monitoring for extended periods.

Divers attached the datalogger units to pilings located at the sampling sites with two stainless steel clamps and a safety cable. The probes were situated 0.4 m above the bottom substrate at both collection sites. The units were retrieved every 14–21 days (de-

[1]The mention of commercial products does not constitute endorsement by the U.S. Environmental Protection Agency.

pending on fouling) for downloading, cleaning, and recalibration. At this time, all electrolytes were replaced as well as the dissolved oxygen low-flow membrane. Laboratory-grade standards were used to calibrate the instruments for salinity and pH. Air calibration was used for the dissolved oxygen probe.

Salinity readings remained within ±0.5 ppt of the calibration standard at the end of each sampling period, and pH readings also showed very little deviation from calibration, with a maximum error of only ±0.1 units. The dissolved oxygen measurements showed the greatest drift in calibration, up to several milligrams per liter. This was primarily due to fouling of the low-flow membrane by algae, silt, and barnacle settlement. In an effort to remedy this situation, a plastic mesh screen was placed around the probe guard to reduce fouling while still allowing water to flow past the probes; also, the sampling period was reduced to 14 days during the warmer months when fouling was greater.

Oyster Collection and Processing

Oysters were collected on (approximately) a monthly basis with hand tongs from October 1991 to October 1992, and additional samples were collected in December 1992 and March 1993. Dates of collection were (1991): October 22, November 19, December 19; (1992): January 21, February 25, March 31, April 21, May 19, June 23, July 21, September 1, September 29, October 26, December 7; and (1993): March 29 for a total of 15 collections over a span of 17 mo (525 days). The September 1 sample is referred to as the August sample in this report. Oysters were selected at an approximate 80-mm height (umbo to edge of bill) and 50-mm length (greatest distance across the shell orthagonal to height) and were placed immediately into coolers containing cold ice packs. The coolers were transported to the Environmental Protection Agency Gulf Ecology Division at Gulf Breeze, FL, and were placed in a refrigerator (4°C) overnight.

Twelve oysters from each site were cleaned of fouling organisms and held at room temperature for 1–2 h. After the total oyster volume was measured, as described below, the shells were notched with a grinder at the margin of the shell and hemolymph was withdrawn from the adductor muscle with a syringe and 22-gauge needle. Hemolymph was used to measure *Perkinsus marinus* infection intensity, described here, and several defense-related characteristics that are reported elsewhere (Fisher et al. 1996).

Condition Index and Wet:Dry Weight Ratio

The total volume of each oyster was estimated by weighing (±2 g) water displaced from a beaker when the oyster was added (assuming 1 g = 1 mL of water). Weighing the displaced water provided greater sensitivity than directly measuring the volume of displaced water. Estimates of shell volume after the removal of all soft tissues were obtained in the same manner, and the difference was calculated as an estimate of the internal shell cavity volume. Soft tissues removed from the shells were blotted and weighed to obtain the total wet weight and then were dissected for histology (see below). The remaining tissues were weighed (partial wet weight), dried at 60°C for 48 h, and then reweighed (partial dry weight). The resulting wet:dry ratio was used to estimate the total dry tissue weight. The condition index (CI) was calculated as (Galtsoff 1964):

$$CI = \frac{\text{total dry tissue wt (g)} \times 100}{\text{internal shell cavity volume (mL)}}$$

Gender and Gonadal Condition

For histologic sectioning, a 3- to 5-mm-thick band of tissue was cut transversely with a razor blade at a distance of one-fourth to one-third of the length of the animal from the umbo in such a manner as to contain portions of mantle, gill, digestive tubule, and gonad. By standard histologic procedures, the dissected tissue was fixed for 24–72 h in Davidson's (formalin) fixative and stored in 70% ethanol before paraffin embedding. After embedding and sectioning with a microtome, slides were stained with hematoxylin and eosin. Gonad portions of the slide were examined with light microscopy to determine gender and were graded for gonadal condition (GC) (Table 1), which was adapted from measurements made by the International Mussel Watch Program (IMW 1980).

Relative Gonad Size

The greatest diameter of adductor muscle was measured with Fowler digital calipers while one end of the muscle was still attached to the half-shell. Gonadal thickness was measured at a consistent site opposite the gills on the band of tissue isolated for histologic sectioning. Gonadal material external to the digestive tubules was measured. These measurements were used to calculate the relative gonad size (Galtsoff 1964):

$$RGS = \frac{\text{gonadal thickness (mm)} \times 100}{\text{diameter of adductor muscle (mm)}}$$

Structure of Digestive Gland and Connective Tissue

Histologic slides were examined (400× magnification) for the structure of digestive diverticulae and vesicular connective tissues. Tubules of digestive diverticulae were measured by the technique of Winstead (1995), with a light microscope (200×) equipped with an ocular micrometer. The area of the section containing digestive tubules was divided into four quadrants, and five tubules from each quadrant were measured. Two sets of measurements

TABLE 1.

A description of gametogenic characteristics observed in histologic sections of oyster gonads and the values assigned for the determination of GC.

Value	Observations
0	Neuter or resting stage with no visible signs of gametes
1	Gametogenesis has begun with no mature gametes
2	First appearance of mature gametes to approximataely one-third mature gametes in follicles
3	Follicles have approximately equal proportions of mature and developing gametes
4	Gametogenesis progressing, but follicles dominated by mature gametes
5	Follicles distended and filled with ripe gametes, limited gametogenesis, ova compacted into polygonal configurations, and sperm have visible tails
6	Active emission (spawning) occurring; general reduction in sperm density or morphological rounding of ova
7	Follicles one-half depleted of mature gametes
8	Gonadal area is reduced, follicles two-thirds depleted of mature gametes
9	Only residual gametes remain, some cytolysis evident
10	Gonads completely devoid of gametes, and cytolysis is ongoing

were taken from each tubule—a lumen diameter and total tubule diameter—and from these values, a tubule ratio was calculated (lumen diameter/tubule diameter), with increasing values indicating more squamous digestive tubule cells. An average digestive tubule ratio (DTR) for an oyster was obtained from the 20 measured tubules.

The tubules also were ranked subjectively according to epithelial layer condition, as judged by cell size and morphology: 1 = large and columnar, 2 = average, and 3 = small and squamous; the morphology of the tubule cells determines the thickness of the epithelial layer and the size of the lumen. These vary within an individual oyster but were sufficiently consistent to be easily classified in this scheme. An earlier study indicated that these two techniques, the quantitative and the semiquantitative, produced similar results (Winstead 1995). Vesicular connective tissue (VCT) was examined for structural and morphological properties that were graded as 1 = lacy, intact, and regular network (organized), 2 = moderate edema and tearing with slight hemocyte response, or 3 = extensive edema and tearing with heavy hemocyte response.

Parasites and Disease

Examinations were made of histologic sections to assess the type and relative intensity of parasitic infections and noninfectious diseases or abnormalities. Each oyster was examined for *Bucephalus* spp. and *Proctoeces* spp. (digenetic trematodes), *Nematopsis* spp. and *P. marinus* (protists), *Tylocephalum* spp. (cestode), rickettsial inclusion bodies, and thigmotrichous ciliate protozoans, as well as noninfectious neoplasias and hemocytic or inflammatory responses.

***Hemolymph Diagnosis of* P. marinus**

The prevalence and infection intensity of the protozoan parasite *P. marinus* was determined by a modification of the method of Gauthier and Fisher (1990). Hemolymph drawn (0.5 mL) from the adductor muscles of individual oysters was centrifuged for 4–5 min at 2,940 × *g* in a microcentrifuge. The supernate (cell-free hemolymph, or serum) was collected and held at 4°C for analyses described in a separate article (Fisher et al. 1996). The pelleted hemocytes were covered with 0.5 mL of Ray's fluid thioglycollate medium to which 2.5 μL of chloromycetin stock solution was added and mixed; then, 100 μL of mycostatin stock solution was layered on top (Ray 1966). These were incubated in the dark for 5–7 days then were centrifuged as described above, incubated for 1 h at 60°C in the presence of 2 M sodium hydroxide, washed twice by centrifugation and suspension in distilled water, and finally resuspended in 0.5 mL of Lugol's iodine solution. Samples were filtered onto 0.22-μm filter paper for microscopic examination (100×) and enumeration.

Statistical Methods

Data were entered into SAS (SAS Inc., Cary, NC) and analyzed by use of the General Linear Models and the Shapiro-Wilk test for normality. Residual plots were examined to assure homogeneity of variance and independence and normality of error terms in the resulting models. *P. marinus* and wet:dry tissue weight data were $\log_{10}$ transformed to achieve compliance with assumptions of analysis of variance (ANOVA). Two-way analysis of variance (ANOVA) was conducted to relate each dependent variable (physiologic measurements) to the main effects of date and site and to test for possible interactions between date and site. The results of all analyses are reported, but main effects are discussed only if there was no significant interaction. Where significant main effects were found, Tukey's post-hoc test was used to differentiate between overall date or site means. For variables that displayed a significant interaction effect, differences due to date for each of the sites were examined with Tukey's post-hoc results, which compared means of all date * site combinations. Correlational analysis (Pearson's procedure) was used to relate collection date salinities with digestive tubule condition and wet:dry tissue weight measurements and to compare qualitative and quantitative (DTR) measures of digestive tubule condition. Levels of significance and high significance were selected by convention as $p \leq 0.05$ and $p \leq 0.01$.

RESULTS

Temperature Variations

Water temperatures were very consistent between the two collection sites, both for temperatures recorded at the time of collection (Fig. 2, top; October 1991 to March 1993) and during the continuous monitoring phase (June 1992 to March 1993), as demonstrated by the daily averages (Fig. 2, bottom). Daily averages rarely varied more than 3°C between sites. Both sites exhibited a seasonal cycle with progressive warming after January and cooling after July. Examination of 30-min data 7 days before monthly collection of oysters (June to December only, not shown) did not reveal any conspicuous fluctuations.

Salinity Variations

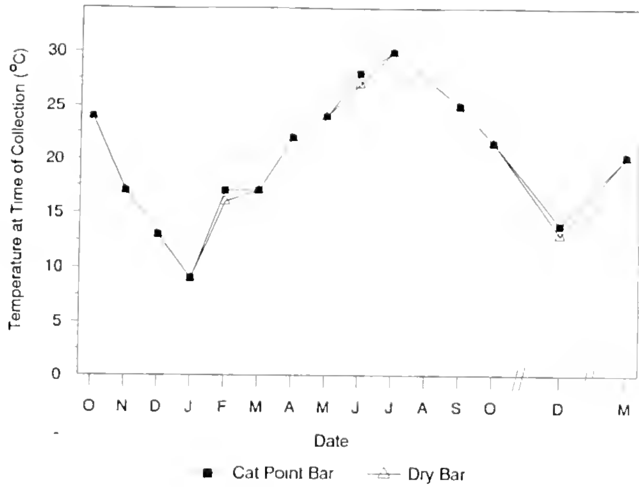
Salinity measured at the time of collection was the same for both sites at only 3 of the 15 collection dates (Fig. 3, top). These measurements displayed an inconsistent profile, with highest salinities reaching 25 ppt and lowest salinities reaching as low as 5 ppt for both Cat Point Bar (February 1992) and Dry Bar (March and December 1992). Neither site exhibited a strong seasonal pattern, although there appeared to be a low-salinity period during winter to spring (January to March).

Continuous salinity monitoring from June 1992 to March 1993 revealed striking differences between sites in daily averages (Fig. 3, bottom); differences were often 10–15 ppt and were sometimes as high as 25 ppt (January 1993). During the 10-mo period, salinities ranged between 35 and 1 ppt. Examination of the 30-min sampling data (not averaged) for both sites revealed large variations in salinity, sometimes varying 10–15 ppt within 1 h. Both sites also exhibited periods of stable salinities, often for 2 wk or longer. These data make clear that salinity measured only at the time of collection can be misleading. For example, ~20 ppt salinity was recorded at the time of collection for Dry Bar in June, even though salinities predominantly ranged from 25 to 35 ppt during this month.

Examination of 30-min data 7 days before each monthly collection of oysters revealed a notable difference between the two sites in December 1992. Before the collection date (December 7), Cat Point fluctuated between 10 and 25 ppt (Fig. 4). During the same 7-day period, Dry Bar salinity fluctuated between 10 and 30 ppt and then declined to 5 ppt by December 7 (Fig. 4).

Dissolved Oxygen, pH, and Tidal Height

During the June 1992 to March 1993 continuous monitoring period, the daily average dissolved oxygen concentration gradu-



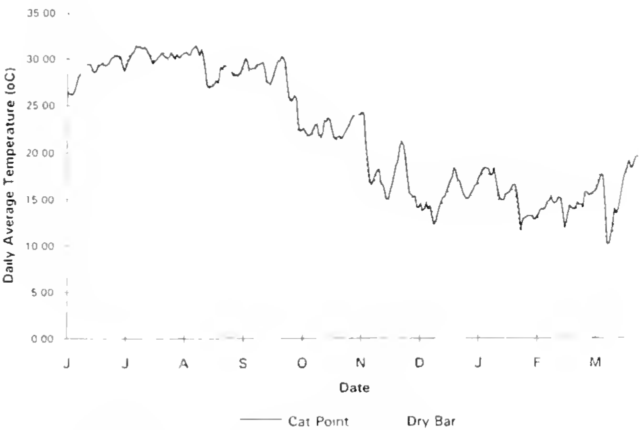
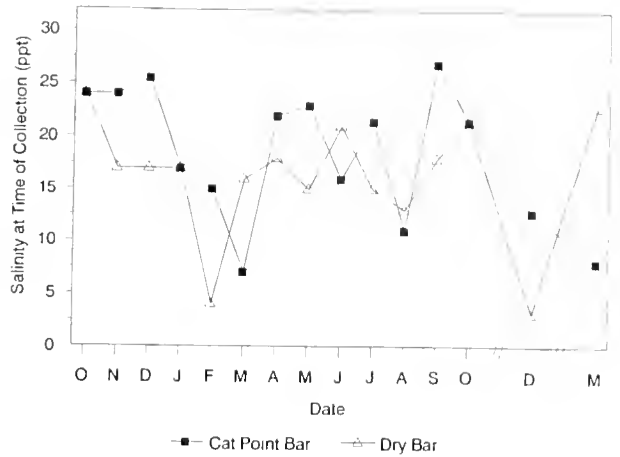


Figure 2. Water temperature of Cat Point Bar and Dry Bar during the period of collection. (Top) Temperatures taken at the time of collection throughout the entire study period. (Bottom) Daily averages of temperatures taken every 30 min from June 1991 to March 1993 only (note scale differences). All temperatures were recorded less than 0.5 m from the bay bottom.



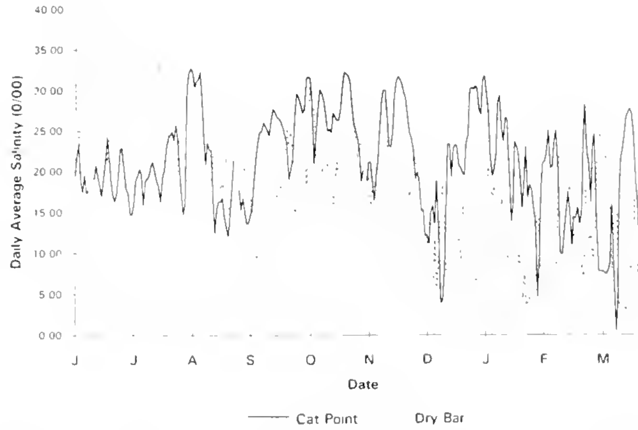


Figure 3. Water salinity of Cat Point Bar and Dry Bar during the period of collection. (Top) Salinities measured at the time of collection throughout the study period. (Bottom) Daily averages of salinities measured every 30 min from June to March 1993 only (note scale differences). All salinities were recorded less than 0.5 m from the bottom of the bay.

ally rose from 4 to 6 mg/L. Similar patterns were exhibited at both sites. The measurement of pH fluctuated from 7.6 to 8.2 during this period, and there were some differences between the two sites. From June to September 1992, the pH at Cat Point remained near 7.8, whereas the Dry Bar pH fluctuated around 8.0. Both sites decreased to 7.7 or below in January 1993 but returned to above 8.0 by February. The tidal height recorded during this period was very similar at both sites. Tide levels fluctuated near 1.4 m in June and gradually declined to 1.2 m by March.

Relative Gonad Size

Average gonad size relative to adductor muscle diameter (Fig. 5)[2] exhibited a significant date * site interaction (Table 2). The significant interaction appeared to be primarily a function of relatively low variability about the mean, because both sites followed a similar temporal pattern (Fig. 5). When analyzed by post-hoc analysis, relative gonad size (RGS) was largest in April 1992 and smallest in October 1991 and September to December 1992 at both sites (Table 3B). Oysters of similar size were collected on all dates, so adductor muscle average diameter ranged only from 12.8 to 18.0 mm for oysters at Cat Point and 16.3 to 19.6 mm for oysters at Dry Bar. Gonad thickness, however, increased from 1.9 (October 1991) to 11.6 mm (April 1992) for Cat Point oysters and from 1.9 (October 1991) to 14.2 mm (April 1992) for Dry Bar oysters. Thus, most of the change in RGS was due to gonadal development rather than changes in adductor muscle size. A significant difference between sites was detected only in November 1991 (Table 3B).

Gender and GC

Histologically rated GC (Fig. 6) exhibited a significant date * site interaction (Table 2), although GC also demonstrated a high degree of synchrony. Site means were significantly different only during May, August, and September 1992 and March 1993

[2]Data are presented in graphic form for convenience; tabular data sets are available upon request to the authors.

Figure 4. Water salinity of Cat Point Bar (solid line) and Dry Bar (dotted line) measured every 30 min during the week before the December 7 (1992) collection of oysters. Dry Bar salinity dropped to ~5 ppt 2 days before collection, whereas Cat Point Bar salinity remained near ~15 ppt.

(Table 3B). The significant interaction appeared to be primarily a function of low variability about the mean, because both sites followed a similar temporal pattern. Gametogenesis (histologic rating = 1) was initiated by February; spawning (=5) began by May and was nearly complete (= 10) by October at both sites (Fig. 6). There was no evidence of gametes during November 1991 to January 1992. However, in the following year, gametogenesis was observed in oysters from both sites by December.

Oysters were essentially neuter (no gametes visible) from October 1991 to January 1992 (Fig. 7). At the onset of gametogenesis (February and March 1992), approximately half of the oysters were males and half were females. The ratio became predomi-

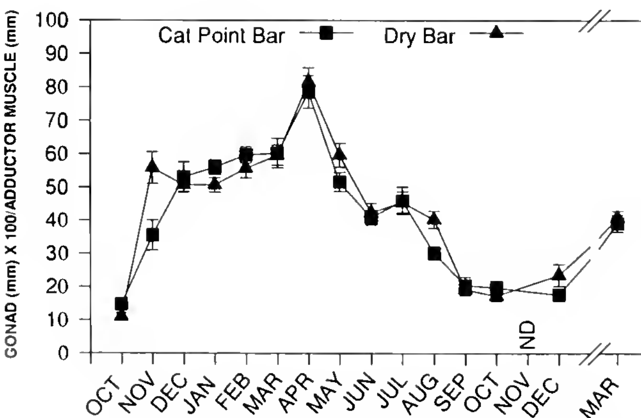


Figure 5. Average RGS measured during October 1991 through March 1993 from oysters collected at Cat Point Bar and Dry Bar (n = 12). Calculation of RGS was the ratio of gonad thickness to adductor muscle diameter × 100. ND, no data; bars indicate standard errors.

TABLE 2.

Results of an interactive ANOVA with date and site as independent variables.[a]

Variable	Date (p<)	Site (p<)	Date*Site (p<)
RGS	0.001[b]	0.072 NS[c]	0.003[d]
GC	0.001[b]	0.178 NS[c]	0.001[b]
CI	0.001[b]	0.004[b]	0.089 NS[c]
Wet:dry weight ($\log_{10}$)	0.001[b]	0.001[b]	0.001[b]
DTR	0.001[b]	0.299 NS[c]	0.001[b]
VCT	0.001[b]	0.001[b]	0.007[d]
Intensity of *P. marinus* infection	0.001[b]	0.551 NS[c]	0.069 NS[c]

[a] All tests were performed from October 1991 through March 1993, except for the diagnosis of *P. marinus*, which was started in December 1991.
[b] Highly significant difference ($p \leq 0.01$).
[c] NS, no significant difference.
[d] Significant difference ($p \leq 0.05$).

TABLE 3.

Study results.

	A		B									
			RGS		GC		W:D		DTR		VCT	
Date	CI	*P. mar*	CP	DB	CP	DB	CP	DB	CP	DB	CP	DB
Oct '91	E	—	IJ	J	A	A	CDEFG	CDEFG	ABC	ABCD	ABCD	BCD
Nov	ABCD	—	EFGH	BC	K	K	EFGHI	GHI	DEF	EF	AB	CD
Dec	ABCDE	ABCD	BCD	BCDE	K	K	EFGHI	GHI	EF	EF	CD	CD
Jan '92	ABCD	A	BC	BCDE	K	K	GHI	EFHGI	DEF	EF	CD	CD
Feb	ABCDE	ABCD	B	BC	JK	JK	FGHI	CDEFG	F	A	CD	D
Mar	ABC	D	B	B	HI	I	DEFGH	HI	F	F	CD	D
Apr	AB	A	A	A	GH	G	GHI	HI	CDEF	EF	BCD	CD
May	ABCDE	ABCD	BCDE	B	CD	EF	EFGHI	EFGHI	F	EF	BCD	BCD
Jun	ABCD	CD	CDEF	CDEF	DEF	CDE	EFGHI	GHI	EF	DEF	BCD	ABCD
Jul	A	AB	BCDEF	BCDEF	CDEF	BC	EFGHI	EFGHI	BCDEF	BCDE	ABCD	BCD
Aug	DE	CD	FGHI	CDEF	C	F	AB	BCDEF	AB	BCDEF	CD	CD
Sep	CDE	ABCD	HIJ	HIJ	C	AB	BCDEF	A	CDEF	EF	A	AB
Oct	BCDE	AB	HIJ	IJ	A	A	ABCD	EFGHI	ABCD	EF	ABC	CD
Dec	CDE	ABC	IJ	GHIJ	JK	K	ABCD	ABC	EF	ABCD	CD	CD
Mar '93	ABCDE	BCD	DEFG	CDEF	J	I	BCDE	GHI	DEF	EF	CD	CD

[a] (A) Significant differences due to sampling date (Tukey's procedure). Matching letters in a single column indicate no significant differences between collection dates for that variable. Analysis was performed with site data combined for variables with no significant date * site interaction (see Table 2). (B) Significant differences between all date * site means. Matching letters in a single column indicate no significant differences. W:D, $\log_{10}$ wet:dry weight; *P. mar.*, level of infection by *P. marinus* in hemolymph.

nantly female during spawning (April to September), with some collections yielding 92% female oysters (Fig. 7).

Oyster Conditian Index and Wet:Dry Weight Ratio

Average condition index varied significantly over collection date and site (Table 2) and had no significant date * site interaction. Values were higher for Dry Bar oysters (Fig. 8), which peaked in July and declined rapidly during the next 2 mo (Table 3A). Oyster tissue wet:dry weight ratios (Fig. 9) exhibited a significant date * site interaction (Table 2). Post-hoc analysis found that wet:dry weight ratios were highest during August to December 1992 for Cat Point Bar and in September for Dry Bar (Table 3B) and that significant differences between sites occurred in September and October 1992 and March 1993.

Structure of Digestive Gland and Connective Tissue

Results from the subjective and quantitative measures of digestive tubule condition were strongly correlated: Pearson correlations between the two tests for all samples were highly significant (r = 0.864, n = 357). The quantitative DTR (Fig. 10) was found to have significant date * site interaction (Table 2). Post-hoc analysis found significant site differences in February, October, and December 1992 (Table 3B). The February 1992 value was the highest for all oysters in the study and contrasted sharply with the low values in preceding and succeeding months at both sites (Fig. 10). Tubule ratios measured from Dry Bar oysters in October and December 1992 were different than those recorded in October and December 1991, respectively.

Subjective ratings of VCT structure (Fig. 11) exhibited signif-

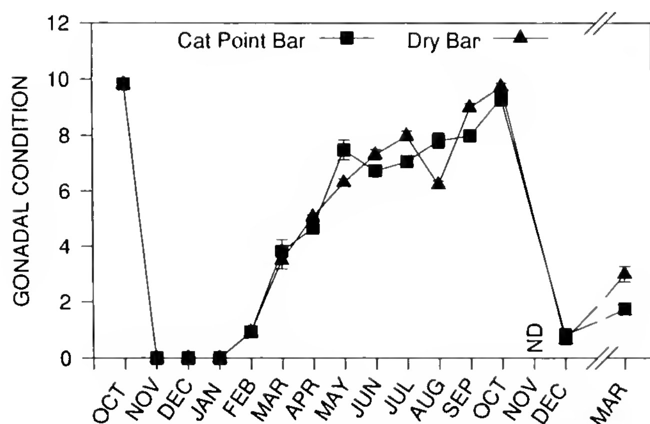


Figure 6. Average GC measured during October 1991 through March 1993 from oysters collected at Cat Point Bar and Dry Bar (n = 12). The assignment of values to gametogenic stages is described in Table 1. ND, no data; bars indicate standard errors.

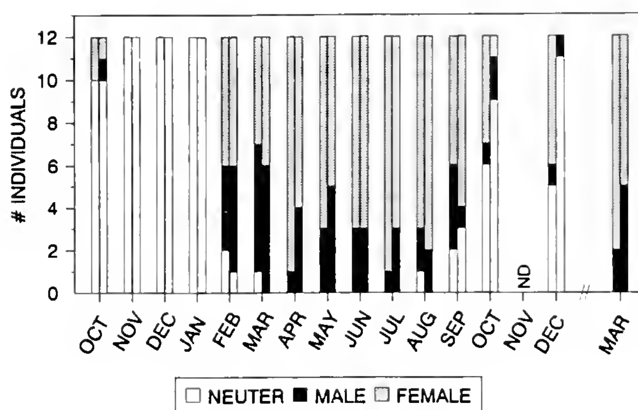


Figure 7. Gender frequency of 12 oysters collected at Cat Point Bar (left) and Dry Bar (right) during October 1991 through March 1993. Gender was determined by microscopic examination of histologic sections. ND, no data.

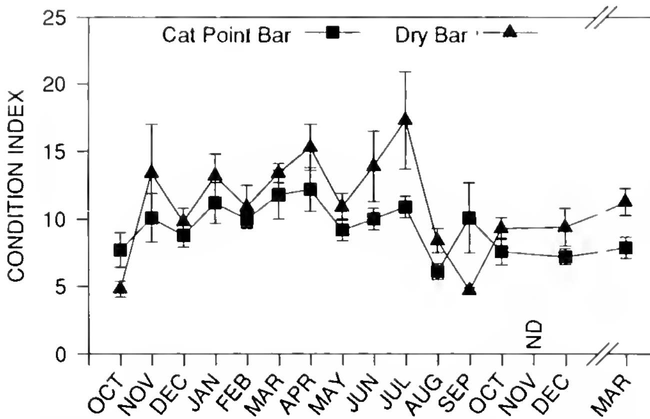


Figure 8. Average CI measured during October 1991 through March 1993 from oysters collected at Cat Point Bar and Dry Bar (n = 12). The calculation of CI was the ratio of dry tissue weight to estimated internal shell cavity volume × 100. ND, no data; bars indicate standard errors.

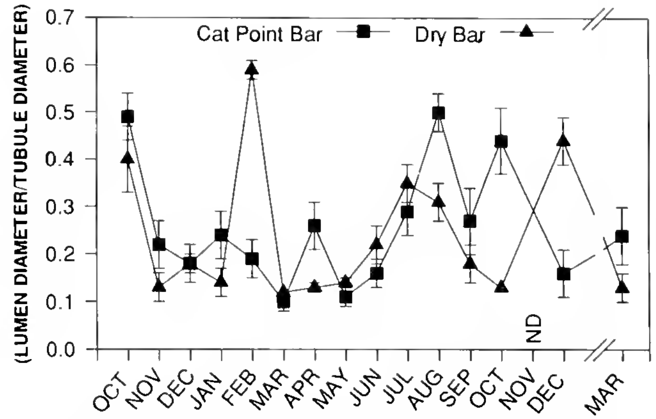


Figure 10. Average ratio of digestive tubule lumen diameter to outer diameter measured during October 1991 through March 1993 from oysters collected at Cat Point Bar and Dry Bar (n = 12). Measurements were made with an ocular micrometer on histologic sections of oyster digestive diverticulae. ND, no data; bars indicate standard errors.

icant date * site interaction (Table 2). Post-hoc analysis found significant differences between sites in November 1991 only (Table 3B). The lowest VCT values (best condition) occurred during December to March, and the highest levels occurred during September at both sites (Fig. 11).

Parasites and Disease

Both sites showed high prevalences of *Nematopsis* spp., *Tylocephalum* spp., and *P. marinus*. *Nematopsis* spp. occurred in >75% of the oysters throughout most of the study period, with exceptions in April (66%) and September (58%) at Dry Bar. Infection by *Tylocephalum* spp. was much more variable. At Cat Point Bar, the highest prevalences (100%) occurred in July and September and the lowest (25%) occurred in March and May. At Dry Bar, the highest prevalences (83%) were found in December, May, June, and October and the lowest (17%) occurred in April. There were no apparent temporal trends or site differences in the prevalences of these parasites.

Hemolymph diagnosis of *P. marinus* found 100% prevalence at both sites throughout the study period, with infection intensities generally less than 1,000/mL of hemolymph (Fig. 12). Infection intensities fluctuated erratically, and significant differences due to collection date were found; however, no significant effects attributable to site or date * site interaction were seen (Table 2). Significant increases and decreases were found between collection dates throughout the sampling (Table 3A).

DISCUSSION

Water temperatures during the study period exhibited a seasonal cycle that was highly coincident at Dry Bar and Cat Point Bar (Fig. 2). Perhaps as a consequence, the reproductive status (RGS and GC) of oysters was relatively synchronous between the two collection sites. This was demonstrated by the lack of significant difference due to site (Table 2), although there was significant date * site interaction. There is evidence that eastern oysters in different locations can have genetically determined environmental requirements for gonadal maturation (Loosanoff 1969, Barber et al. 1991) and contrasting evidence that they are controlled primarily by temperature (Ruddy et al. 1975, Butler 1955). In this study, any genetic differences that might exist between oysters at

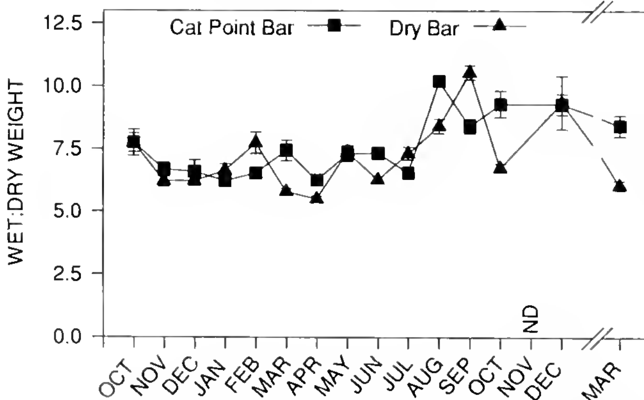


Figure 9. Average ratio of tissue wet weight to dry weight measured during October 1991 through March 1993 from oysters collected at Cat Point Bar and Dry Bar (n = 12). ND, no data; bars indicate standard errors.

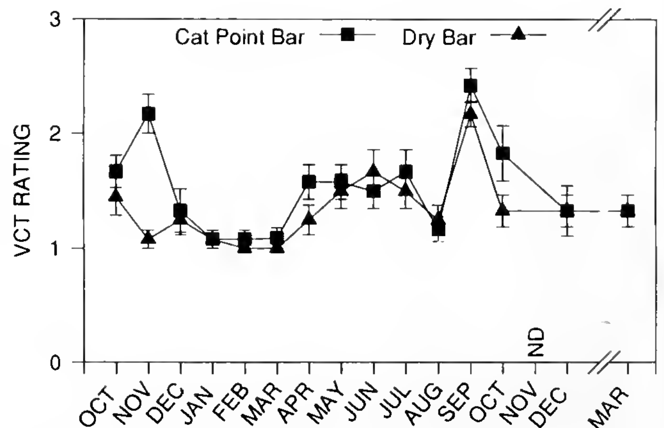


Figure 11. Average rating for VCT structure examined in histologic sections during October 1991 through March 1993 from oysters collected at Cat Point Bar and Dry Bar (n = 12). ND, no data; bars indicate standard errors.

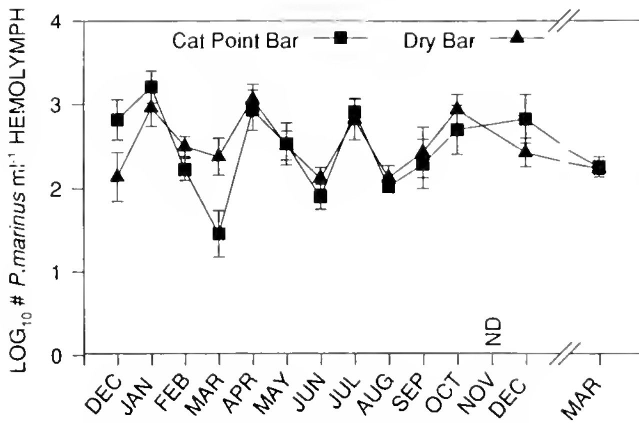


Figure 12. Average of $\log_{10}$-transformed estimates of *P. marinus* intensity in 1 mL of oyster hemolymph. Oysters were collected at Cat Point Bar and Dry Bar (n = 12) during October 1991 through March 1993. Protozoan meronts were counted after culture in fluid thioglycollate medium and staining with Lugol's iodine solution. ND, no data; bars indicate standard errors.

Cat Point and Dry Bar did not appear to interfere with their reproductive synchrony (Figs. 5 and 6).

In contrast to temperature, salinities at the two sites were quite different. Data recorded at the time of collection (Fig. 3, top) found salinities to vary as much as 10 ppt between sites even though there was a general pattern of low salinity at both sites during winter to spring and high salinity during fall. Salinity data from continuous monitors deployed from June 1992 to March 1993 (Fig. 3, bottom) showed dramatic fluctuations and differences between sites. These differences occur because of various influences from river flow, tidal action, and wind. The Apalachicola Bay system is an area of mixed tides, ranging from 0.3 to 0.7 m, that alternate between the semidiurnal tides of southwestern Florida and the diurnal tides of northwestern Florida (Dawson 1955, Gorsline 1963). Net water movement in the system is generally east to west (Ingle and Dawson 1953, Conner et al. 1982), with currents governed primarily by the astronomical tides. These can be influenced, however, by strong prevailing winds (Estabrook 1973). The Apalachicola River is the primary source of fresh water in the bay, carrying an annual mean discharge of approximately 15,000 (range, 9,300–80,000) cubic feet per second (U.S. Army Corps of Engineers 1978). Because of these factors, river flow dominates salinities at Dry Bar whereas tidal exchange may equal the river's influence at Cat Point Bar. During periods of light wind or an east wind, the influence of the river was primarily toward the southwest and Dry Bar. Winds from the west shifted the river's influence so that fresher water was found at Cat Point Bar. Local rainfall during the study period did not appear to have an immediate effect on salinity at either site.

The wet:dry tissue weight ratio (Fig. 9) of oysters increased near the end of spawning (Fig. 8) and was generally the inverse of the CI. Both characteristics were probably associated with degraded oyster condition typical for oysters during this period (Soniat and Ray 1985). The lower CI in autumn for Apalachicola Bay oysters is similar to that reported for eastern oysters from some Virginia estuaries (Chesapeake Bay; Austin et al. 1993) and from Galveston Bay, TX (Soniat and Ray 1985), but contrasts with other studies in Chesapeake Bay (Engle 1951) and Louisiana (Hopkins et al. 1954) that have shown CI to be low in summer and high during fall and winter. This apparent lack of synchrony among different oyster populations, even when the sites are relatively close, may be related to the availability or quality of nutrients (Soniat and Ray 1985, Austin et al. 1993). If so, the utility of CI as a biologic indicator (Scott and Middaugh 1978, Scott and Vernberg 1979, Lawrence and Scott 1982, Roper et al. 1991) must be limited to situations where nutrient input was closely monitored. Nutrient differences may also explain why Dry Bar oysters had a significantly higher CI than Cat Point Bar oysters throughout the study period.

Digestive tubule atrophy in bivalves has been associated with xenobiotic exposure (Lowe et al. 1972, Bayne et al. 1976, Chagot et al. 1990, Weis et al. 1995) and environmental stressors such as temperature, spawning, salinity, or nutrition (Thompson et al. 1974, Bayne et al. 1981, Couch 1985, Widdows and Johnson 1988). Stress, regardless of the source, appears to cause epithelial atrophy through the formation of autolysosomes in tubule cells. In this study, the condition of digestive tubules (DTR; Fig. 10) may have been influenced by temperature or temperature-driven physiologic changes because tubule atrophy (high DTR) was greater toward the end of active spawning, at least for Cat Point Bar oysters (Table 3B). Conspicuously high DTR were found in February and December 1992 for Dry Bar oysters (Fig. 10) and may have been related to acute salinity changes. Salinities at Dry Bar were low at those collection dates (Fig. 3, top), and data from the continuous water monitors showed a rapid decrease just before the December 7 sampling date (Fig. 4). An acute decrease in salinity may have caused oysters, which are osmoconformers, to close their shells and stop feeding. Winstead (1995) has recently demonstrated that digestive tubules in unfed oysters can significantly atrophy, compared with fed controls, within 2 days after the cessation of feeding. Alternatively, changes in phytoplankton quantity or quality could create the same effect and be related to salinity changes (e.g., river flow). Regardless, factors in digestive tubule variability cannot be accurately assessed by monthly monitoring if changes occur as rapidly as indicated by Winstead (1995).

VCT structure appeared to relate to the changing condition of the oyster reproductive cycle, showing the poorest structure (highest ratings) near the end of spawning, with recovery by early winter (Fig. 11). A significant difference between sites was found in November 1991, when Cat Point Bar oysters had poor connective tissue structure relative to Dry Bar oysters. This could be related to the smaller gonad size for Cat Point Bar oysters on that date (Fig. 5), but the factors responsible are unknown.

The low *P. marinus* infection intensities observed in Cat Point Bar oyster hemolymph in March 1992 (Fig. 12) coincided with low salinity for that date and site (Fig. 3, top). This is relevant because *P. marinus* require salinities above 6 ppt to sporulate (Perkins 1966, Chu and Greene 1989) and transmission in nature is reduced at salinities below 12 ppt (Andrews and Hewatt 1957, Paynter and Burreson 1991). However, the salinity was even lower at Dry Bar in February 1992 and December 1993 (Fig. 3) and there was no decrease in *P. marinus* intensity. The duration of low-salinity conditions may be critical to infection intensity. The infection prevalences of *P. marinus* (100%) and other parasites such as *Tylocephalum* (17–100%) and *Nematopsis* (58–75%) were much higher than the less than 5% prevalences previously found at other Gulf of Mexico estuaries (Couch 1985).

RGS (Fig. 5) was greatest at the initial stages of spawning (April), diminished as spawning proceeded, and recovered after spawning was completed. In 1991, gonads were enlarging in No-

vember and December even though new gametes were not visible in the follicles until February 1992. This means that gonads develop long before gametogenesis or that early gametogenesis was undetectable by these histologic methods. Gonad sizes in November and December 1992 were not as large as those in 1991, indicating annual variability; this has also been shown by Loosanoff (1965) using Long Island oysters.

It can also be inferred from the monitoring of gonadal condition that spawning during 1992 was continuous; the depletion of the follicles was relatively constant from May through October (Fig. 6). Individual oysters may have had spawning peaks during this period, but there were few departures (e.g., August sample from Dry Bar) from the regular depletion of mature gametes. There was no indication of a second gametogenic cycle at any time during the year, even though evidence for such a possibility was previously noted for Galveston Bay oysters (Soniat and Ray 1985).

The data generated by this study offer some insights to the variability of several physiologic characteristics of Apalachicola Bay oysters. Seasonal cycling in most of these characteristics can be largely attributed to effects of temperature (excluding potential differences in endogenous rhythms). Temperature may have affected oyster physiologic characteristics directly or indirectly through control over reproductive activities. Other environmental factors, particularly salinity, may have been involved in the differences demonstrated between sites at specific collection dates. Because not all differences could be explained by the water quality measurements of this study, other factors (particularly nutrition) must be considered to understand the variability.

Successful interpretation of bivalve physiologic characteristics as indicators of their health or as biologic indicators of environmental contamination demands thorough knowledge of natural sources of variability in the measurements. The physiologic characteristics of oysters (and other bivalves) may effectively reflect anthropogenic stress, provided that variability in the indicators is either limited or well defined and correctable (Huggett et al. 1992). Some of the characteristics measured here were significantly different at the same sampling month for different years (Table 3), likely as the result of discrepancies in temperature or other environmental conditions. These data represent only monthly samples during a year-long period for a limited geographic region, so the interpretations must be verified for different years and different locations.

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HEMATOLOGIC AND SEROLOGIC VARIABILITY OF EASTERN OYSTERS FROM APALACHICOLA BAY, FLORIDA

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ABSTRACT Eastern oysters (*Crassostrea virginica*) were collected monthly from two sites approximately 15 km apart in Apalachicola Bay, FL, during a 1-y period. Hematologic and serologic measurements were made on hemolymph withdrawn from the adductor muscle. The two sites experienced nearly identical temperature patterns during the study period, but salinity and other physical factors fluctuated. Significant differences attributable to sampling date were found for circulating hemocyte density, phagocytic activity, and superoxide anion (O_2^-)–producing ability and for serum protein, lysozyme, and agglutinating activity, with data from both sites combined. This variability was most likely related to temperature or temperature-influenced reproductive cycling. Oyster hemocyte locomotion did not vary significantly with time over the study period, nor were significant differences found between sites. Significant differences between site means (combined for all dates) were found for O_2^-, protein and lysozyme, and significant date * site interactions were found for phagocytosis, agglutination, and lysozyme, indicating that local conditions, such as salinity fluctuations, influenced these measurements. An accurate description of variability in oyster defensive functions will require more frequent sampling and a better understanding of local environmental influences.

KEY WORDS: Eastern oysters (*Crassostrea virginica*), bivalve immunology, invertebrate hematology, invertebrate serology

INTRODUCTION

Several hematologic and serologic parameters have been measured to indicate the status of the defense (immune) system of bivalve molluscs (for reviews, see Chu 1988 and Feng 1988). Bivalve defensive cells and hemolymph molecules are influenced by changes in ambient temperature and salinity (Fisher 1988), which may account for the high variability in the defense responses of oysters collected at different sites and/or times of year (Fisher et al. 1989, Oliver and Fisher 1995). This variability is a critical concern for research that attempts to understand the relationship of defense processes with infection and resistance in oysters (*Crassostrea virginica*) afflicted by protozoan parasite diseases (Ford 1986, Ford 1988, Chu and La Peyre 1989, Chu and La Peyre 1993a, Chu and La Peyre 1993b, Kanaley and Ford 1990, Chintala and Fisher 1991, Anderson et al. 1992b, Chu et al. 1993, Ford et al. 1993, Chintala et al. 1994). Also, there is continued interest in describing the effects of xenobiotic exposure on bivalve defense mechanisms or in using measurements of defense activities to characterize environmental conditions (Ruddell and Rains 1975, Fries and Tripp 1980, Anderson et al. 1981, McCormick-Ray 1987, Cheng 1988, Cheng 1990, Cheng 1993, Seiler and Morse 1988, Suresh and Mohandas 1990, Sami et al. 1992; also see reviews by Anderson 1988 and Anderson 1993). Such applications of defense characteristics demand a better understanding of their ranges and sources of variability.

A major source of variability in bivalve biology is the annual reproductive cycle, driven primarily by temperature (Galtsoff 1964). Many physiologic and defense characteristics are influenced by this dominating activity (Eble 1966, Swift and Ahmed 1983, Fisher and Newell 1986a, Fisher et al. 1989), but intermittent changes in other environmental factors such as salinity, dissolved oxygen, nutrients, toxicants, parasites, and disease also exert some influence. The combined effects of these exogenous factors modify the inherent defense capabilities of each organism.

Variability in defense characteristics has been demonstrated in vitro after various alterations in salinity and temperature conditions (Fisher and Newell 1986b, Fisher 1988, Fisher and Tamplin 1988). Several studies have shown variable hemocyte responses of oysters collected at several locations or held in the laboratory under different controlled conditions (Fisher and Newell 1986a, Fisher et al. 1989, Chu and La Peyre 1993a, Chu et al. 1993, Cheng et al. 1993, Oliver and Fisher 1995). Yet, for estuarine oysters that can experience diurnal tidal fluctuations, frequent and sometimes drastic alterations in nature make it difficult to extrapolate laboratory findings to field conditions.

Oysters from two bars in Apalachicola Bay (Franklin County, FL) were studied from October 1991 to March 1993 to examine variations in hemocyte and hemolymph characteristics. The oyster bars lie within 15 km of each other in a bay system protected by a chain of barrier islands (Gorsline 1963). Temperature differences between these sites during the collection period were minimal, and oyster reproductive cycles were nearly identical (Fisher et al. 1996). However, currents, tides and river inflow created smaller-scale and intermittent environmental diversity in other factors such as salinity. The goals of this research were to monitor and describe variability in oyster defense activities and to distinguish contributions of seasonal temperature cycles from site-specific factors. Presented here are monthly averages for circulating hemocyte number, locomotive activity, phagocytic activity, and superoxide anion (O_2^-) generation, as well as hemolymph protein concentration, lysozyme concentration, and agglutinin titer to horse erythrocytes.

MATERIALS AND METHODS

Background Information

The Apalachicola Bay system is a highly productive lagoon/barrier island complex. The eastern oyster is the most important commercial invertebrate in Apalachicola Bay and comprises nearly 90% of the oysters harvested in Florida. Because of relatively mild temperatures in the area, rapid oyster growth is sustained throughout the year. Harvestable oysters, those larger than 3 inches (76 mm), have been grown from spat in as little as 39 wk, and the spawning season is reportedly one of the longest in the United States (Ingle and Dawson 1952). Oysters in Apalachicola Bay, like most oysters throughout the Gulf of Mexico, exhibit a nearly 100% prevalence of the protozoan pathogen *Perkinsus marinus* (Craig et al. 1989).

Site Characteristics

The two sites for oyster collection, Cat Point Bar and Dry Bar (also called St. Vincent's Bar), were previously described in relation to hydrology, water quality, and value to the oyster industry (Fisher et al. 1996). A history of water quality data exists through the efforts of the Florida Department of Environmental Protection (Thompson et al. 1990). The two sites are less than 15 km apart and are southeast (Cat Point Bar) and southwest (Dry Bar) of the mouth of the Apalachicola River (Fig. 1). Both sites lie within the chain of protective barrier islands, but the physical and chemical characteristics of the water are influenced by wind, tide, and river currents (Gorsline 1963). For this study, temperature and salinity were measured at the time of oyster collection. Also, continuous (30-min interval) monitoring of water quality was initiated in June 1992 and continued through December 1992 with Hydrolab[1] Datasonde 3 dataloggers attached to pilings at the collection sites (Fisher et al. 1996).

Oyster Collection and Processing

Oysters were collected with hand tongs on (approximately) a monthly basis from October 1991 to October 1992, and additional samples were collected in December 1992 and March 1993. Dates of collection were (1991): October 22, November 19, December 19; (1992): January 21, February 25, March 31, April 21, May 19, June 23, July 21, September 1, September 29, October 26, December 7; and (1993): March 29, for a total of 15 collections over a span of 17 mo (525 days). The September 1 sample is referred to as the August sample in this report. Oysters ranging from 50 to 90 mm in height (umbo to beak) were collected and placed immediately into coolers containing cold ice packs. The coolers were transported to the Environmental Protection Agency Gulf Ecology Division at Gulf Breeze, FL, and placed in a refrigerator (4°C) overnight.

Twelve oysters from each site were allowed to warm to room

[1]The mention of product names does not signify a recommendation or endorsement by the Environmental Protection Agency.

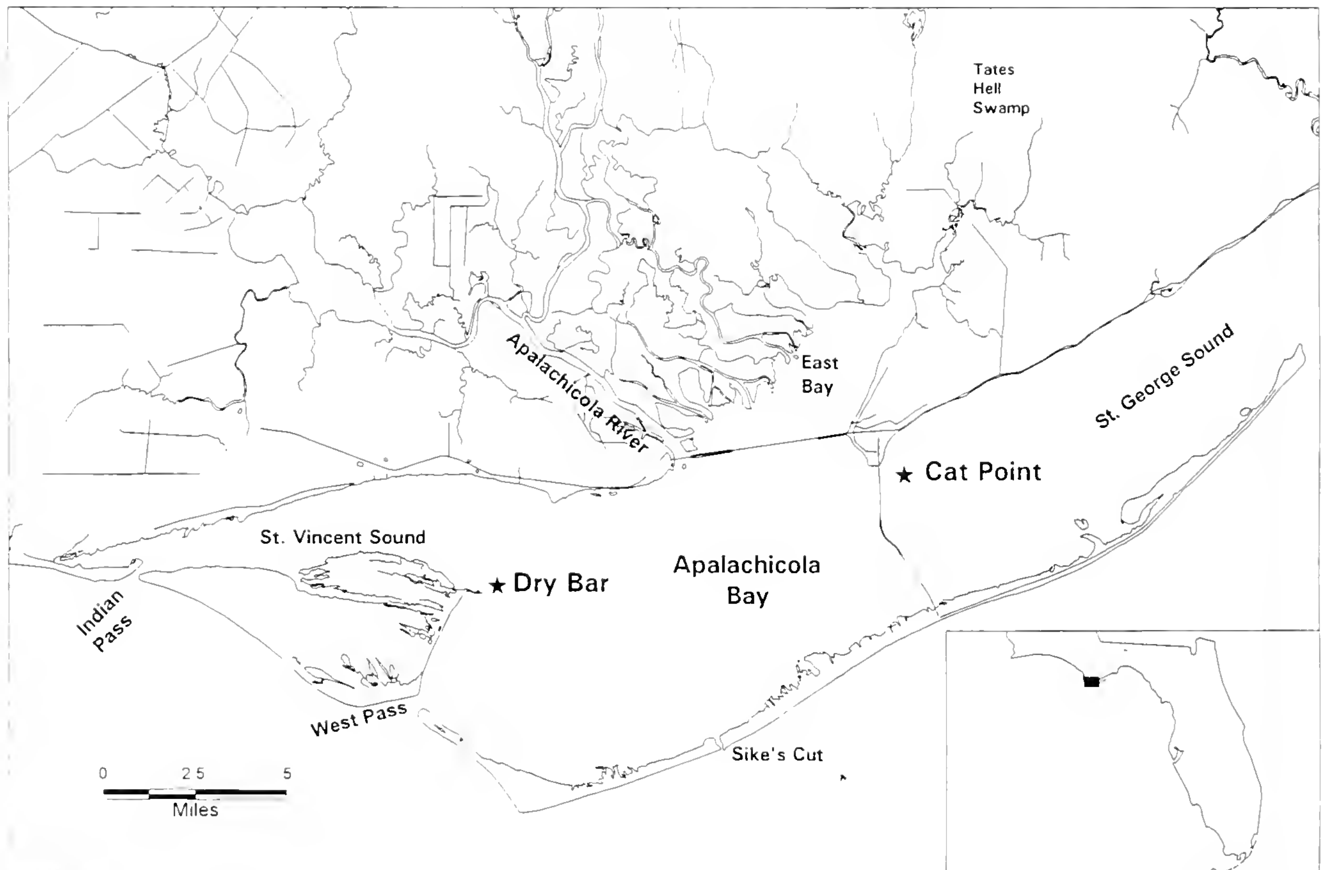

Figure 1. The Apalachicola River and Bay system with the two oyster collection sites, Cat Point Bar and Dry Bar (also known as St. Vincent's Bar), noted with stars. The sites are in northwest Florida (inset).

temperature for 2–4 h. They were then scrubbed clean of fouling organisms, and a grinder was used to notch the shells at the posterior edge adjacent to the adductor muscle. The mantle cavity was rinsed thoroughly with filtered (0.22 µm pore size) seawater to remove debris. Hemolymph was withdrawn from the adductor muscle with a 3-mL syringe and a 22-gauge needle. Hemolymph was used to measure various hematologic and serologic characteristics, as described below, and to diagnose *P. marinus* infection intensity (see Fisher et al. 1996). These characteristics were measured for different durations during the course of the study (see Table 1).

Circulating Hemocyte Density

Hemolymph withdrawn from oysters was mixed gently to ensure sample homogeneity; then, one drop was dispensed onto each side of a hemacytometer counting chamber for duplicate counts. The remaining hemolymph sample was placed on ice immediately after hemacytometer loading to minimize hemocyte aggregation and activity.

Hemocyte Mobility and Rate of Location

Two drops of hemolymph were placed in a single well of a Lab-Tek 8 chamber slide containing 200 µL of filtered (0.45 µm pore size) water collected from the site. Hemocytes were maintained at 27°C for 30–60 min to allow hemocyte settling, attachment, and locomotion along the glass slide surface. For each oyster, the movement of 12 to 15 adherent hemocytes was tracked with transparent overlays on a video monitor attached to an Nikon inverted microscope under phase contrast (Fisher and Newell 1986b). Tracings were measured with electronic digital calipers. The average rate for at least 10 mobile hemocytes was recorded as the rate for each individual oyster. Hemocytes that did not move were also recorded, and the percentage of mobile hemocytes was calculated.

Particle-Binding (Phagocytic) Index

The ability to bind a foreign particle (phagocytic index, PI) was determined in vitro by the addition of a calculated volume of hemolymph containing 30,000 hemocytes to wells of Lab-Tek 8 chamber slides that had been preloaded with 30,000 test particles (yeast, *Saccharomyces cerevisiae;* Sigma Chemical Co.) suspended in filtered seawater sampled from the collection site (see Oliver and Fisher 1995). The proportion of [yeast:hemocyte] was maintained at [1:1] because phagocytic activity is dependent on particle availability. After 60 min of incubation at 27°C, the slides were gently dipped in filtered seawater to remove unbound particles and nonadherent hemocytes, fixed for 2 min in absolute methanol, and then dipped in deionized water and fresh methanol before air drying. Examination of slides was performed on a Nikon inverted microscope with 40× objective under phase contrast. A minimum of 200 hemocytes was examined per well to determine the PI, which was expressed as:

$$\frac{\text{(number of hemocytes displaying bound or ingested yeast)} \times 100}{\text{(total number of hemocytes observed)}}$$

Hemocyte Superoxide Anion Production

Superoxide anion (O_2^-) generation by hemocytes from individual oysters was quantified by measuring the reduction of nitroblue tetrazolium (NBT) dye to formazan. The colorimetric method described by Anderson et al. (1992a) was scaled for an analysis of individual oysters as described by Oliver and Fisher (1995). Hemocyte production of O_2^- was measured under both unchallenged and yeast-challenged conditions.

Hemolymph Serum Protein

Hemolymph (0.25–0.5 mL) from each individual was placed in a plastic microfuge tube and centrifuged at 2,900 × *g* for 5 min. The resulting pellet was cultured in Ray's fluid thioglycollate medium (Ray 1966) to diagnose the intensity of *P. marinus* disease (Fisher et al. 1992), and the serum (supernate) was held at 4°C for less than 1 wk before being tested for protein, lysozyme, and agglutinin content. Levels of serum protein were measured with the Pierce BCA Protein Assay kit with samples diluted 10-fold in deionized water. Protein concentrations for each sample were calculated from a standard curve generated from dilutions of bovine serum albumin.

TABLE 1.

Results of an interactive ANOVA with date and site for the variables HC, percent mobile hemocytes (% mobile), rate of hemocyte locomotion (RHL), PI, reduction of NBT, unchallenged (NBT-UNCH) and challenged with yeast (NBT-CHALL), hemolymph serum protein content, agglutinin content, and lysozyme content.[a]

Variable	Duration	Date (p<)	Site (p<)	Date * Site (p<)
HC	Dec '91–Mar '93	0.001[b]	0.916 NS[c]	0.167 NS[c]
% Mobile	Dec '91–Mar '93	0.182 NS[c]	0.674 NS[c]	0.858 NS[c]
RHL	Dec '91–Mar '93	0.161 NS[c]	0.636 NS[c]	0.104 NS[c]
PI	Jan '92–Mar '93	0.001[b]	0.224 NS[c]	0.015[d]
NBT-UNCH	Mar '92–Mar '93	0.001[b]	0.007[b]	0.082 NS[c]
NBT-CHALL	Mar '92–Mar '93	0.001[b]	0.021[d]	0.171 NS[c]
Protein	Oct '91–Mar '93	0.001[b]	0.001[b]	0.259 NS[c]
Agglutinin	Oct '91–Oct '92	0.001[b]	0.338 NS[c]	0.001[b]
Lysozyme	Apr '92–Mar '93	0.001[b]	0.011[d]	0.002[b]

[a] Tests were performed for different durations over the study period.
[b] Highly significant (p < 0.01).
[c] NS, not significant.
[c] Significant (p < 0.05).

Agglutination by Hemolymph Serum

Hemolymph serum samples were tested for the agglutination of horse erythrocytes (red blood cells [RBC]) obtained from Cocalico Biologicals, Inc. (Reamstown, PA) and diluted 1:1 in Alsever's solution. Serial twofold dilutions of 50 μL of serum in 96-well microtiter plates were assayed with 50 μL of a 2% RBC suspension in 8.5 ppt artificial seawater, according to previously published methods (Fisher and DiNuzzo 1991, Fisher 1992). Microtiter plates were examined after 2–3 h of incubation at 24°C, and titers were recorded as the reciprocal of the highest dilution showing positive agglutination. Titers were recorded as $\log_2$ values to reflect the twofold serial dilutions.

Hemolymph Serum Lysozyme

Lysozyme was quantified by measuring the ability of oyster serum to degrade a suspension of bacteria *Micrococcus lysodeikticus* (Sigma Chemical Co.). These procedures were originally described by Shugar (1952) and modified for oyster serum by Rodrick and Cheng (1974). Hemolymph serum samples were stored in the refrigerator for 1–2 days before quantification. A 0.2 mg/mL bacterial suspension was prepared by adding 0.005 g *M. lysodeikticus* to 25 mL of 0.5% glycylglycine buffer (Gly) (Gly = 1.65 g of glycylglycine [Aldrich Chemical Co.] plus 0.625 g of sodium chloride in 125 mL of deionized water). The pH of Gly was adjusted to 5.5 with 1N hydrochloric acid. The bacteria were resuspended in Gly, resulting in a uniformly turbid solution, and the optical density was adjusted to 0.7 absorbance units at 540 nm with a Shimadzu spectrophotometer. A disposable microcuvette was loaded with 20 μL of serum, followed by 0.5 mL of *M. lysodeikticus*. The decrease in turbidity in the microcuvette was traced for 4 min at 540 nm in the kinetic mode of the spectrophotometer. The rates of degradation were compared with those of standard solutions prepared from hen egg white lysozyme (Sigma Chemical Co.).

Statistical Methods

Data were entered into SAS (SAS Institute, Cary, NC) and analyzed with General Linear Models and the Shapiro-Wilk test for normalcy. Residual plots were examined to check for homogeneity of variance, independence, and normality of error terms in the resulting models. Two-way analysis of variance (ANOVA) was conducted to relate each dependent variable (hemocyte and hemolymph measurements) to the main effects date and site and to test for possible interactions between date and site (= date * site). The results of all analyses are reported, but main effects are discussed only if there was no significant interaction. Where significant main effects were found, Turkey's post-hoc test was used to differentiate between overall date or site means. For variables that displayed a significant interaction effect, differences due to date for each of the sites were examined with Tukey's post-hoc results, which compared means of all date * site combinations. Levels of significance and high significance were designated as $p \leq 0.05$ and $p \leq 0.01$, respectively.

RESULTS

Temperature and Salinity Conditions

Water temperatures measured when oysters were collected were very consistent for the two collection sites (Fig. 2, top), and close agreement in daily values was confirmed by Hydrolab data for part of the study period (Fig. 2, bottom). Both sites experienced progressive warming after January and cooling after July. In contrast, salinity measured at the time of collection varied greatly between the two sites (Fig. 3, top); data collected continuously from June 1992 to March 1993 revealed differences between the two sites' daily averaged salinity values that were often 10–15 ppt and sometimes as high as 25 ppt (Fig. 3, bottom). Even within a given day, 10–15 ppt salinity variation at one site was not uncommon.

Summary of Oyster Physiology

Measurements of oyster physiology (see Fisher et al. 1996) showed that oysters collected from both sites were reproductively similar; gametogenesis was first visible in histologic sections in February 1992, and spawning was apparently continuous from April through September. Oysters collected were predominantly female (80%) throughout most of the study period, even though the gender ratio was approximately equal during February and March. The condition index of oysters was lowest during autumn, and the wet:dry tissue weight ratio (used to calculate total tissue weight for the condition index) generally reflected the inverse of condition index.

Circulating Hemocyte Density

The effect of collection date on circulating hemocyte density (HC) was highly significant, whereas no significant effect due to site or date * site interaction was found (Table 1). Temporal changes were very similar for the HC of oysters from both sites (Fig. 4).[2] For both sites, the highest HC was measured in May, after which it declined through the summer to the lowest level, measured in August (Table 2A); the level then increased in September and October.

Hemocyte Locomotion

No significant effects were found for percent mobile hemocytes (Fig. 5) or hemocyte rate of locomotion (Fig. 6) as the result of date, site, or date * site interaction (Table 1).

Particle-Binding (Phagocytic) Index

A [1:1] yeast:hemocyte challenge resulted in 20 to 35% phagocytic hemocytes throughout the study (Fig. 7). The effect of collection date on PI was highly significant, and the interactive effect of date * site was also significant (Table 1). Hemocytes from both sites showed a significant decrease in particle binding between April and June and recovery by August (Table 2B).

Hemocyte Superoxide Anion Production

Unchallenged and yeast-challenged hemocytes produced superoxide anion (O_2^-) at nearly the same levels throughout the study period (Fig. 8). Levels from challenged hemocytes were significantly correlated with those from unchallenged hemocytes (r = 0.861, n = 224, Pearson's procedure). For unchallenged and challenged O_2^- production, the main effects of both date and site were significant and no significant interactive date * site effect was found (Table 1). The overall seasonal pattern was similar for both sites, with progressively lower measurements obtained from

[2]Data are presented in graphic form for convenience; tabular data sets are available upon request to the authors.

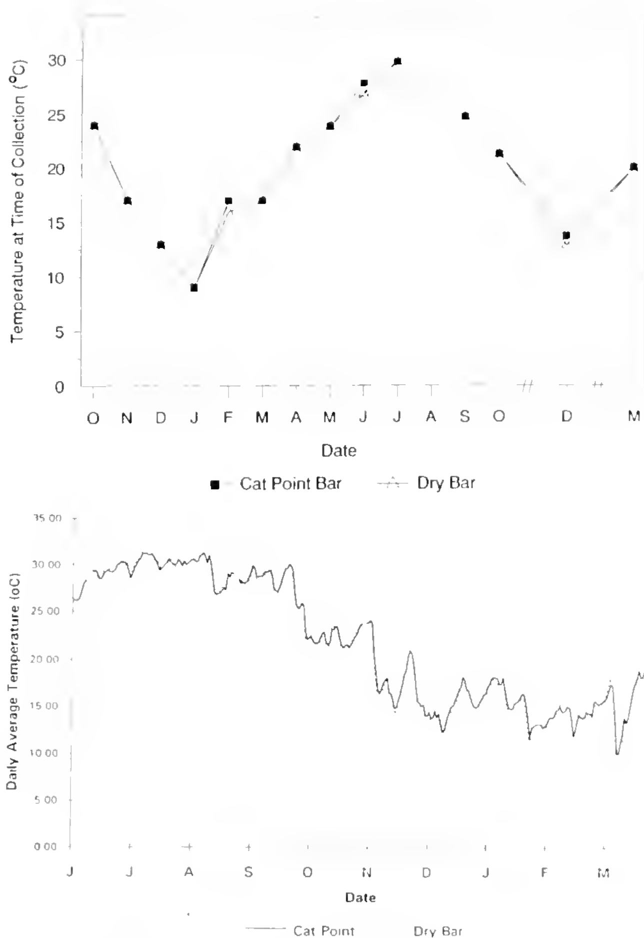

Figure 2. Water temperature of Cat Point Bar and Dry Bar during the period of collection. (Top) Temperature taken at the time of collection throughout the entire study period. (Bottom) Daily averages of temperatures taken every 30 min from June 1992 to March 1993 only (note scale differences). All temperatures were recorded less than 0.5 m from the bay bottom.

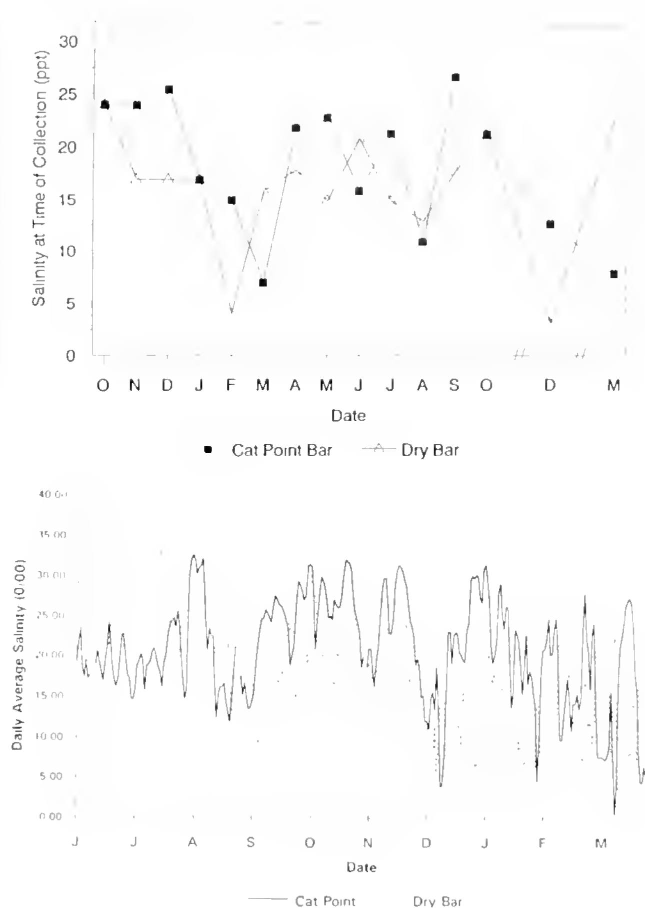

Figure 3. Water salinity of Cat Point Bar and Dry Bar during the period of collection. (Top) Salinities measured at the time of collection throughout the study period. (Bottom) Daily averages of salinities measured every 30 min from June 1992 to March 1993 only (note scale differences). All salinities were recorded less than 0.5 m from the bottom of the bay.

spring to summer, following by increased activity during the fall and winter months (Table 2A). The mean response over all dates for both unchallenged and challenged O_2^- production was significantly higher for Dry Bar oysters.

Serum Protein Concentration

Highly significant differences in protein content were attributable to both date and site, and no significant date * site interaction was found (Table 1). Average protein content throughout the study period was significantly higher for Dry Bar oysters. With the exception of relatively low March 1992 results, the highest protein concentrations were measured during winter and spring (December to April 1992) and the lowest were measured during the late summer (Table 2A; Fig. 9).

Serum Agglutinotion Activity

Differences in the ability of oyster sera to agglutinate horse erythrocytes were significant for date effect and date * site interaction, but were not significant for site effect (Table 1). Although a trend appears consistent at both sites (Fig. 10), there were few significant differences at either site over time when all date * site combinations were considered (Table 2B).

Serum Lysozyme Concentrotion

The effects of collection date, site, and date * site interaction on oyster serum lysozyme concentration variability were all significant (Table 1). Lysozyme levels increased significantly from May to July for Cat Point Bar oysters, and although a similar trend occurred for Dry Bar oysters (Fig. 11), it was not significant (Table 2B).

DISCUSSION

Natural variations due to season and habitat cannot be overlooked if measurements of oyster defense mechanisms are to be properly interpreted in the context of physiology or disease susceptibility or as biologic indicators of pollution. To achieve some insight to the natural variation of oyster hematologic and serologic characteristics, oysters from two relatively pristine sites in Apalachicola Bay were examined monthly during a 1-y period. This period encompassed the oyster reproductive cycle, which is strongly regulated by seasonal temperatures. Most of the measured defense traits varied significantly throughout the year (Table 1,

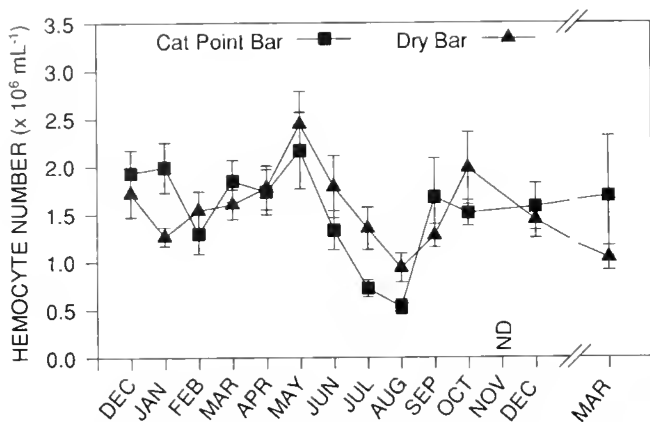

Figure 4. Circulating HC in hemolymph of oysters collected from two sites, Cat Point Bar and Dry Bar, during the period from December 1991 through March 1993. ND, no data; bars indicate standard errors.

variability by date), and some of these exhibited apparent annual patterns that may reflect changes in seasonal temperatures or oyster reproductive condition. Water temperature and reproductive condition were nearly identical for the two sites during the study period (Fisher et al. 1996). However, significant site effects and interactive date * site effects (Table 1) demonstrated the influence of unique local conditions, which could be any combination of physical, chemical, and biologic factors that occurred independently at each site. The relationship of salinity with site effects could not be determined in this study, but it is possible that changing estuarine salinities were partly responsible for the site effects because salinity fluctuated widely between sites (Fig. 3) and is known to have an explicit effect on defense mechanisms in vitro (Fisher and Newell 1986b, Fisher 1988, Fisher and Tamplin 1988, Fisher et al. 1989). Alternatively, salinity fluctuations may have tracked other hydrographic changes that affected oyster biology (e.g., changes in turbidity, oxygen, nutrient availability and composition, contaminants, or other factors that might be associated with changes in river flow or currents).

Variation in oyster HC and type has been demonstrated in other studies: Chu and La Peyre (1993b) found differences among oysters at three sites in Chesapeake Bay, and Oliver and Fisher (1995), as an adjunct to this project, found differences between hemocyte morphology and activities in oysters from Chesapeake Bay and Apalachicola Bay (Cat Point oysters) in March and October 1992. In this study, HC was lowest in July and August (Fig. 4), coinciding with the highest water temperatures during the year and active spawning by the oysters. Higher HC recorded in September could have been related to decreasing temperature and the end of spawning, when they resorb gonadal tissue. The lack of significant site or date * site effects implies that variation in HC was associated more with seasonal temperatures than with local conditions. This pattern contrasts with the relatively high HC found for oysters held in warm temperatures by Chu and La Peyre (1993b) and with earlier observations made by Feng (1965), who related increased temperature to a higher oyster heart rate, which propelled a larger number of hemocytes into circulation. Because those studies used oysters from the mid- and north Atlantic, it is possible that the contrasting results are due to higher mean summer temperatures in the Gulf of Mexico or to the unique physiologic characteristics of each oyster population.

Hemocyte mobility (percent mobile hemocytes and rate of locomotion) had no significant variability attributable to date or site. Earlier work (Fisher et al. 1989) with oyster hemocyte samples from an oceanic and an estuarine environment also found no effect on the rate of locomotion by warm summer temperatures, even though hemocyte-spreading capacity was retarded. Acute in vitro increases in salinity have been shown to depress hemocyte locomotion in laboratory studies (Fisher and Newell 1986a, Fisher and Tamplin 1988, Fisher et al. 1989), but it is not likely that such effects could be detected with a monthly monitoring regimen.

The phagocytic ability (PI) of oyster hemocytes varied significantly by date, but a clear seasonal pattern was not evident (Fig. 7). Phagocytic activity by Dry Bar oyster hemocytes was significantly lower in March 1993 compared with March 1992 (Table 2B), demonstrating the likelihood of year-to-year variability. The significant date * site interaction (Table 1) indicates that local conditions exerted an inconsistent influence on PI measurements.

The challenged and unchallenged production of O_2^- by hemocytes, measurements to reflect phagocytosis-associated killing capacity, demonstrated an apparent seasonal pattern (Fig. 8), as well as a significant site difference (Table 1). It appears that O_2^-

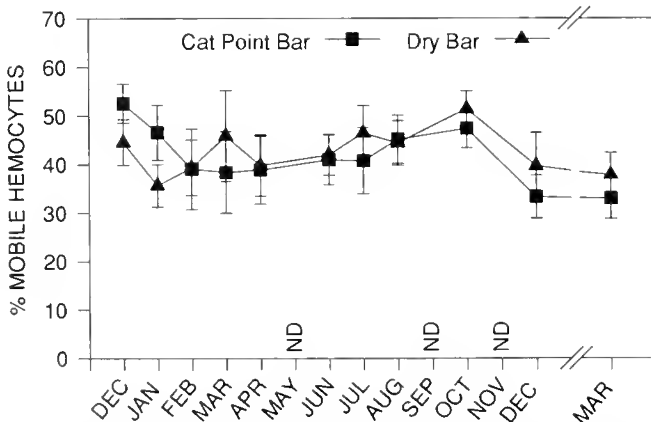

Figure 5. Percentage of circulating hemocytes that exhibited mobility. Hemocytes were withdrawn from oysters collected from two sites, Cat Point Bar and Dry Bar, during the period from December 1991 through March 1993. ND, no data; bars indicate standard errors.

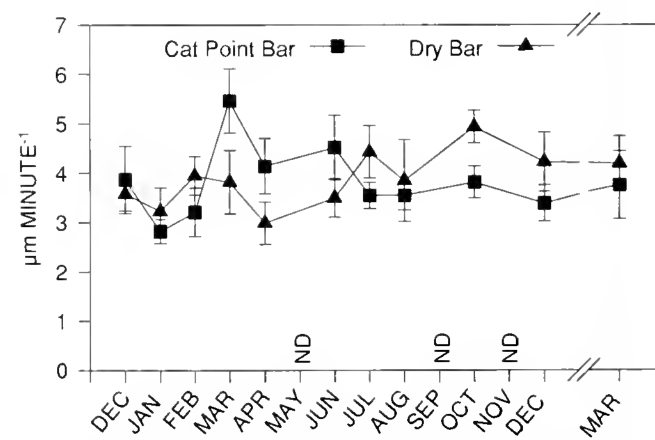

Figure 6. Rate of locomotion of circulating hemocytes drawn from oysters collected from two sites, Cat Point Bar and Dry Bar, during the period from December 1991 through March 1993. ND, no data; bars indicate standard errors.

production by oyster hemocytes was closely related to temperature because significant increases occurred in August and September, just as temperatures began to decline. Oysters at both sites were still spawning through September, so the effect cannot be clearly linked to reproductive cycling. In contrast, Anderson et al. (1992a) reported that the O_2^- production of hemocytes from Chesapeake Bay oysters, measured by NBT reduction of hemocytes pooled from several oysters, was significantly greater in those samples collected from waters at 21–29°C as compared with those at 2–13°C. Again, because those studies used oysters from the mid-Atlantic, it is possible that the contrasting results are due to higher mean summer temperatures in the Gulf of Mexico or to the unique physiologic characteristics of each oyster population. For example, Oliver and Fisher (1995) found O_2^- production by Chesapeake Bay oyster hemocytes to be twice that of Apalachicola Bay oyster hemocytes in both March and October 1992.

Volety and Chu (1995) presented evidence that the oyster pathogen *P. marinus* could suppress the production of reactive oxygen intermediates (including O_2^-) in vitro, even though hemocytes taken from oysters with heavy *P. marinus* infections were found to have increased O_2^- production compared with individuals with light infections (Anderson et al. 1995). In this study, the prevalence and intensity of *P. marinus* (measured by hemolymph assay) changed significantly over time (Fisher et al. 1996), but there was no obvious trend or pattern that might be related to the changes in O_2^- production observed. Obviously, the factors contributing to variability in this important defense activity must be further elucidated.

Serum protein concentrations appeared to exhibit an annual pattern for oysters at both sites, with the highest levels recorded in February and the lowest recorded in August (Table 2). A similar phenomenon was described in a study of Chesapeake Bay oysters (Fisher and Newell 1986a) that suggested that the high levels were related to the final stages of gamete maturation. Chu and La Peyre (1989) also found peak serum protein concentrations in Chesapeake Bay oysters from February to March, with lower values in June and July. It is not known why protein concentrations were higher in serum from Dry Bar oysters.

Site-specific factors, evidenced by a significant date * site interaction (Table 1), may have contributed to the variability observed in the agglutination of horse RBC by oyster serum (Fig. 10). Agglutinating molecules are believed to function in bivalve defense by opsonizing foreign particles for phagocytosis (Tripp 1966, Tripp and Kent 1967, Anderson and Good 1976, Olafsen 1988, Olafsen et al. 1992). On the basis of correlational observations, such a role was hypothesized for *C. virginica* as a defense against disease caused by *Haplosporidium nelsoni* (Chintala and Fisher 1991); however, this could not be confirmed in field studies (Chintala et al. 1994). Agglutinins may also be secreted by epithelial mucous cells to selectivity bind nutrient particles for ingestion (Fisher 1992). If so, then, nutrient composition and availability at different sites could have played an important role in the variability observed.

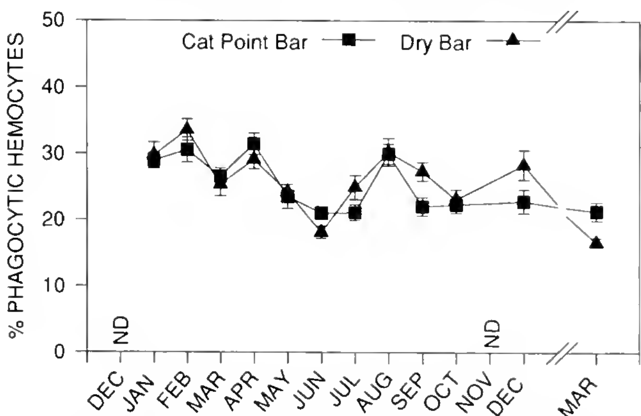

Figure 7. Percentage of circulating hemocytes that were phagocytic in particle-binding tests (PI). Hemocytes were drawn from oysters collected at two sites, Cat Point Bar and Dry Bar, during the period from January 1992 through March 1993. ND, no data; bars indicate standard errors.

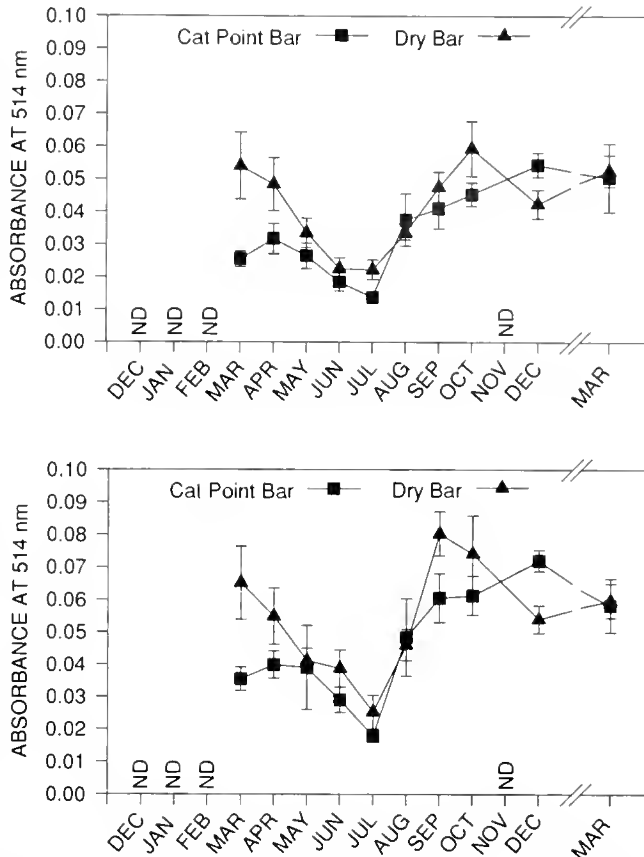

Figure 8. Spectrophotometer absorbance readings representing the amount of NBT reduced (O_2^- produced) by oyster hemocytes. (Top) Unchallenged hemocytes. (Bottom) Hemocytes challenged with yeast. Hemocytes were drawn from oysters collected at two sites, Cat Point Bar and Dry Bar, during the period from March 1992 through March 1993. ND, no data; bars indicate standard errors.

Delaware Bay oysters (Feng and Canzonier 1970) and Chesapeake Bay oysters (Chu and La Peyre 1989) have exhibited very low, sometimes undetectable concentrations of hemolymph lysozyme during summer months. Similarly, Chu and La Peyre (1993a) have demonstrated in the laboratory, using Chesapeake Bay oysters, that serum lysozyme has an inverse relationship with temperature. In contrast, results for Apalachicola Bay oysters high lysozyme levels during the warmest month (July) and lower levels during fall and winter (Fig. 11). High lysozyme levels during summer could be explained by increased secretion from hemocytes due to *P. marinus* invasion. Alternatively, high levels could represent a breakdown of lysosomes due to stress (decreased lyso-

TABLE 2.

Study results.[a]

	A				B					
					PI		AGGL		LYSO	
Date	HC	NBT-UN	NBT-CH	PROT	CP	DB	CP	DB	CP	DB
Oct '91				D			ABC	C		
Nov				CD			ABC	AB		
Dec	AB			ABCD			ABC	A		
Jan '92	AB			AB	ABCDEFG	ABCDE	ABC	ABC		
Feb	ABC			A	ABC	A	BC	ABC		
Mar	AB	AB	ABC	BCD	ABCDEFG	BCDEFGH	BC	ABC		
Apr	AB	AB	ABC	ABC	AB	ABCDEF	ABC	ABC	CDE	BCDE
May	A	BC	BCD	CD	CDEFGHI	BCDEFGHI	ABC	AB	CDE	BCDE
Jun	ABC	C	CD	BCD	GHI	HI	ABC	ABC	ABC	BCD
Jul	BC	C	D	ABCD	GHI	BCDEFGH	AB	ABC	A	AB
Aug	C	ABC	ABC	D	ABCDE	ABCD	ABC	A	BCDE	ABC
Sep	ABC	AB	A	CD	EFGHI	ABCDEFG	AB	ABC	CDE	CDE
Oct	AB	A	A	BCD	DEFGHI	CDEFGHI	A	AB	CDE	ABC
Dec	ABC	A	A	ABCD	CDEFGHI	ABCDEFG			E	DE
Mar '03	BC	A	AB	BCD	FGHI	I			BCDE	ABCD

[a] (A) Significant differences due to sampling date (Tukey's procedure). Shared letters in the column indicate no significant differences between collection dates for that variable. Analysis was performed with site data combined for variables with no significant date * site interaction (see Table 1). (B) Significant differences between means from all date-site combinations with significant interaction (see Table 1). Shared letters between any date-site combinations indicate no significant differences. NBT-UN, reduction of NBT, unchallenged; NBT-CH, reduction of NBT, challenged with yeast; PROT, protein concentration; AGGL, agglutination of horse erythrocytes; LYSO, lysozyme concentration; CP, Cat Point Bar oysters; DB, Dry Bar oysters.

somal latency) or increased secretion associated with feeding and spawning (Eble 1966). The variability described here and the striking contrast with studies of mid-Atlantic oysters present intriguing questions related to the source and/or function of serum lysozymes.

Much of the variability described in these defense-related measurements could stem from changes in hemocyte composition rather than actual changes in hemocyte activity. For example, it is generally considered that granular hemocytes are more phagocytic than agranular hemocytes (Renwrantz et al. 1979) and generate more O_2^- (Anderson et al. 1992a). There is some evidence that circulating hemocyte composition changes over time: McCormick-Ray and Howard (1991) found that the percent granulocytes in Chesapeake Bay oyster hemolymph decreased from January to May. Also, Oliver and Fisher (1995) demonstrated a disparity between the hemocyte composition of oysters from Chesapeake Bay and Apalachicola Bay, as well as a change in the composition at both sites between March and October. However, identifying changes in hemocyte composition is complex because of polymorphic cells and varying definitions for morphological observations, including "granularity" (M. Auffret, Universite Bretagne Occidentale, pers. comm.). For Gulf of Mexico oysters, the capacity to identify cell types is more difficult because hemocytes generally have less cytoplasmic volume with few morphological distinctions.

A major role for oyster hemocytes is to respond defensively to

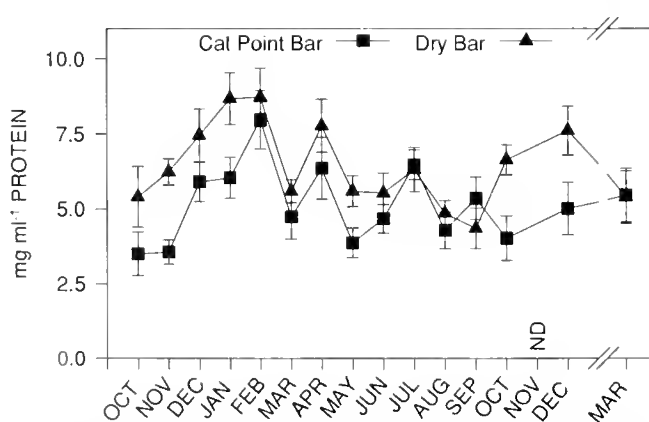

Figure 9. Protein concentrations in hemolymph of oysters collected at two sites, Cat Point Bar and Dry Bar, during the period from October 1991 through March 1993. ND, no data; bars indicate standard errors.

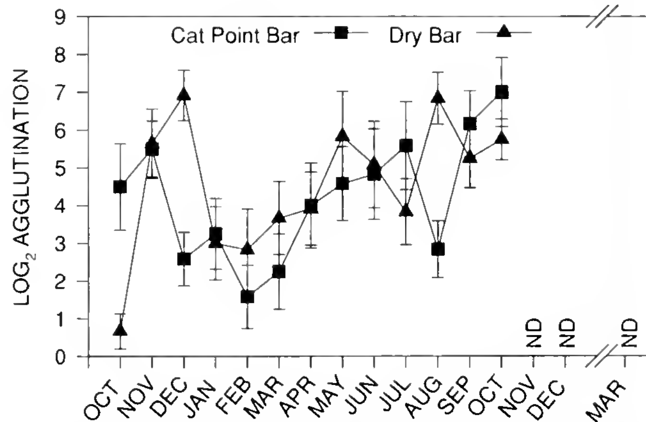

Figure 10. Agglutination titers (log_2) for oyster serum and horse RBC. Oyster serum was drawn from oysters collected at two sites, Cat Point Bar and Dry Bar, during the period from October 1991 through October 1992. ND, no data; bars indicate standard errors.

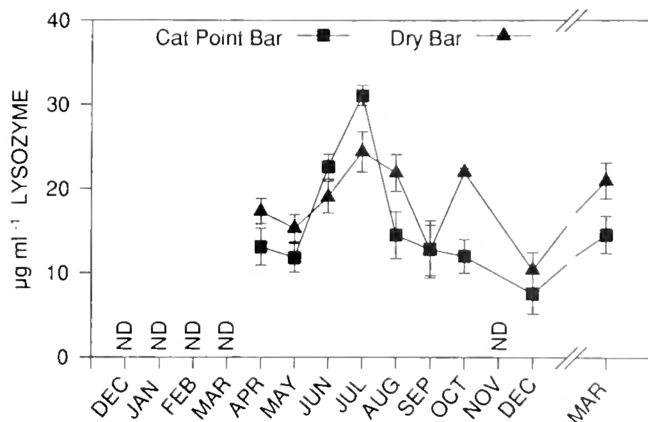

Figure 11. Lysozyme concentrations in hemolymph of oysters collected at two sites, Cat Point Bar and Dry Bar, during the period from April 1992 through March 1993. ND, no data; bars indicate standard errors.

intrusions by parasites and potential pathogens. Hence, hemocyte activities are influenced by the presence and type of parasites present. The possible effects of *P. marinus* on O_2^- production have been noted (Anderson et al. 1995, Volety and Chu 1995). We have recently recorded increased hemocyte activities in oysters infected by a metacercarian (J. Winstead pers. comm.) parasite. Yet, there were no obvious trends or site differences in the prevalence of *P. marinus*, *Tylocephalum* spp., or *Nematopsis* spp. (Fisher et al. 1996) in Cat Point and Dry Bar oysters that might easily explain the measured variability.

The monthly monitoring of oysters from two sites with similar temperature regimens revealed significant variability attributable to collection date in hemocyte density, NBT reduction, and serum protein concentrations. It is not clear whether these changes were directly influenced by temperature, physiologic consequences of the temperature-controlled reproductive period, other seasonally changing environmental factors such as nutrient availability and composition, or some combination of these. The significant effect of site for O_2^- production and protein, as well as the interactive date * site effects for other characteristics, emphasizes the need to consider local effects in assessing defense activities, even for closely situated sites with similar temperature regimens.

The effect of local conditions may be better evaluated if sampling is conducted with greater frequency than this study; many important short-term effects of site-specific factors may be impossible to detect with a monthly monitoring scheme. Similarly, we emphasize that the measurements described here represent only a single year with unique and specific physical, chemical, and biologic factors. Reasonable interpretations and generalizations on oyster defense variability will have to be made with higher frequency, longer term studies performed at several geographic locations.

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A RETROSPECTIVE TIME SERIES ANALYSIS OF OYSTER, *CRASSOSTREA VIRGINICA*, RECRUITMENT (1946–1993)

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ABSTRACT Temporal patterns of eastern oyster, *Crassostrea virginica* (Gmelin 1791), spatfall in the Virginia tributary rivers to the Chesapeake Bay showed a decline in all rivers from 1946 through the early 1970s, with a subsequent leveling off. The decline was most severe in the James and less so moving north to the York and Rappahannock Rivers; it was least severe in the Potomac River. Yearling patterns generally mirrored the spat. Cluster analyses grouped the bars naturally by up- and downriver spatfall patterns. They also clustered this way when between-river comparisons were made. Spatfall showed a significant cross-correlation with yearlings a year later in all Virginia rivers, which suggests that the "yearling" designation was accurate and that spat counts may be used to predict yearling abundance. The relation of spat to later seed was significant for the James River at 2 and 3 y, but none was found between spat and market oyster. James River seed demonstrated a slightly significant relation to market oyster 4 y later. Regression analyses between spat counts and spring and summer water temperatures and river discharge produced little explanation of spat variation. There was, however, a significant relation between spat count and the Palmer Drought Index. The drought index is a combination of rainfall, soil type, and evapotranspiration. When the period of the greatest change in the drought index was correlated with spatfall, there was found to be a significant 2- to 4-y lag. We suggest that this reflects a response by the ecosystem to changing environmental conditions.

KEY WORDS: Oyster, *Crassostrea virginica*, recruitment, spat, yearling oyster

INTRODUCTION

The Chesapeake Bay estuarine system has, since colonial times, produced the highest harvest of oyster, *Crassostrea virginica* (Gmelin 1791), in the United States. These harvests reached a peak during the late 1880s, when Maryland and Virginia annually produced some 20,000,000 Bu (Hargis and Haven 1988). After the turn of the century, the landings declined; then, after the early 1960s, there was a dramatic decline, primarily on the private leased bottoms in Virginia's higher salinity waters of the lower bay. The cause(s) of the decline has been a major focus of many studies (reviewed by Richkus et al. 1992) and recommendations (Haven et al. 1978, Newell and Barber 1992).

The Virginia Institute of Marine Science (VIMS) has, since 1946, collected abundance data on both weekly and annual spatfall and annual yearling oyster abundance on the public rocks ("Baylor Survey"). This brackets the time when the oyster stock in the Chesapeake Bay declined dramatically. Although there have been numerous studies over the years examining the spatfall results of VIMS (Haven and Fritz 1985), none have examined them in their 45-y entirety. Further, most studies have not taken advantage of recent advances in time series analyses. Chai (1988) investigated the spat and market oyster relationship in Maryland's rivers using time series analysis (autoregressive integrated moving average, ARIMA), but no such analysis has been performed on Virginia's stock.

Virginia commits significant resources to the annual monitoring of the spatfall, yearling oyster, and market oyster abundance on the public rocks. Data on spatfall are collected during the summer on shellstrings and again in the fall as surviving spat-on-shell, but there has been no systematic examination of the spat relation to yearling or market oysters by use of time series analyses. Management agencies (ASMFC, MAFMC, PRFC, and VMRC) use juvenile indices as predictors of later life stages and/or adult brood stock as the "spawner" in spawner-recruit relationships (Richkus et al. 1992). Although this has been established and tested for many finfish species, there still remains the validation of spatfall as a predictor of later oyster abundance. The Chesapeake Bay Stock Assessment Committee (CBSAC) has outlined the need for an examination of the indices of oyster recruitment (CBSAC 1988). Further, the Chesapeake Bay–wide (Maryland and Virginia) bistate oyster management plan (CEC 1994) cites the need for an analysis of the relationship between the abundance of juvenile and subsequent life stage oysters. The objectives of this study are to (1) describe deterministic and stochastic fluctuations in spatfall and yearling oyster abundance on Virginia's public oyster rocks; (2) correlate spatfall with subsequent yearling, seed, and market counts; (3) run cluster analyses of spat between oyster rocks and rivers; and (4) examine possible physical environmental forces that may drive the fluctuations in spat abundance.

MATERIALS AND METHODS

The VIMS oyster program has collected data on spatfall since the summer of 1946. Counts are made weekly of spat on summer shellstrings and late fall spat-on-shells and live oyster dredged from the bottom. Shellstrings are hung cup side down in the water column at representative public oyster rock (Fig. 1) for a week at a time starting in June each summer. They are removed, and spat that have "struck" on the smooth lower surface are counted. Fall surveys incorporate counts of spat-on-shell from a Virginia bushel of shell collected by standard oyster dredge. Also counted during the fall survey are yearling, "small" oyster (<3", 7.62 mm), and market oyster. All data are stored in the VIMS Fisheries Data Management Unit and are available by request.

DATA TRANSFORMATION:

Biologic count data are frequently skewed. A logarithmic transformation produces data that are more nearly normally dis-

VIMS Contribution No. 2032.

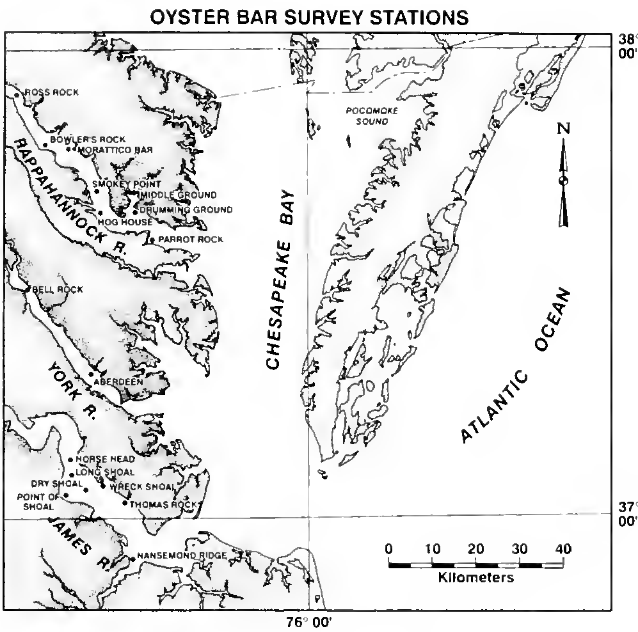


Figure 1. Locations of Virginia Chesapeake Bay Oyster Reefs.

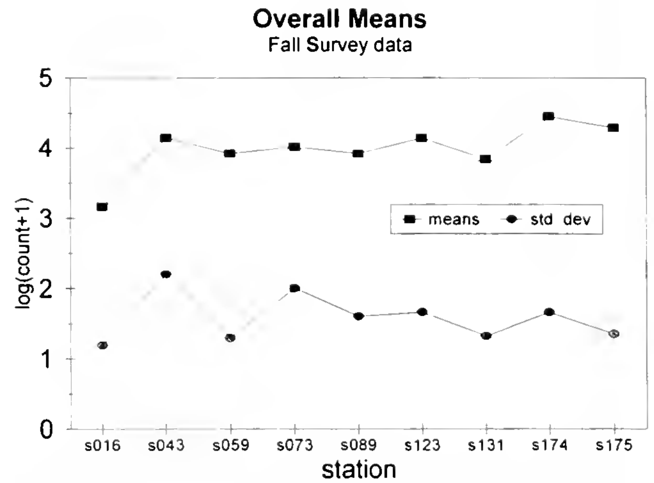


Figure 3. Overall means and standard deviations of fall survey data, 1946–1995.

tributed and allows the use of standard statistical procedures and inferences. The transformation used here is of the form log(X + 1). We plotted the means and standard deviations over time of the logarithmically transformed data. Figures 2 and 3 show the overall means and standard deviations for a group of stations and demonstrate the interstation variation for the shellstring data and the fall survey data, respectively (1946–1993). For both sets of data in the James River (shellstring and fall survey), the means are relatively constant from station to station, as is the standard deviation. This may be an indication that the time variation at stations in the James show a degree of synchronicity.

Shellstring data are sporadic during the period from 1947 to 1952, nonexistent for many bars through 1963, and fairly complete from that date through the present. Even so, however, only three or four bars have an unbroken record from 1963 to 1993. The fall survey continuity for spat-per-bushel and larger oysters is better, and the length of the series is longer (1946 to present). Following our examination of the length and completeness of the records, it was decided to focus on the fall surveys and not to consider the shellstring data. Fall yearling data were also available for all of the oyster rocks from which we had spat data.

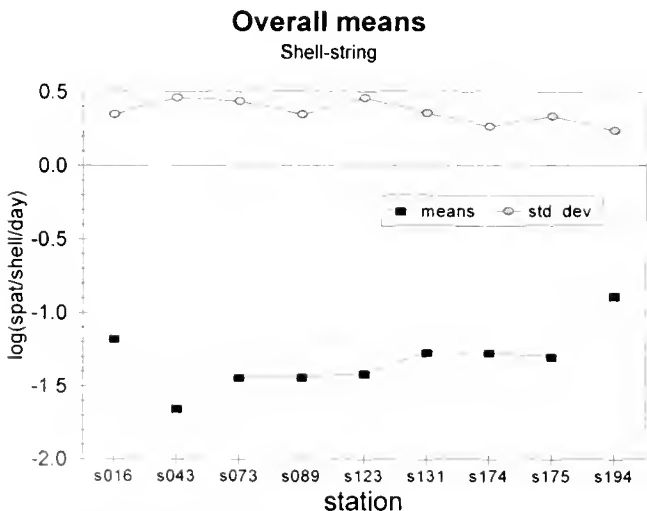


Figure 2. Overall means and standard deviations (std. dev.) of shell-string data, 1946–1995.

The length of the time series varied widely from station to station, ranging from almost continuous coverage since 1947 to measurements made in only a few years. It was decided to select a group of stations that contained the most usable information. The data were originally organized in computer files, with each file containing the total time series of observations for a single station. Each line in a file contained the following data items: year, the log transform of the spat count, the log transform of the yearling count. As noted above, the length of the time series varied considerably from station to station. Because the formats of all files were identical, the size of the file in bytes is a good measure of the quantity of information contained, i.e., the number of years for which data were collected at that station. Time series analyses require sequences that should be at least about 30 points in length and that are continuous, that is, have no gaps. The total size of all 67 data sets was 67,665 bytes. One-half of the information was contained in 14 data sets, with sizes ranging from 2,772 to 1,953 bytes. It was determined that these stations should be subjected to detailed analysis. Most of the selected stations are fall survey data. A further six files were over 1,500 bytes in size. Although Chai (1988) used an ARIMA to fill in missing data for Maryland spatfall, and Austin et al. (1993) used a linear extrapolation for Virginia oyster condition indices, the annual variability of our data is such that neither method is generally applicable, although in a few cases, a 1- or 2-y interpolation was appropriate. The quantity of data aggregated over all stations consists of 1,009 yearly measurements, although not all years have both spat and yearling data. Of this total amount of information, the stations selected for analysis comprise 518 yearly data values organized into 14 time series, all of which exceed 30 y in length.

Data for landings (seed and market) for the James River (1963 to present) were obtained from the Virginia Marine Resources Commission, as were the number of boat days (1983–1995). Spatfall data for the Potomac River were obtained from Chai (1988) for 1940–1985.

All station data and the yearly environmental data (tempera-

ture, river flow, and Palmer Drought Index [PDI]) were incorporated into a QUATTRO PRO FOR WINDOWS® spreadsheet. Most of the analyses were run with QUATTRO or MINITAB® for WINDOWS. Where appropriate, the following statistical analyses were used: Pearson correlation coefficient, linear regression (including multiple regression), cross-correlation, agglomerative cluster analyses, *loess* smoothing, differencing.

It is appropriate to make a comment here on the use of the *loess* procedure. Almost all of the time series in the data set show features with different timescales, short-term fluctuations from year to year that are superimposed on long-term trends that span decades. These features are separated by smoothing the data to produce the trend; the signal with a shorter term variation is produced by subtracting the trend from the original data. A common technique for smoothing is a moving weighted average filter. This procedure has the disadvantage that the smoothed series is necessarily shorter in duration than the original. The degree of smoothing necessary for the data in this study would require the order of the filter (the number of points averaged together) to be so high as to cause a severe loss of data at the extremities of the time series. Another common technique, recursive filtering, also has similar end effects, as well as imparting a phase shift to the data. The smoothing technique initially known as LOWESS (LOcally WEighted Scatterplot Smoother) (Cleveland 1979) does not suffer from these problems and in addition is robust (is not unduly affected by outliers). This smoothing procedure is sufficiently well accepted to have been incorporated into a number of well-known statistical packages, including SPSS and Minitab. The method does not lend itself to a simple formulaic statement, and neither can it be described in a single paragraph; consequently, a more detailed description is presented in an appendix. The originator of the method, W. S. Cleveland, has renamed the procedure "*loess*" (Cleveland 1993); this usage will be adhered to in all subsequent references to the method.

RESULTS AND DISCUSSION

General Temporal Patterns

Plots of the mean spatfall and yearling counts for each river were generated and inspected visually. In many cases, there were insufficient or incomplete data at any given bar or reef to maintain the time series. By combining them, however, and developing a mean annual index for each river, a robust data set was generated. Initially, we attempted to use a 5-point moving average to examine both long-term trends and periodic bay-wide cycles. The *loess* procedure, however, provided a better representation of a combination the 5-y moving average and the long-term trend. Consequently, we used *loess* as implemented in MINITAB. Although there were some area-wide coherent events, such as the droughts of the mid-1960s and early to mid-1980s, and a general post-1960s decline, spatfall in the four rivers exhibited a temporally independent pattern of set.

James River

The 0.2 degree of smoothing *loess* filter for spatfall in the James River showed a stable period before 1960 and then a period of decline (1960–1975). The later decade (1966–1975) of this decline was characterized by wide interannual variation. Although after 1975 there was a period of renewed set (Fig. 4a), it never returned to the pre-1960 levels. The long-term trend, revealed by the 0.7 smoothing filter, depicts a long-term decline from the mid-1940s through the early 1970s, followed by a leveling off of the decline. Spatfall ranged from five to eight spat per bushel during the pre-1960 decline, one to five during the 1960s–1970s, and three to six during the late 1970s and 1980s. Patterns of yearling abundance mirrored the spat (Fig. 4b), although the decline during 1950 to the early-1970s is more pronounced. Yearling dropped from a high of around 6 per bushel to a variable number of around 1.5–5/Bu after the decline.

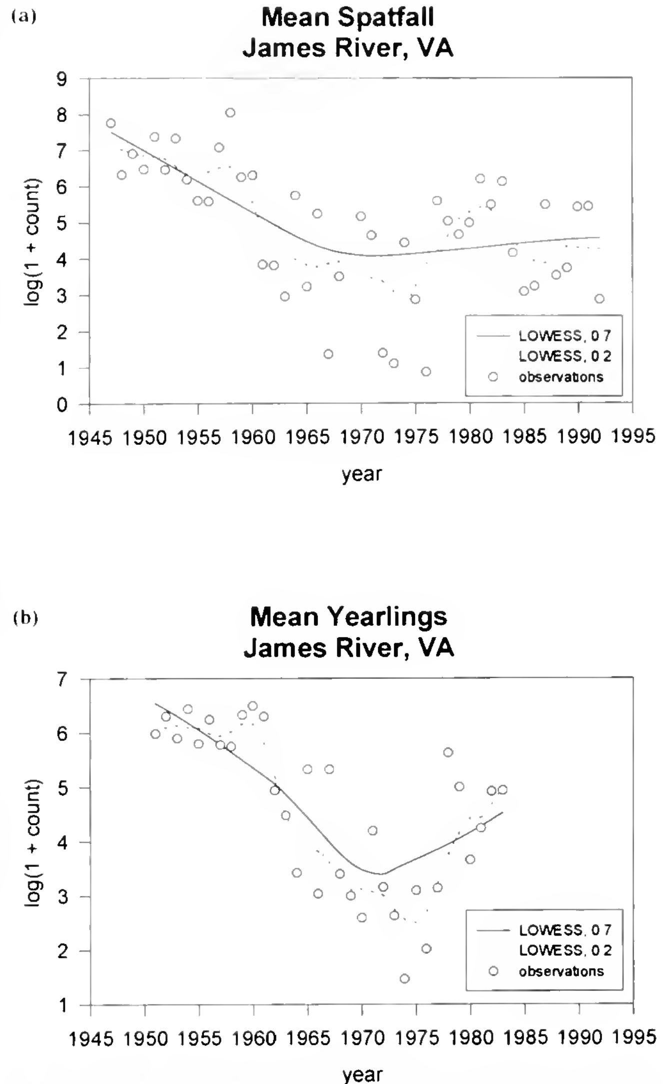
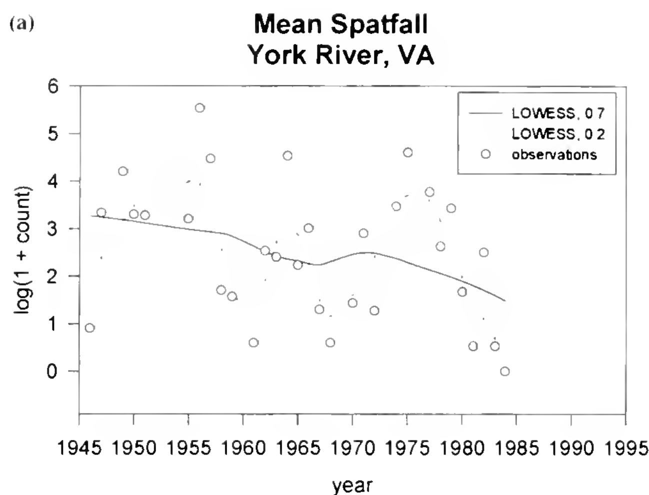


Figure 4. (a) Mean spatfall (number of spat-on-shell per bushel), James River, VA, 1946–1992; *loess* filters at the 0.2 and 0.7 degrees of smoothing. (b) Mean yearlings (number per bushel), James River, VA, 1948–1983; *loess* filters at the 0.2 and 0.7 degrees of smoothing.

York River

The empirical York River (Fig. 5a) spat-on-shell data exhibit no obvious pattern of set, although the *loess* 0.7 smoothing filter suggests a steady decline between 1950 and 1990, interrupted at 7- to 8-y intervals. Wide interannual fluctuations are apparent from 1946 through 1970. The 7- to 8-y periodic cycle is strikingly similar to the pattern in condition index described by Austin et al. (1993). The yearling oyster abundance (Fig. 5b) in the York River



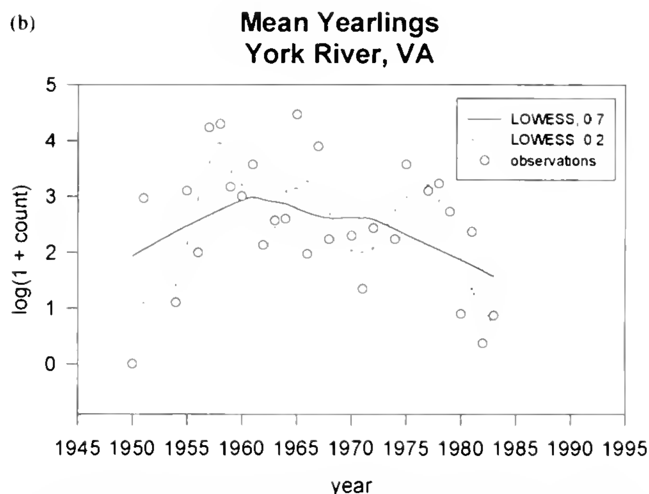
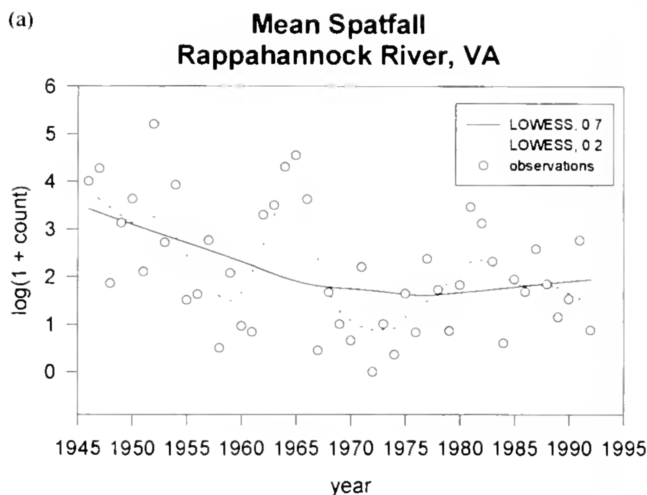


Figure 5. (a) Mean spatfall, York River, VA, 1946–1992, *loess* filters at 0.2 and 0.7 degrees of smoothing. (b) Mean yearlings, York River, VA, 1950–1982, *loess* filters at 0.2 and 0.7 degrees of smoothing.



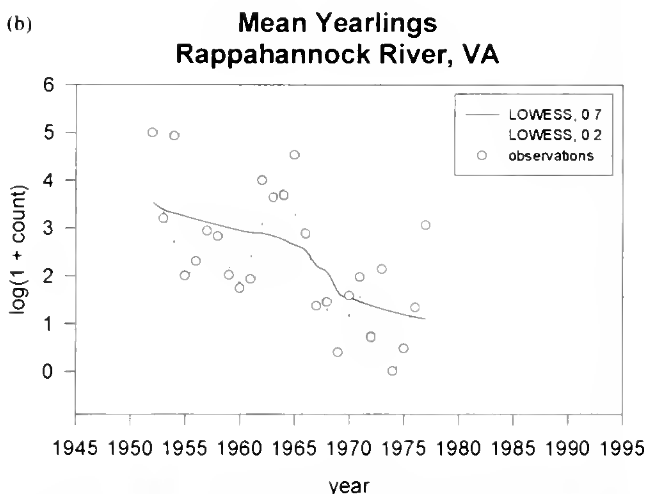


Figure 6. (a) Mean spatfall, Rappahannock River, VA, 1946–1992, *loess* filters at 0.2 and 0.7 degrees of smoothing. (b) Mean yearlings, Rappahannock River, VA, 1946–1977, *loess* filters at 0.2 and 0.7 degrees of smoothing.

exhibit peaks around 1957–1958, 1965 and 1975, and followed a general pattern similar to that of the spat.

Rappahannock River

The spat pattern in the Rappahannock (Fig. 6a) shows a degree of coherence with the James: high values (two to five) but quite variable before 1955, with a decline through 1961 (less than one), then a significant "recovery" (greater than three) during the mid-1960s drought, a return to poor set (one to two) by 1970, and finally, a slight increase through 1990. There is also a short response (1981–1983) to the drought during the early 1980s. The yearling abundance patterns parallel that of the spat, exhibiting a decline from 1950 through the early 1970s, followed by a slight recovery (Fig. 6b).

Potomac River

The Potomac spatfall (Fig. 7) has remained fairly constant since 1950, with short 1- to 3-y responses to the droughts in the 1960s and 1980s. The *loess* filters show no "1960s decline," only a moderate increase during the 1960s drought, and a subsequent decline through the early 1970s. It is possible that the decline from 1950 through 1972 was interrupted and partially masked during the drought. An apparent recovery is seen from the early 1970s through 1985.

Interreef and Intrareef Coherence

The coherence of the cumulative abundance of annual spatfall patterns was examined between oyster reefs within river and between rivers by use of the MINITAB Agglomerative cluster analyses and Pearson correlation. The analyses were run on James, Rappahannock, and Potomac River reefs. There was an insufficient number of either reefs or unbroken data strings of sufficient length in the York to allow comparisons in that river.

The degree to which two time series exhibit the same features of temporal variation can be measured with the simple Pearson correlation coefficient between the two sets of data. Correspondingly, a visual comparison can be made from a scatterplot where each year is represented by a point, the (x, y) coordinates of which are given by the respective observations at the two stations. If the series from the two stations are approximately synchronous, the scatterplot will show the pattern associated with two well-correlated variables.

These more complex relationships are conveniently explored

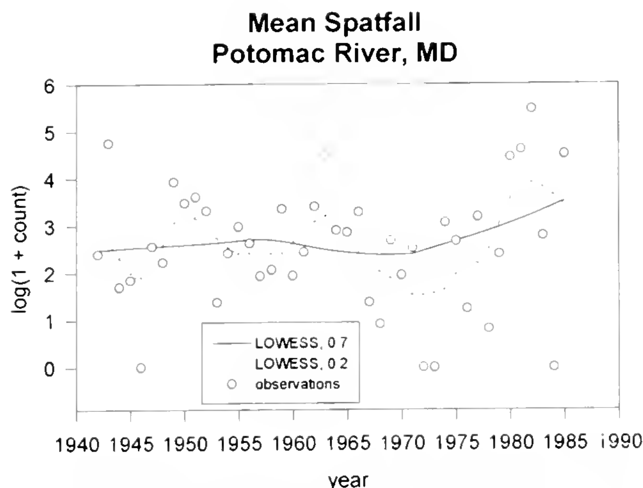


Figure 7. Mean spatfall, Potomac River, MD, 1942–1986, *loess* filters at 0.2 and 0.7 degrees of smoothing.

by use of the cluster analysis technique. The starting point is the calculation of a distance matrix, D, the elements of which, d_{ij}, are given by:

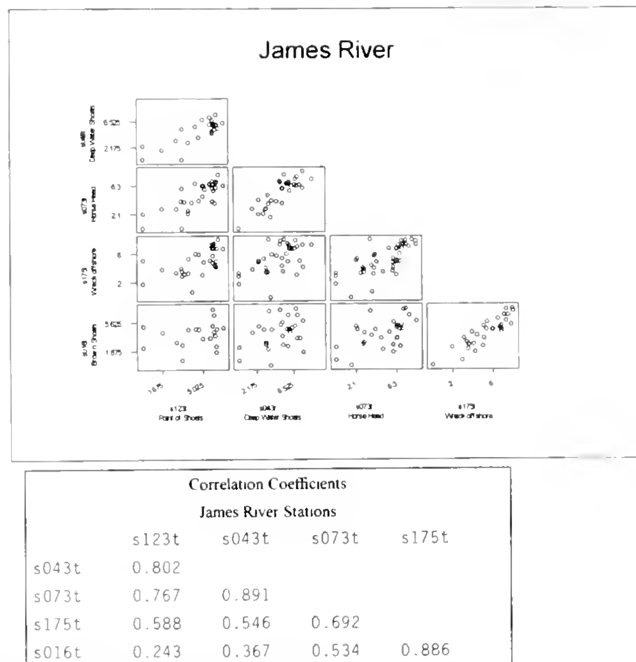
$$d_{ij} = 1 - r_{ij}$$

where r_{ij} is the correlation coefficient between stations *I* and *j*. The closest pair of stations, using this distance measure, is combined into a single cluster. The distance matrix is recalculated for the new set of stations (now one less in number), and the closest pair are combined into a cluster. The process can be repeated until a single cluster is produced. The results are best displayed in a dendogram, as shown, for example, in Figure 9, which clearly demonstrates the clustering hierarchy and the presence of two distinct groups of stations with regard to their temporal structure of spatfall counts.

James River

Figure 8 is a matrix of scatterplots for all 10 possible pairs of the five stations in the James River. The corresponding correlation coefficients are shown in a parallel matrix representation. It is seen that the highest correlation of 0.891 (d_{ij} = 0.109) occurs for the Horse Head–Deepwater Shoals pairing, with a slightly smaller value, 0.886, for the pair Wreck Shoals–Brown Shoals (d_{ij} = 0.114). A third station, Point of Shoals, also shows a high correlation of 0.802 (d_{ij} = 0.198) with one of the first pair, Deepwater Shoals, and a slightly less value, 0.767 (d_{ij} = 0.233) with the other station of the first pair, Horse Head.

The cluster analysis (Fig. 9) shows that James River stations fell into two groups. Stations along the southwest shore (Group I), Deepwater Shoals and Horse Head, demonstrated a high degree of similarity (95), as shown in the previous paragraph. These two were also similar to Point of Shoals (90). A second similarity grouping (Group II) was exhibited between Wreck Shoals and Brown Shoals, but at a lesser degree of similarity (88). This second group included the stations along the northeast shore. The same rankings and similarity were also independently described by the Pearson correlation coefficients (Fig. 8). Haven and Fritz (1985), looking at the synchrony of setting pulses in the James, found identical groupings of reefs for Groups I and II; Austin et al.



Correlation Coefficients

James River Stations

	s123t	s043t	s073t	s175t
s043t	0.802			
s073t	0.767	0.891		
s175t	0.588	0.546	0.692	
s016t	0.243	0.367	0.534	0.886

Figure 8. Cross-correlation matrix for spat on James River oyster reefs.

(1993), looking at oyster condition indices, found the same Group I and Group II classifications.

Rappahannock River

The results of a similar analysis on the stations in the Rappahannock River are shown in Figures 10 and 11. Downriver and midriver reefs, Smokey Deep and Hog House, were closest in resemblance (d_{ij} = 0.253), followed by their grouping with Drumming Ground (0.386). Upriver reefs, Morattico and Bowlers Rock, were independent and showed no similarity to other reefs.

James Versus Rappahannock

When the stations from both rivers were analyzed together, the same groupings emerged (Figures 12 and 13). Southwest shore James (Group I) reefs, Deepwater Shoals and Horse Head, formed their own similarity cluster, including Point of Shoals in the James. A second, more diverse group was composed of mid- and

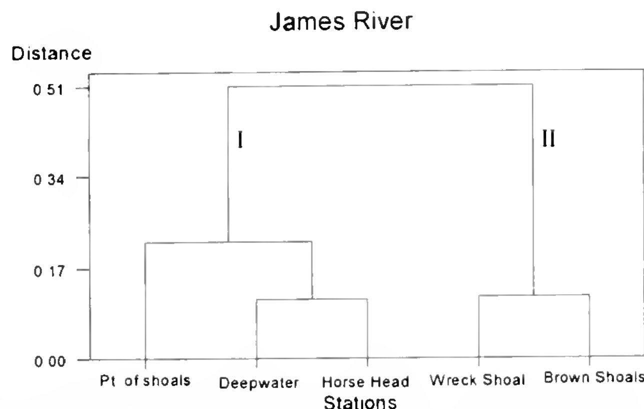
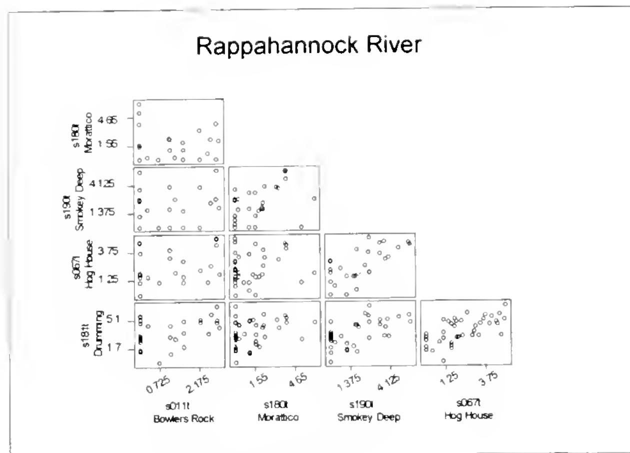
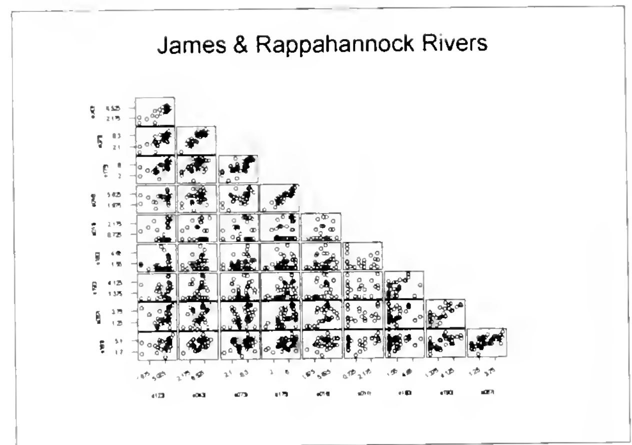


Figure 9. Agglomerative cluster analysis of oyster reefs, James River. Pt., Point.



Correlation Coefficients Rappahannock River Stations				
	s011t	s180t	s190t	s067t
s180t	0.084			
s190t	0.307	0.533		
s067t	0.404	0.266	0.747	
s181t	0.382	0.297	0.589	0.638

Figure 10. Cross-correlation matrix for spat on Rappahannock River oyster reefs.



Correlation Coefficients James & Rappahannock Rivers								
	s123t	s043t	s073t	s175t	s016t	s011t	s180t	s190t
s043t	0.802							
s073t	0.767	0.891						
s175t	0.588	0.546	0.692					
s016t	0.243	0.367	0.534	0.886				
s011t	0.170	0.080	0.039	0.194	0.125			
s180t	0.175	0.261	0.220	0.149	0.129	0.084		
s190t	0.305	0.352	0.243	0.410	0.349	0.307	0.533	
s067t	0.161	0.307	0.247	0.474	0.471	0.404	0.266	0.747
s181t	0.383	0.425	0.318	0.450	0.333	0.382	0.297	0.589

Figure 12. Cross-correlation matrix for spat on James River and Rappahannock River oyster reefs.

lower-Rappahannock and lower-James (Group II) reefs. This included Smokey Point Deep (Rappahannock) and Wreck Shoals (James); Hog House (Rappahannock), Brown Shoals (James), and Drumming Ground (Rappahannock). The upper-Rappahannock reefs, Morattico and Bowlers Rock, again demonstrated no coherence with either other Rappahannock or James River oyster reefs.

Potomac River

The stations in the Potomac River grouped by distance from the mouth of the river. Not all stations had sufficient data and were rejected by the cluster analysis. Figures 14 and 15 show the composition of the similarity groups. Popes Creek, the only station north of the Route 301 bridge, grouped with the "midriver" stations (Cobb Island, Cedar Island, Heron Point, and Swan Island). The three downriver stations, Jones, Ragged Point, and Cornfield Harbor, were grouped, with Ragged and Cornfield being the most similar.

All Rivers

When the cluster analysis was run on the two James groups (I and II), mean Rappahannock, and six Potomac stations, several new groups aligned (Figs. 16 and 17). The two James groups clustered with Jones Shoal, Potomac River, and the mean of the Rappahannock stations clustered with the midriver Potomac. Cornfield Harbor, at the mouth of the Potomac River, did not group with any other station(s).

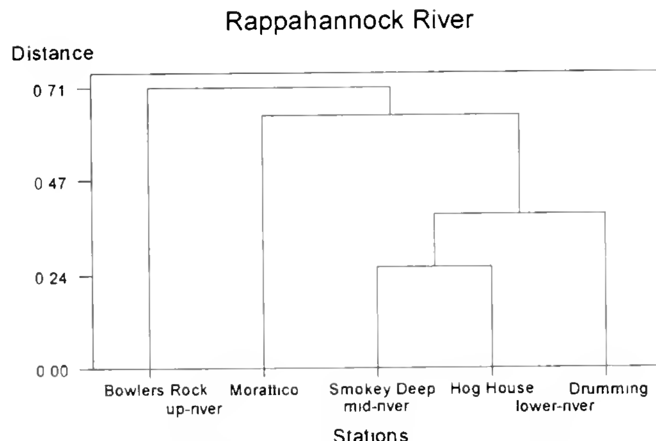


Figure 11. Agglomerative cluster analysis of oyster reefs, Rappahannock River.

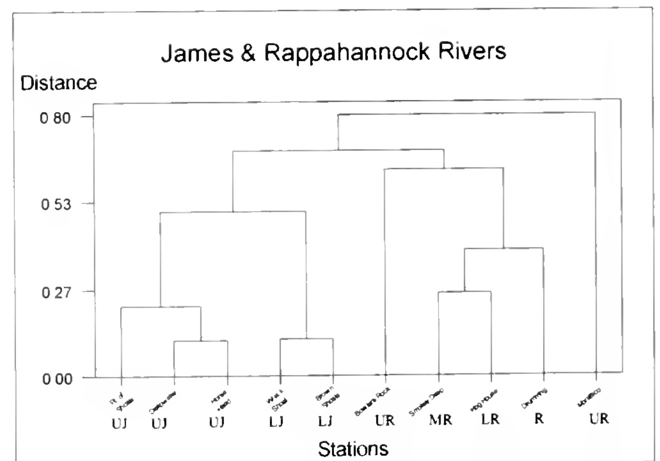
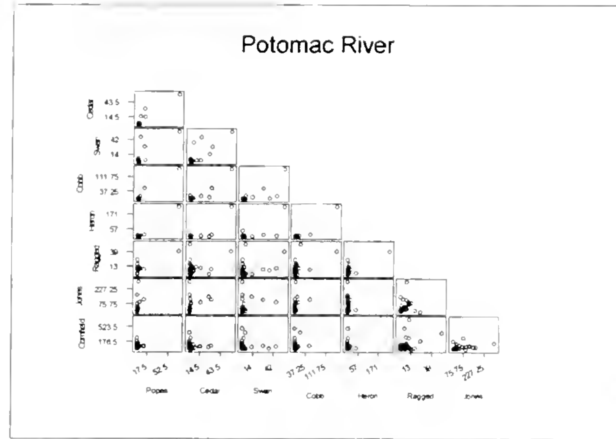


Figure 13. Agglomerative cluster analysis of oyster reefs, James and Rappahannock Rivers.

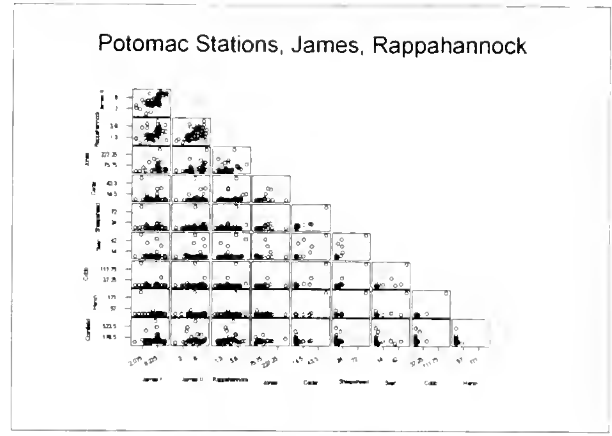


Correlation Coefficients

Potomac River

	Popes	Cobb	Cedar	Heron	Swan	Jones	Ragged
Cobb	0.979						
Cedar	0.930	0.913					
Heron	0.993	0.953	0.821				
Swan	0.759	0.747	0.787	0.693			
Jones	0.309	0.258	0.303	-0.125	0.210		
Ragged	0.764	0.550	0.348	0.737	0.337	-0.037	
Cornfield	-0.037	0.090	-0.078	0.122	-0.125	0.253	0.400

Figure 14. Cross-correlation matrix for spat on Potomac River oyster reefs.



Potomac Stations, James, Rappahanock

Correlation Coefficients

	James I	James II	Rapp	Jones	Cedar	Sheepshead	Swan	Cobb	Heron
meanII	0.621								
meanrapp	0.354	0.458							
Jones	0.151	0.436	0.201						
Cedar	0.197	0.093	0.275	0.303					
Sheepshead	0.284	0.067	0.266	0.333	0.920				
Swan	0.203	0.148	0.245	0.210	0.787	0.776			
Cobb	0.261	0.046	0.200	0.258	0.913	0.964	0.74[illegible]		
Heron	0.330	0.043	0.192	0.125	0.821	0.932	0.693	0.953	
Cornfield	0.053	0.072	0.136	0.253	0.078	0.079	0.125	0.090	0.122

Figure 16. Cross-correlation matrix for spat on James, Rappahannock, and Potomac oyster reefs.

Relationship Between Spat and Subsequent Cohort Stages

Counts of spatfall have been maintained by Virginia and Maryland since 1946. The original purpose of the spatfall monitoring in Virginia was to provide the state's oyster growers with information on the location and timing of peaks in spatfall to allow them to broadcast shell to receive best the annual "strike." Over the years, and after the prolonged decline in market oyster landings that coincided with the decline in spat abundance, the annual spatfall report became a forecast for the status of the Virginia oyster harvest (e.g., Barber 1991). This relationship was never documented.

Spat Versus Yearling

The relationship between spat and subsequent cohort stages can be conveniently investigated by obtaining the cross-correlation function (ccf) for the spat and the yearling times series. Figure 18a shows the ccf for the James River data. The structure in the function is primarily due to the long-term trend in the data. It appears that, superimposed on this background, there is an enhancement at a lag of 1 y, indicative of the expected relationship between spat density and yearling density in the following year. This relationship is revealed more clearly by removing the long-term trend from both data sets and computing the ccf of the residuals (Fig. 18b).

The long-term trends are estimated by use of the *loess* smoothing technique. The ccf of the residual series is shown in Figure 18b. It is seen that there is a significant correlation between the series when lagged by 1 y (i.e., spat in year t are compared with yearling in year $t + 1$) and that the correlation does not extend

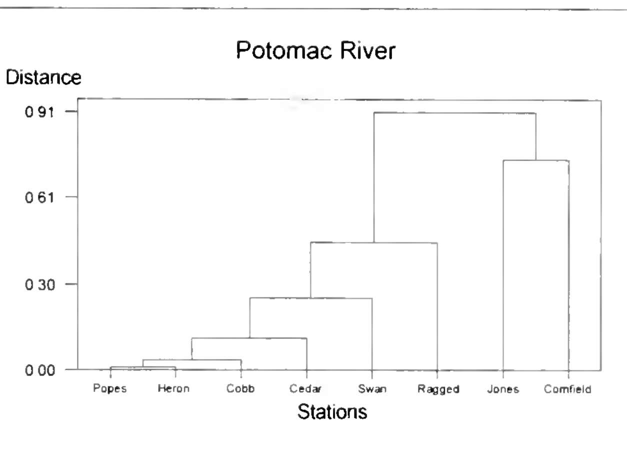


Figure 15. Agglomerative cluster analysis of oyster reefs, Potomac River.

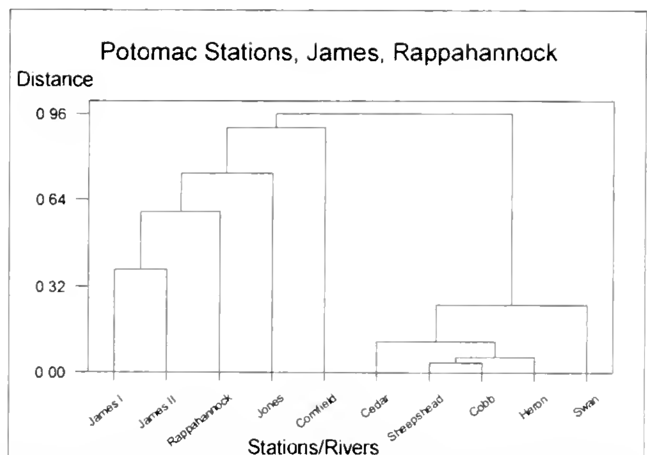


Figure 17. Agglomerative cluster analysis of oyster reefs, James, Rappahannock, and Potomac Rivers.

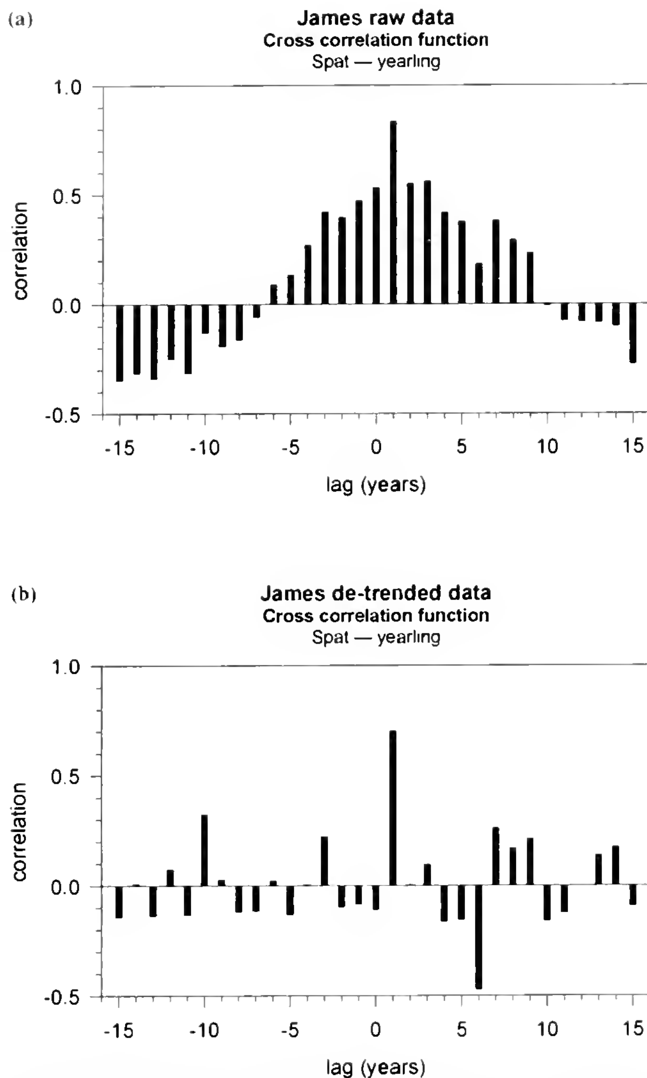


Figure 18. (a) ccf for James River spat and yearling. (b) ccf for James River spat and yearling (detrended).

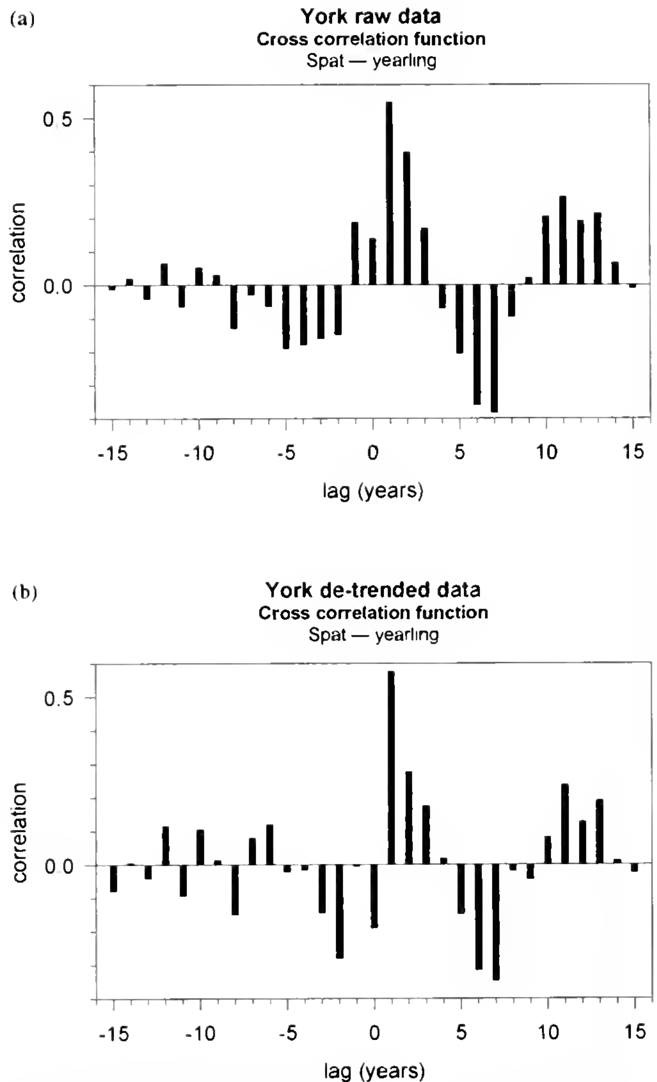


Figure 19. (a) ccf for York River spat and yearling. (b) ccf for York River spat and yearling (detrended).

beyond 1 y. This can be considered to be a confirmation of the accuracy of designating "yearlings."

Similar results are found in the York and Rappahannock Rivers. The ccf values for the raw and detrended data, respectively, are shown in Figure 19 for the York River and Figure 20a and for the Rappahannock River. In both rivers, there is a significant ccf at a lag of 1 y.

Having established the presence of a 1-y lag relationship between spat and yearling with no other lags having a significant effect, one may use linear regression between the mean spatfall values for each river, lagged a year, and the mean yearling data. This relationship accounted for a significant percentage of the variation in the James (R^2 = 0.73; Fig. 21a), roughly half in the Rappahannock (R^2 = 0.48, Fig. 21b), and less than 15% in the York (R^2 = 0.14, Fig. 21c).

The disparity in the coefficient of determination between rivers may be explained in several ways. The James oyster reefs are located in a more geographically compact area, but with a diverse environment strongly influenced by the gravitational circulation (salinity driven deeper "salt intrusion"; Pritchard 1952), which results in a retentive circulation pattern in the lower river (Kuo et al. 1990). It has also been suggested that the proximity of the lower James to the ocean provides a "healthier" environment than the up-bay tributaries (Kuo and Neilsen 1987). Rappahannock spat survival may be less because of summer and late fall hypoxia (Officer et al. 1984, Kuo and Neilsen 1987). Spatfall in the James has a higher chance of retention, survival, and reaching the yearling stage, whereas survival in the Rappahannock and York is less. Further, repletion efforts (shelling the bottom and seed planting) in these river have been shown to affect results (Ulanowicz et al. 1980, Chai 1988).

James River Spat Versus Seed

The logical progression for predicting future harvest, with a significant spat-yearling relationship, would be to examine the yearling-seed relation. This was not possible, however, because of the deficiency in the length of the yearling data collection period, which ended in 1984. Further, catch per unit effort (CPUE) data for seed and market oyster are not available until 1983, and then only for the James. This allows only a 1-y overlap, in only one river. Consequently, we examined the James River spat-seed/day

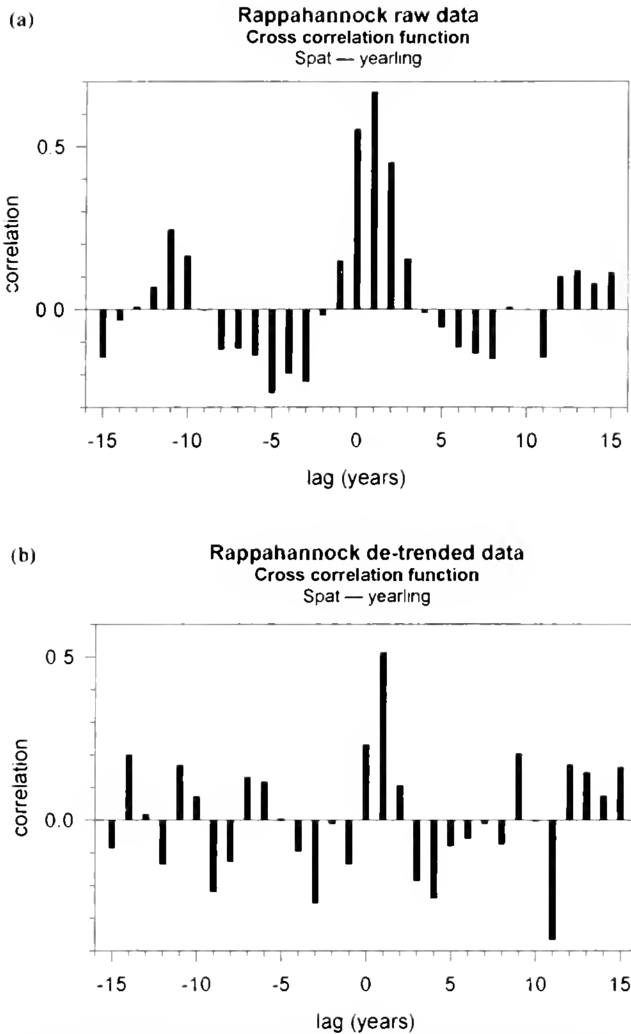


Figure 20. (a) ccf for Rappahannock River spat and yearling. (b) ccf for Rappahannock River spat and yearling (detrended).

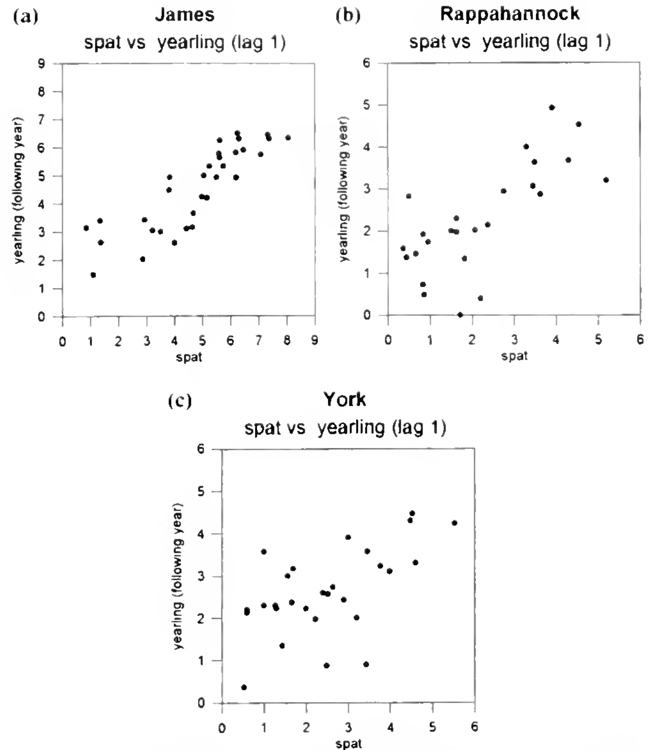


Figure 21. (a) Regression of James River spat versus yearling (lag 1 y). (b) Regression of Rappahannock River spat versus yearling (lag 1 y). (c) Regression for York River spat versus yearling (lag 1 y).

relationship. Unfortunately, the short time period of the CPUE data prevented reliable differencing, or detrending, so analyses were conducted with the trend present in the data. Spat and yearling data were collected by fishery-independent surveys, seed from fishery-dependent commercial harvest data reported to the VMRC by watermen.

Pearson correlations were run between log(seed/day) and the spat value lagged 1 through 4 y as a mean of narrowing the field of observations for subsequent regression analyses. Significant correlations were found at lags of 2 and 3 y (Table 2), and to a

TABLE 1.

Station data files (stations selected for analysis contain 50% of all available information).

Station	Size of File (bytes)	Cumulative (bytes)	River	Station Name
S073	2,772	2,772	James	Horse Head
S175	2,772	5,544	James	Wreck Offshore
S180	2,772	8,316	Rappahannock	Morattico Bar
S181	2,772	11,088	Rappahannock	Drumming Ground
S001	2,583	13,671	York	Aberdeen Rock
S067	2,583	16,254	Rappahannock	Hog House Bar
S190	2,457	18,711	Rappahannock	Smokey Point Deep
S043	2,268	20,979	James	Deepwater Shoals
S179	2,268	23,247	York	Bell Rock
S050	2,142	25,389	Piankatank	Ginney Point
S109	2,142	27,531	York	Pages Rock
S016	2,016	29,547	James	Brown Shoals
S123	2,016	31,563	James	Point of Shoals
S011	1,953	33,516	Rappahannock	Bowlers Rock

TABLE 2.

Pearson correlation coefficients and linear regression of James River spat versus seed/day.

Parameter	Pearson Correlations					Linear Regression	
	Log seed/day	Spat	Spat1	Spat2	Spat3	p	R^a (%)
Spat	−0.130						
Spat1	0.097	0.098					
Spat2	0.676	0.035	0.125				
Spat3	0.681	0.107	0.061	0.097			
Spat4	0.517	0.282	0.104	0.066	0.103		
Equations							
Log seed/day = 2.610 + 0.078 spat1						0.790	0.9
Log seed/day = 0.606 + 0.507 spat2						0.032	45.8
Log seed/day = 0.565 + 0.520 spat3						0.30	46.4
Log seed/day = 1.040 + 0.408 spat4						0.126	26.7

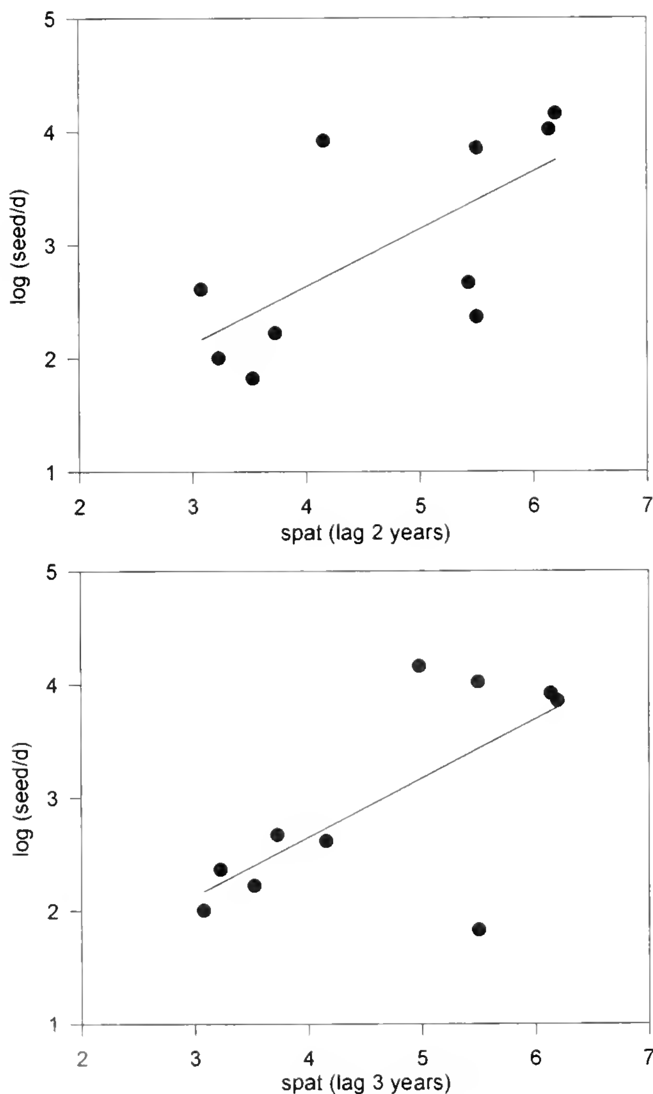


Figure 22. Regression for James River spat (lagged 2 and 3 y) versus James River market oysters.

lesser degree at 4 y. Regressions of seed/day and spat lagged 1 to 4 y were also run. The 2- and 3-y lag was found to be significant at the $p = 0.05$ level (Table 2; Fig. 22).

Spat Versus Market Oysters

The relationship between James River spat and subsequent years' market oyster landings was examined. As with the spat/seed analysis, only James River spat/market was examined because the James River landings are not "contaminated" by oyster repletion and because the spat and market/day are from the same river. The short CPUE data series (market/day) precluded differencing. Pearson correlations were run for spat against market/day 1–4 y later. None were significant (Table 3), although there was a negative correlation between market/day and spat 2 y earlier, which is probably an artifact of the short, nondetrended market data.

Krantz and Merritt (1977) found their best correlation between spat and commercial harvest at a lag of 6–8 y and cited this as further evidence to ". . . sustain the theory that a period of successive years of low spat set will require between six to eight years before the period of poor recruitment is reflected in the commercial harvest." Ulanowicz et al. (1982), using a multivariate analyses, found a correlation between spat and seed at a lag of 4 y, and using cross-correlation analyses, found a peak in the correlation between spat and commercial harvest at a 9-y lag. This period, they speculated, could be due to a ". . . possible natural oyster cycle . . ." or an ". . . unexplained environmental variable."

These 4-, 6-, 8-, and 9-y lags found by Krantz and Merritt and Ulanowicz et al. may be artifacts of the cross-correlation because ". . . interpretation of the sample cross-correlation function can be fraught with danger unless one uses the prefiltering procedure . . ." (Chatfield 1989). Neither study detrended the raw

TABLE 3.

Pearson correlation coefficients for James River market oysters/day versus spat lagged 1–4 y.

Lag Year	*r*
Spat1	−0.283
Spat2	−0.696
Spat3	−0.490
Spat4	−0.125

TABLE 4.

Pearson correlation coefficients and linear regression coefficients and equations for James River seed/day versus market/day.

Parameter	Pearson Correlations					Linear Regression	
	Log market/day	Seed1/day	Seed2/day	Seed3/day	Seed4/day	p	R^2 (%)
Seed1/day	−0.063						
Seed2/day	0.160	0.732					
Seed3/day	0.513	0.387		0.802			
Seed4/day	0.716	0.028		0.554	0.820		
Equations							
Log market/day = 1.53 + 0.238 log seed3/day						0.194	26.3%
Log market/day = 1.17 + 0.355 log seed4/day						0.071	51.2%

data, so it is quite possible that the 4- to 9-y lags that they found are artifacts of this lack of differencing.

Their multivariate analysis revealed that spat densities and seed planting accounted for 56% of the variation in commercial harvest. The removal of significant volumes of seed from the James River, and their transplantation to the Rappahannock and Potomac Rivers, has no doubt affected the statistical results of our spat versus seed and market analysis.

James River Seed Versus Market Oyster

The James River abundance of seed was analyzed relative to James River market oyster CPUE. Normally, one would expect that the market oyster catch is composed of several year classes or cohorts. This is true for the James (Mann, unpublished data); however, with the current level of fishing pressure, depleted stocks, small minimum size limit (3", 76.2 mm), and slow variable growth rates, it is likely that the commercial harvest, although composed of several year classes, is supported primarily by only a year class or two, most probably, age four. Nevertheless, Pearson correlations were run on log(market/day) by log(seed/day) lagged 1–4 y (Table 4). Because of the short overlap period with effort (boat days, 1983–1994), the data were not differenced. There was a significant correlation between market oyster and seed, lagged by 3 and 4 y (0.513, 0.716), and a slightly significant regression ($p = 0.071$, $R^2 = 51.2\%$) with seek laged 4 y (Table 3; Fig 23). This relationship appears to be fortuitous and is probably due to the strong downward trend in seed after 1985 and the pulse of market landings in the later 1980s, also followed by a dramatic decline.

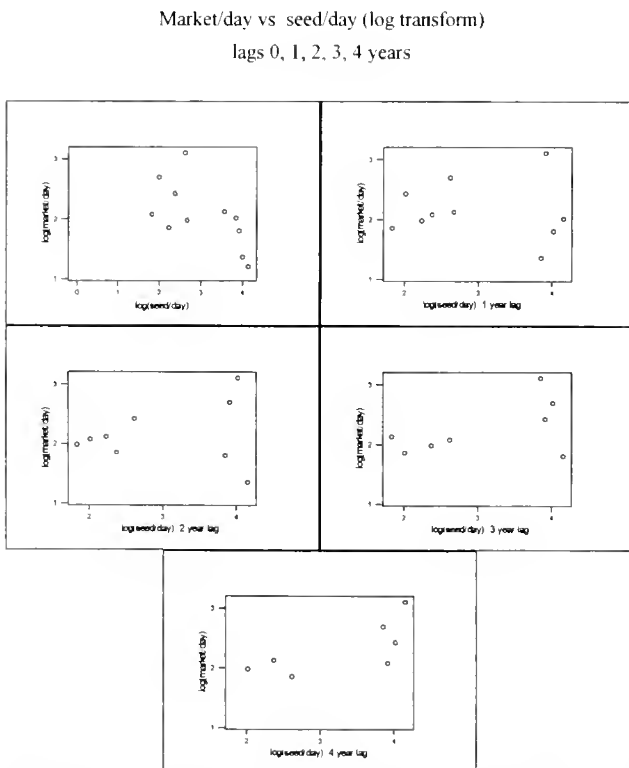


Figure 23. Regression for James River market/day versus lagged seed/day.

Predictions With Spat

Because spat (age "zero-plus") show a statistical relationship to seed (age two and three), intuitively, one might expect that there would be a relation between seed (2–3 y) and market oyster (age three and four) a year later. However, there is no significant relation between spat and any market size, and the seed-to-market relationship is between the age two and three seed and age six to seven market oyster.

It is our conclusion that spat abundance can be used to predict the abundance of subsequent yearling oyster abundance and can form the basis for a method of predicting abundance of seed

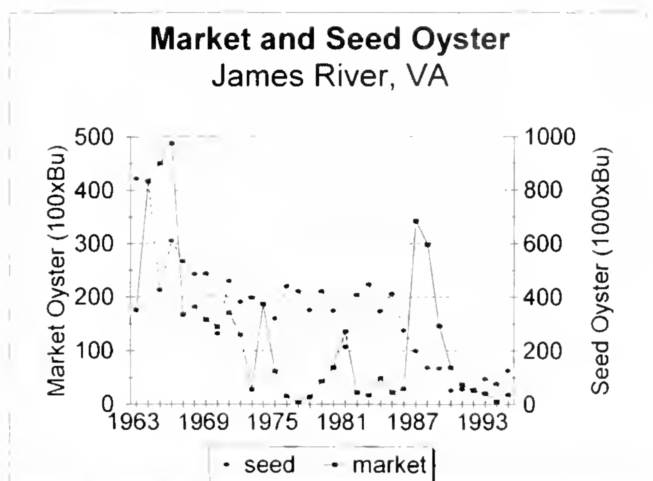


Figure 24. Total market and seed oyster harvest, James River, VA.

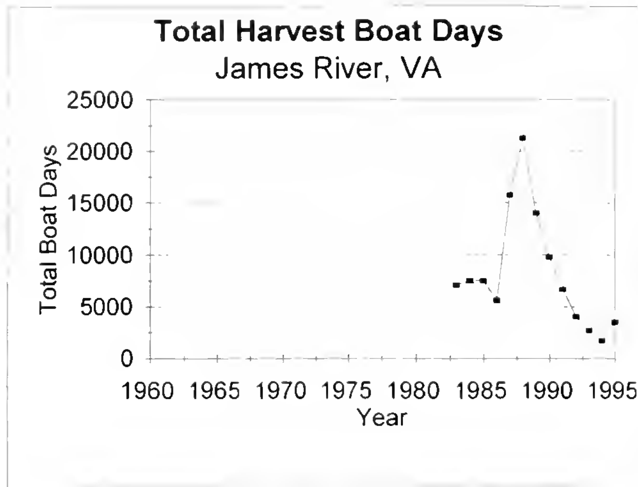


Figure 25. Number of boat days, James River, VA.

(CPUE) 2–3 y later. It does not appear, at this time, that spat can be used to predict future market oyster harvest. It may be that when the catch/day data set is longer, it will be possible to make a correlation after detrending. Further, although there is an apparent relation between seed CPUE and the market CPUE 4 y later, we feel that this may be due more to the overall trend of the data rather than to biologic cause and effect. Further, the multiple cohorts in the market catch and problems with CPUE data for seed and market oyster make this examination questionable. We will discuss the additional problems with seed and market data as to how they relate to CPUE when calculated with boat days. With this in mind, any examination of seed or market landings must be made with caution.

James River Seed and Market Harvest

The VMRC has maintained monthly harvest statistics since 1963 for seed, and market (currently, 3″, 76.2 mm) oyster, and since 1983 the number of boat days fished in the James. Figure 24 (Table 4) depicts the annual harvest of seed and market oyster in the James River since 1963. Figure 25 shows a dramatic increase

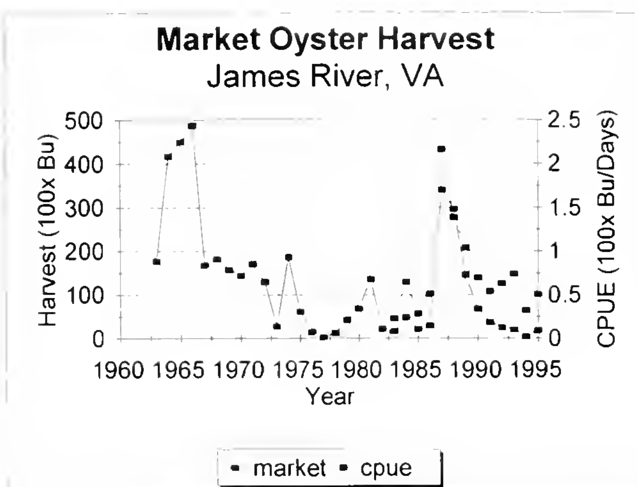


Figure 26. Total market harvest and CPUE harvest, James River, VA.

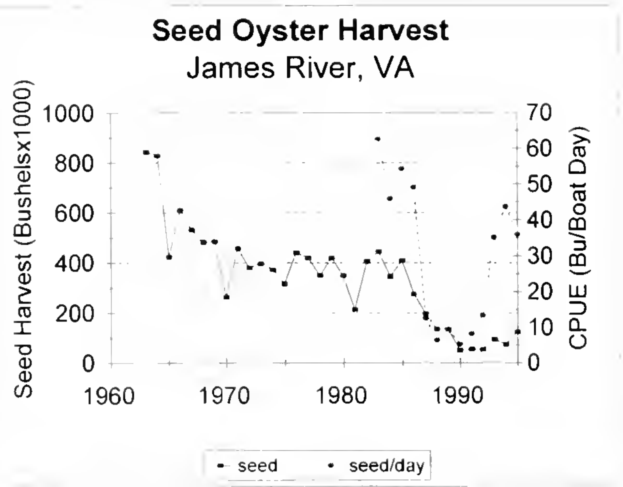


Figure 27. Seed oyster harvest and CPUE, James River, VA.

in boat days (effort) in the James through, and peaking in, 1988 and an equally dramatic decline thereafter. This variation is due to the scarcity of oyster bay-wide, except in the James, a subsequent migration of the watermen from the less productive waters of the

TABLE 5.

James River seed and market oyster harvest 1963–1995 (all data from VMRC).

Year	Seed (×1,000 Bu)	Market (×100 Bu)	Seed/Day	Market/Day	Boat Days
1963	844	175.7			
1964	830	417.4			
1965	424	450.0			
1966	611	487.9			
1967	533	167.0			
1968	484	182.0			
1969	487	157.7			
1970	264	143.8			
1971	459	170.8			
1972	381	129.7			
1973	396	27.4			
1974	373	186.3			
1975	317	61.6			
1976	441	14.6			
1977	420	3.3			
1978	350	13.2			
1979	420	42.7			
1980	350	68.4			
1981	214	136.0			
1982	406	21.5			
1983	445	16.1	62.8	0.2	7,087
1984	346	48.8	45.9	0.6	7,533
1985	410	21.5	54.4	0.3	7,537
1986	277	28.8	49.2	0.5	5,625
1987	199	341.4	12.6	2.2	15,754
1988	136	297.2	6.4	1.4	21,305
1989	135	146.2	9.6	1.0	14,027
1990	51	68.2	5.2	0.7	9,810
1991	55	36.5	8.2	0.5	6,698
1992	54	25.6	13.4	0.6	4,032
1993	95	20.0	35.2	0.7	2,698
1994	75	5.5	43.7	0.3	1,715
1995	126	17.7	36.0	0.5	3,500

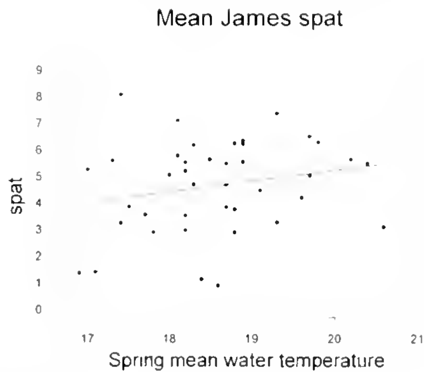


Figure 28. Regression of mean James River spat versus spring VIMS pier temperatures.

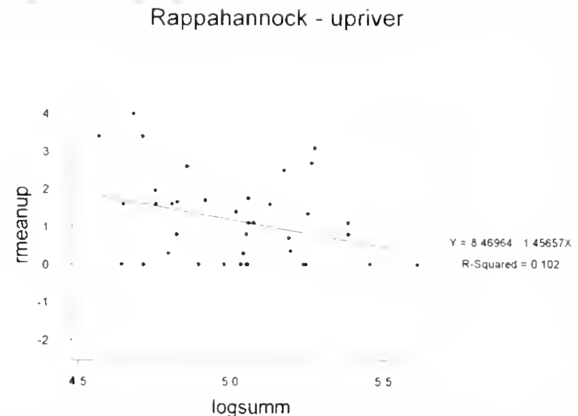


Figure 29. Regression of upper Rappahannock River spatfall versus summer river flow.

Rappahannock and Potomac Rivers into the James for both seed and market oysters, and a decline in effort as catch dropped off. Figure 26 depicts the market oyster and CPUE since 1983, with data derived from the market oyster harvest and both days (Bushels Market oyster/boat days). Reduced oyster stocks and active management by the marine Resources Commission combined to result in a post-1990 reduction in effort.

Both harvest of market oyster and CPUE of market oyster parallel boat days. This is because watermen would rather focus their efforts toward harvesting $30/Bu of market oyster, than $4/Bu of seed. After 1990, however, as stocks of market oyster became seriously depleted, significant effort was redirected toward the harvest of seed, the remaining resource. A quota system for seed was introduced in 1993–1994, permitting the harvest of 80 kBu, but the limit was increased at the watermen's insistence to 120 kBu in 1994–1995. Although CPUE for seed increased in 1993–1995, the total seed harvest has remained relatively stable since 1990 (Fig. 27).

A significant note of caution should be introduced. Although the number of boat days has been recorded monthly since the 1982–1983 season, they were not separated between seed harvest days, market harvest days, and which days were a split between the two activities. In other words, of the 5,625 boat days in 1986, it is not possible to determine how many of these were spent harvesting seed and how many were spent harvesting market oyster. Consequently, the CPUE calculations for seed and market were made with the unlikely assumption that equal numbers of days were spent on each fishery. In short, although the calculations have been made, we would not place great reliability on them because the data are so "spongy." This results in the Fisheries Management Axiom: Are "spongy" better than none?

Other factors may influence the results here in a way that cannot be estimated. The first is that the James River is the source of seed for the Virginia repletion program that transports seed oyster from the James to nonproducing areas of the Virginia tributaries. This movement of seed may result in changes in abundance both in the James (Downward) and the other rivers (upward) that are not reflected in our count data. The second factor is the spread of disease, which has been responsible for much of the mid- and late-1980s decline in market oyster (Bureson and Ragone Calvo 1996). After reaching 19–45 mm, when 2–3 y of age, the seed oyster in the lower, more saline regions of the James River become susceptible to the diseases MSX (*Haplosporidium nelsoni*) and Dermo (*Perkinsus marinus*). Burreson and Ragone Calvo (1996) have found this to be particularly severe on Wreck Shoal in the James River, where mortality has been 100% for several years. The removal of seed and market oyster from the stock by either disease or repletion will obviously affect our results, but we are unable to estimate to what degree this has occurred. It is our conclusion that the seed and market CPUE data, as currently collected, cannot be used to examine the effect of seed abundance on subsequent market landings.

TABLE 6.

Regression analysis for James and Rappahannock upriver spat abundance versus spring and summer river flow.

River	Season	p	R^2
James River	Spring	0.089	0.034
James River	Summer	0.018	0.074
Rappahannock River	Spring	0.102	0.052
Rappahannock River	Summer	0.023	0.102

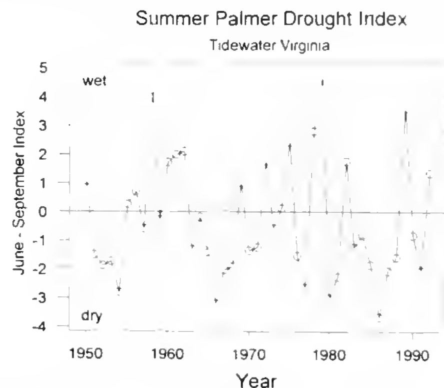


Figure 30. Summer PDI, Tidewater, VA.

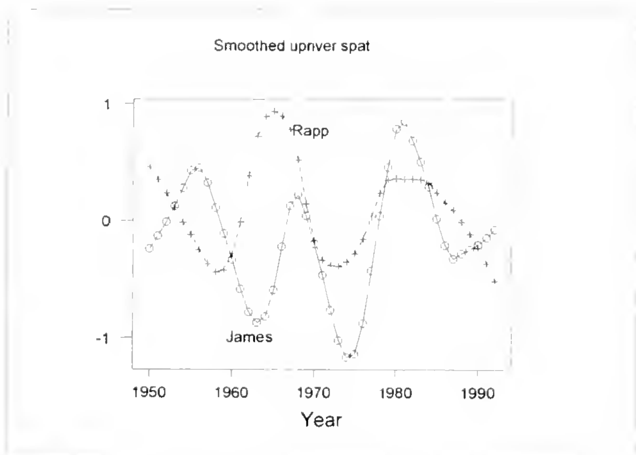


Figure 31. Detrended, smoothed upriver spatfall for James and Rappahannock (Rapp.) Rivers.

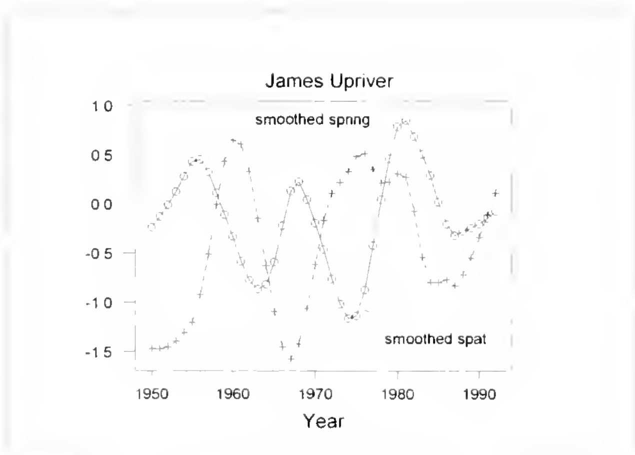


Figure 33. Detrended, smoothed upriver James spatfall and detrended, smoothed spring PDI.

Relation of Spat to Its Physical Environment

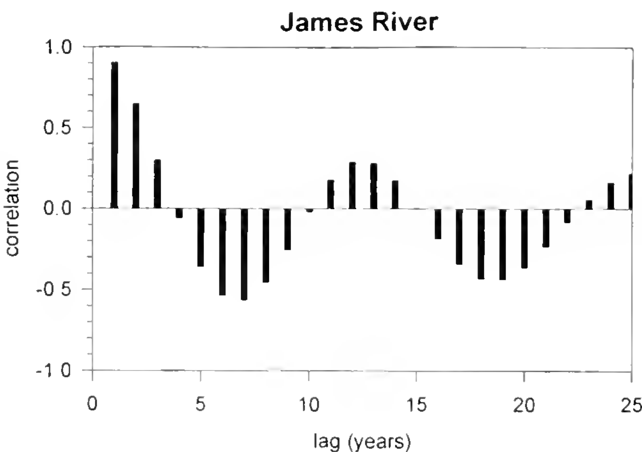
Most marine organisms, particularly those attached to the bottom, are susceptible to fluctuations in the physical environment. Numerous articles addressing these oyster-environment relationships have been published (Ulanowicz et al. 1980, Haven 1982, Chai 1988, Austin et al. 1993). Generally, they have pointed to temperature and salinity (or its proxy, river discharge) as the controlling physical variables.

Temperature Effects on Spat

The water temperature data measured at the VIMS pier at the mouth of the York River constitutes an almost continuous data set since 1952 and was used as surrogate data for all of the rivers. The effects were examined of mean spring temperature (May through July) and mean summer temperature (July through September) on the mean spat from the James and from the Rappahannock Rivers. In no case did the value of R^2 exceed 2.1%, and none of the regressions were significant (Table 5). As an illustration of the lack of relationship, the data and regression line for the "most significant" ($p = 0.18$) regression between James River spat and spring temperature are shown in Figure 28. The conclusion is that the water temperature during the spring and summer preceding the spat measurement has minimal effect on the spatfall.

River Discharge Effects on Spat

River discharge is monitored by the U.S. Geological Survey and the NOAA Office of Hydrology. We used data from the monitoring stations located on the fall line of the Rappahannock and



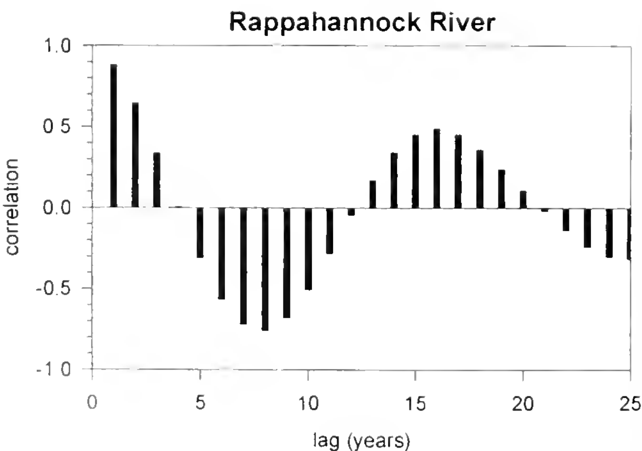


Figure 32. cff for detrended and smoothed James River and Rappahannock River spatfall.

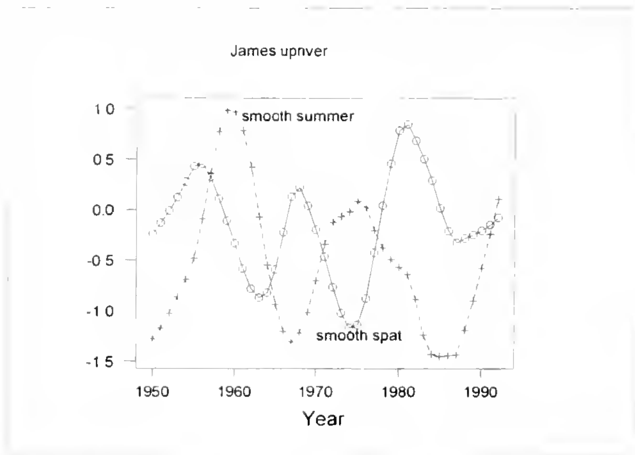


Figure 34. Detrended, smoothed upriver James spatfall and detrended, smoothed summer PDI.

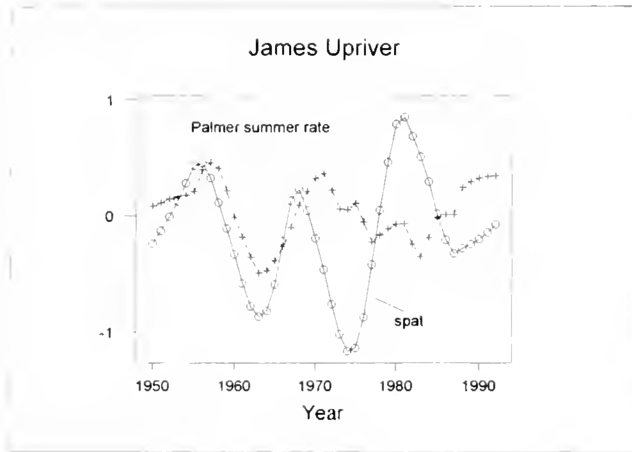


Figure 35. Detrended, smoothed upriver James spatfall and detrended, smoothed summer period of maximum rate of change in PDI.

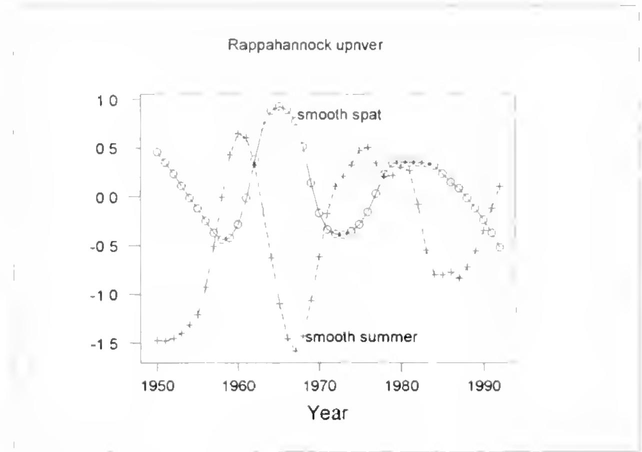


Figure 37. Detrended, smoothed upriver Rappahannock spatfall and detrended, smoothed summer PDI.

James Rivers and selected "upriver" stations by using agglomerative cluster analysis characterizations of the oyster reefs. It was expected that stations furthest up stream would be those most likely to reflect fluctuations in stream flow (Haven 1982). These included the Group I James River reefs (Deepwater Shoals, Horse head, and Point of Shoals) and both Morattico and Bowlers Reefs in the Rappahannock River.

We looked at both spring (May to July) and summer (June to September) mean discharge patterns for the James and Rappahannock Rivers and regressed them (log flow) against the mean spatfall abundance for the two upriver populations. It is obvious from the results in Table 6 that with the exception of the Rappahannock summer flow (Fig. 29), spring/summer river discharges alone did not produce a significant variation in spatfall patterns.

Andrews et al. (1959) noted that the significant 1957 spat set was largely wiped out during the 1958 winter-spring freshets. Although the fall survey count showed a large set in 1957, mortality was high during the following May to June period, when the previously overwintering dormant spat became active in the low-salinity James. They also reported that although this occurred in the James, they did not notice a similar effect in the Rappahannock. Haven (1982) reported that the prolonged periods of low salinity during the fall, winter, and spring of 1979–1980 produced extensive mortalities of the 1979 set in the James River. Yearling were affected to a lesser degree, and market size oyster exhibited the lowest mortality. This is reflected by a lower fall count of yearling oyster in the James River in 1980 (Fig. 4b).

PDI Relation to Spat Abundance

Neither temperature nor river discharge (proxy, salinity) data gave significant relationships with spat abundance, in spite of historic reports and an intuitive assumption that they should. In search of an alternative environmental variable, we considered the PDI, a combination of air temperature, precipitation, and soil type as a possible integrated environmental signature. The index is in standard usage by climatologists and is published monthly by the Office of the Virginia State Climatologist at the University of Virginia. Precipitation data alone do not always reflect river discharge and, consequently, salinity, because it is often the rate of the precipitation that influences the amount of runoff that ultimately results in river discharge. Rain soaks in, while rain showers often exceed the soil's absorption capacity and result in runoff to the creeks and rivers. The PDI is computed for four areas of the state, depending on the temperature, precipitation, and soil type regimens. We considered that the Tidewater index was appropriate for this study.

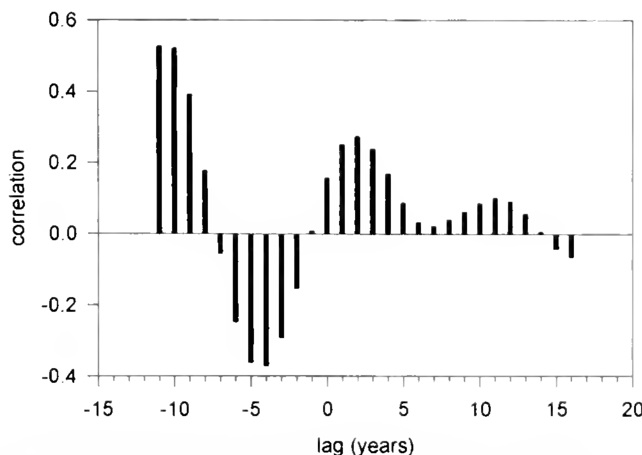


Figure 36. Cross-correlation of James River spat and smoothed summer period of maximum rate of change in PDI.

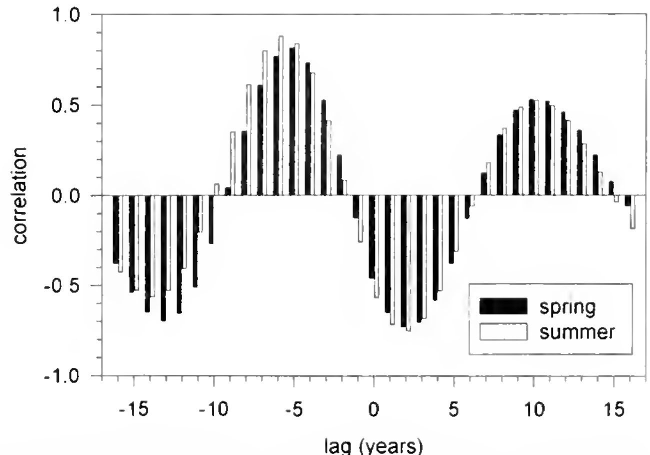
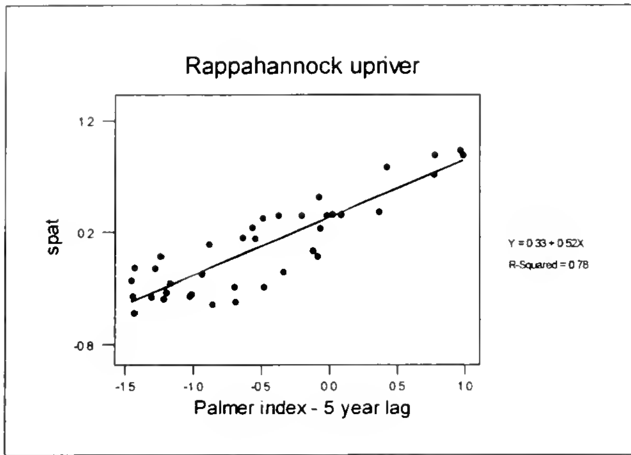


Figure 38. Cross-correlation of spring and summer Rappahannock River spat and PDI.



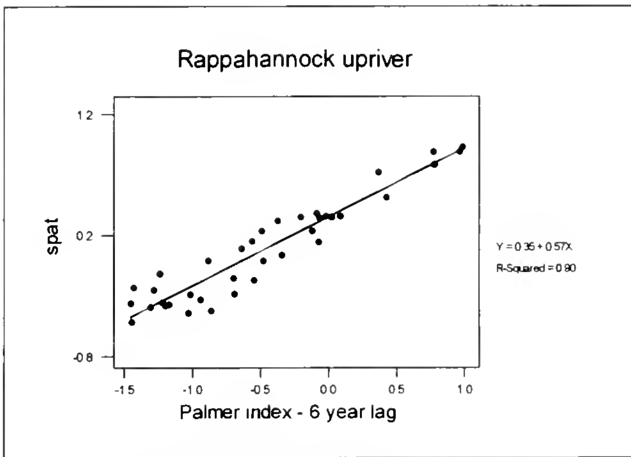


Figure 39. Regression of Rappahannock River spat and PDI lagged 5 and 6 y.

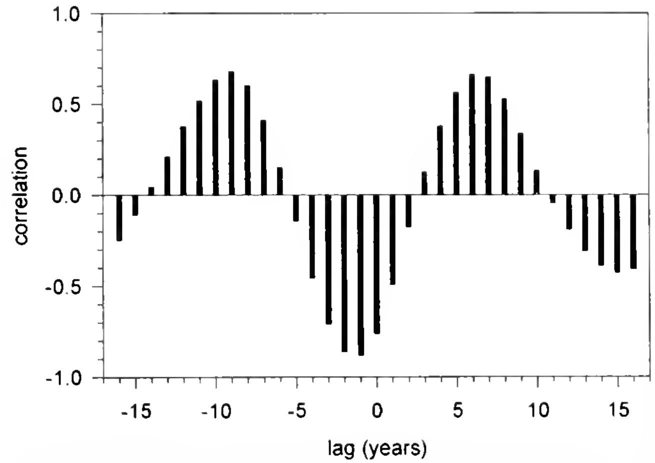


Figure 41. Cross-correlation between Rappahannock River spat and summer period of maximum Rate of change in PDI.

The PDI is a negative or positive deviation from normal. A positive index represents wet conditions (e.g., 1979, >4.0), and a negative index represents dry or drought conditions (e.g., 1986, > -3.5). Figure 30 depicts the summer index, which is reasonably representative of both the spring and the summer. The "dry" or drought conditions of the mid-1960s and mid-1980s are readily apparent, as is the trend away from drought to "wet" conditions during 1966 through 1979. There were no "wet" periods of over 2 y during the period of measurement, 1950 and 1994.

We computed a spring (May to July) and a summer (June to September) mean index. The ccf between the summer and spring PDI was 0.845, and the regression coefficient was 0.71.

As discussed above, if there is a relationship between fall spat-on-shell and river discharge (i.e., salinity) and/or temperature, it should be most readily apparent at the stations furthest upriver. Using the agglomerative cluster analysis characterizations of the oyster reefs, we picked the James and Rappahannock "upriver" groups for analysis. Strong long-term trends in spat data, particularly those following the post-1960 decline, were apparent (Figs. 4–7). Cross-correlation was the analysis tool planned for exploring the relationship between spat and river discharge. As we have observed earlier, the results of this type of analysis can be severely corrupted by the presence of long-term trends (Chatfield 1989). Such trends were therefore removed by use of the *loess* filter in MINITAB, with the adjustable parameter set to remove all but the lowest frequencies (parameter value = 0.7). The residuals from this smoothing constitute the detrended data. The random year-to-

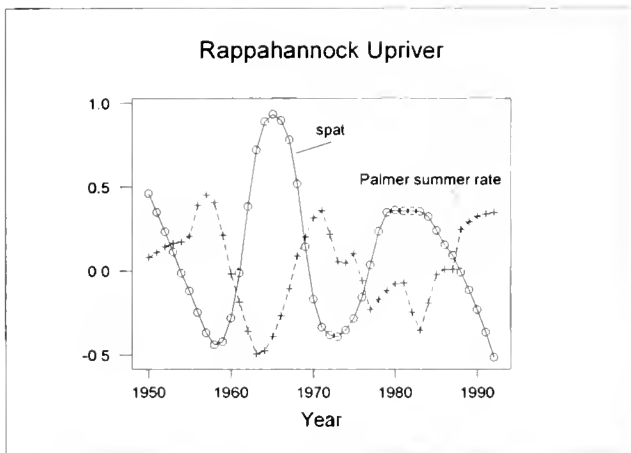


Figure 40. Detrended, smoothed upriver Rappahannock spatfall and detrended, smoothed summer period of maximum rate of change in PDI.

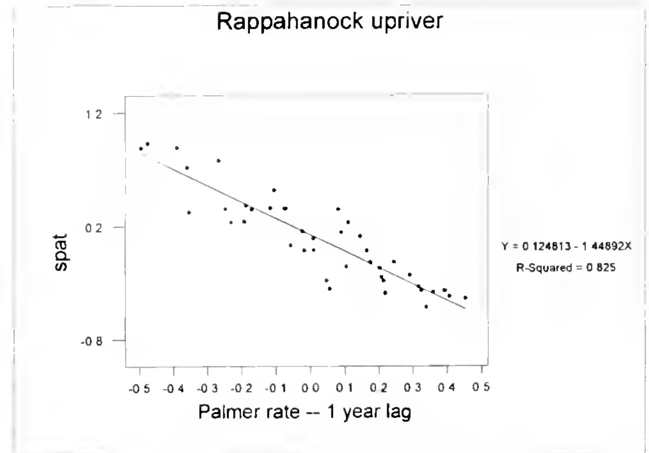


Figure 42. Regression of detrended, smoothed upriver Rappahannock spatfall versus detrended, smoothed summer period of maximum rate of change in PDI, lagged 1 y.

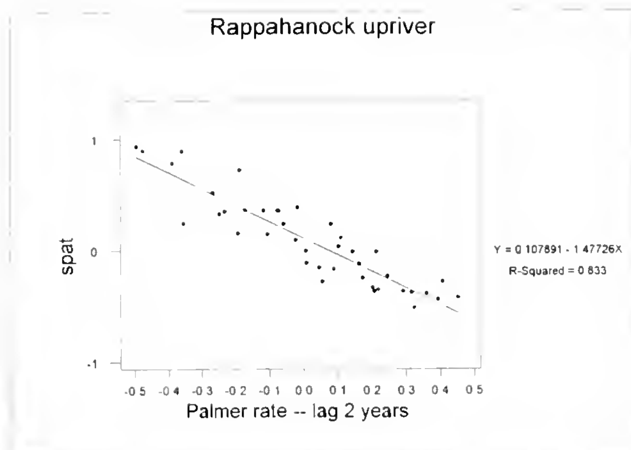


Figure 43. Regression of detrended, smoothed upriver Rappahannock spatfall versus detrended, smoothed summer period of maximum rate of change in PDI lagged 2 y.

year fluctuations in these data were smoothed by a further application of the *loess* filter with a parameter of 0.3. The overall effect of these procedures was a bandpass filtering of the original data whereby long-term trends and short-term fluctuations were removed. Figure 31 shows these smoothed data for both rivers through time. By visual inspection, the James River spat exhibited an 11- to 12-y cycle and the Rappahannock exhibited a 15-y cycle. This observation is confirmed by an inspection of the ccf (Fig. 32).

James River

Figure 33 shows the James upriver smoothed spat and spring PDI, and Figure 34 shows the spat and summer PDI. In each case, it is apparent from inspection that the summer precipitation deficits are more profound than the spring, but that both seasons move in synchrony. Also, visual inspection shows that spat and PDI fluctuate out of phase. If, however, one considers the period of greatest change in PDI (ΔPDI), as opposed to the actual values, it is apparent that they are in phase (Fig. 35). The peaks and valleys of the spat data are in phase with the periods of greatest ΔPDI. Regression of the summer ΔPDI against spat yielded an R^2 of only 14.2%. However, when the period of greatest ΔPDI was cross-correlated with spat, the greatest correlation was found at a lag of 4 y (−0.372) (Fig. 36).

Rappahannock River

The Rappahannock smooth spat and summer PDI demonstrated a greater degree of visual synchrony (Fig. 37) than the James, and the R^2 was 26.9%. Cross-correlation analyses showed a significant lag of 6 y (0.880) between spat and summer PDI, and a 5-y lag (0.817) with the spring PDI (Fig. 38). The R^2 for the regression between spat and the 5- and 6-y lag of the summer PDI were 0.77 and 0.90 (Fig. 39).

When the rate of change in the PDI (ΔPDI) (Fig. 40) was cross-correlated with spat (Fig. 41), the greatest correlation was found at a lag of 1 and 2 y (−0.881 and −8.62). R^2 for the lags were 0.825 and 0.833, respectively (Figs. 42 and 43). The responses of the spatfall to the changes in the PDI are reflected both in the 1960s, as conditions evolved from "damp" to "drought," and in the more prolonged "drying" period of the mid-1970s to mid-1980s, as the spatfall reflect a short and a longer period of increased set (Fig. 40).

The James River, because of its proximity to the mouth of the Chesapeake Bay, is more under the influence of oceanic-salinity gravitational circulation (Pritchard 1952, Neilson and Kuo 1989) than the Rappahannock. This circulation regimen has been suggested in the past (Austin et al. 1993) to be the cause of some interriver variations in oyster condition index. As such, it is not unexpected that the upper Rappahannock showed the greatest response to fluctuations in freshwater input. It must be pointed out, however, that Andrews et al. (1959) found no such James versus Rappahannock differences when examining extremely low flow patterns.

Spat and PDI Linkages

Statistically, high cross-correlations and/or regression coefficients between PDI and spatfall at 7 or 8 y do not make ready biologic sense. Yet, it is coincidence that this is the same lag period found to be statistically significant by Krantz and Merritt (1977) and Ulanowicz et al. (1980) for spat to harvest? Stepwise multiple regression by Ulanowicz et al. also showed that "drought episodes," cumulative (sustained) excessive salinity, extreme rainfall during the previous season, and harvest all caused direct variations in spat density. In this study, however, the "depth of the drought" or "peak period of rainfall/runoff" might not be expected to show a direct cause and effect with spatfall because the long lag is unexplained biologically. On the other hand, if one considers the period of greatest PDI change (ΔPDI), that period when the environment passes from one temperature/precipitation regimen to another, it makes biologic sense that the populations, after a lag, will begin to show change; then, change will occur rapidly as the population shifts toward equilibrium with the "new" environment. The cyclic nature of the physical (PDI) results in rapid and cyclic changes in the spatfall. Only during the extended drought of the early- to mid-1980s did the spatfall rates have a chance to equilibrate. Allen et al. (1977) and Legendre et al. (1985), looking at succession of species within a community, said that ecological succession evolves in steps, instead of smoothly, shifting from one structure to another, produced by intermittent shifts in the environmental structure.

CONCLUSIONS

Spatfall in the Virginia tributaries to the Chesapeake Bay and the Potomac River show a pattern of declining set from the late 1949's through the early 1970s, followed by a moderate recovery after 1975. The decline is not apparent in the Potomac. Counts of yearling oyster follow a similar pattern, except in the York River, where they increased, followed by a steady decline. Patterns of spatfall tended to partition into upriver and downriver clusters.

Spatfall levels, as indexed by counts-on-shell, can be used to predict the abundance of seed oyster 2–3 y later, but are not a good predictor of market oyster abundance. This lack of a predictive spat-market capability may be, in part, the result of the movement of seed through the oyster repletion program.

Although spat did not show a direct statistical relation to temperature or salinity, there was a significant relationship with the PDI, particularly when the index was shifting from wet to dry or vice versa. We attribute this to a shift in the environmental structure of the rivers and the response of the oyster recruitment.

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EPIZOOTIOLOGY OF THE PARASITE, *PERKINSUS MARINUS* (DERMO) IN INTERTIDAL OYSTER POPULATIONS FROM LONG ISLAND SOUND

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ABSTRACT The recent reported occurrence of *Perkinsus marinus* in oysters from Long Island Sound prompted this study of the epizootiology of the parasite in this region. The monthly prevalence and infection intensity of *P. marinus* were determined for three intertidal populations of eastern oysters, *Crassostrea virginica*, during the period of 1993–1996. Total numbers of infected oysters were highest at the Bridgeport site, followed by the Westport and Milford locations. Disease prevalence was greater in 1995 than in 1994 at all sites studied Numbers of infected oysters and parasite burdens peaked in the late fall and declined dramatically in the winter/early spring. Prevalence was highest (20–100%) at the Bridgeport, CT, site in every month sampled, and the weighted prevalence (Mackin's scale: 0–5) reached a maximum of 3 2 in November 1994. Temperature and salinity data available for the Bridgeport location indicate that conditions were reportedly favorable for parasite proliferation ($\geq$20°C; $>$10 ppt) from June through September.

KEY WORDS: *Perkinsus marinus*, Dermo, prevalence, intensity, Long Island Sound, temperature

INTRODUCTION

Perkinsus marinus (commonly known as "Dermo"), a protozoan pathogen of the Apicomplexan group, is generally considered the most serious oyster pathogen in Chesapeake Bay and the Gulf of Mexico. *P. marinus* was first identified as a disease-causing agent in the eastern oyster in the Gulf of Mexico in 1947 by Mackin et al. (1950), and its occurrence was quickly documented throughout the southeastern United States (Ray 1954). By 1949, this pathogen was discovered in the lower Chesapeake Bay (Andrews and Hewatt 1957, Andrews 1988) and in the mid- to late 1980s, it began to spread to locations farther and farther north in the estuary. *P. marinus* is now present on nearly all oyster bars in Virginia and Maryland and has recently become established in Delaware Bay (Smith and Jordan 1993, Ragone Calvo and Burreson 1995). This apparent range extension northward has continued, and between 1991 and 1992, infected oysters were found from Long Island Sound to Cape Cod. MA. By 1995, the infection of oysters by species of the genus *Perkinsus* (probably *P. marinus*) had been reported as far north as the Damariscotta River in Maine (Kleinschuster and Parent 1995).

Regional differences in the seasonal cycle of *P. marinus* have been well documented. They have been attributed largely to variations in seasonal temperature patterns between northern and southern limits of the parasite's range. In the south, the parasite exhibits high prevalences throughout the year, declining very little during the winter months (Andrews and Ray 1988; Soniat and Gauthier 1989, Crosby and Roberts 1990, O'Beirn et al. 1994). In more northerly waters, epizootics display a more seasonal pattern in which the prevalence and intensity of the disease increase in the late spring, peak in the late summer/fall, and decline over the winter/early spring months of the year (Andrews and Hewatt 1957, Andrews and Ray, 1988, Burrell et al. 1984). A detailed understanding of the periodicity of Dermo disease in areas in which it has been recently reported, however, is not yet available.

Since the first reported cases of *P. marinus* in Long Island Sound appeared in the early 1990s, the disease has reached epizootic levels in numerous locations throughout Long Island Sound, affecting both cultured and wild stock (Brousseau et al. 1994, Brousseau 1995, Brousseau unpubl.). To date, information about Dermo disease in this area has been generated largely by work on intensively cultured commercial beds. Oyster culling and harvesting activities, however, subject these "populations" to constant disturbance, making it difficult to study the natural course of disease in this setting. This investigation was initiated to determine the epizootiology of *P. marinus* in western Long Island Sound in three undisturbed natural populations. The data presented here represent the first long-term study (31 mo) of diseased oysters in this region.

MATERIALS AND METHODS

Oysters (*Crassostrea virginica*) were collected from three intertidal sites in western Long Island Sound: Milford Pt., Milford, CT; Black Rock Harbor, Bridgeport, CT; and Saugatuck River, Westport, CT (Fig. 1). Oysters from the Milford site were collected monthly from September 1993 to August 1994. The

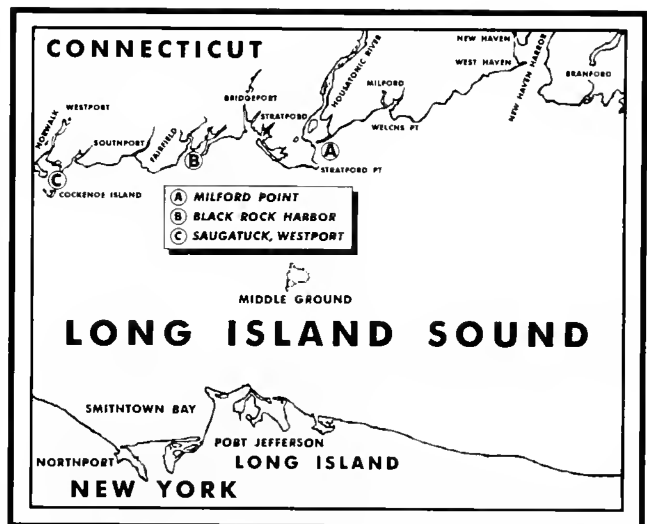
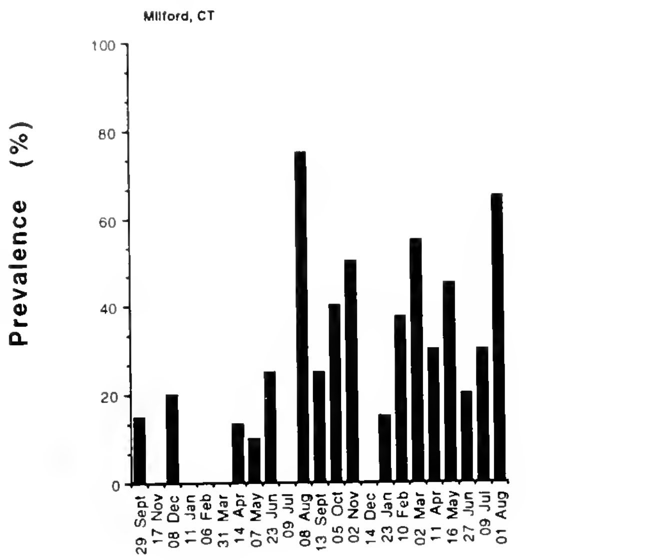
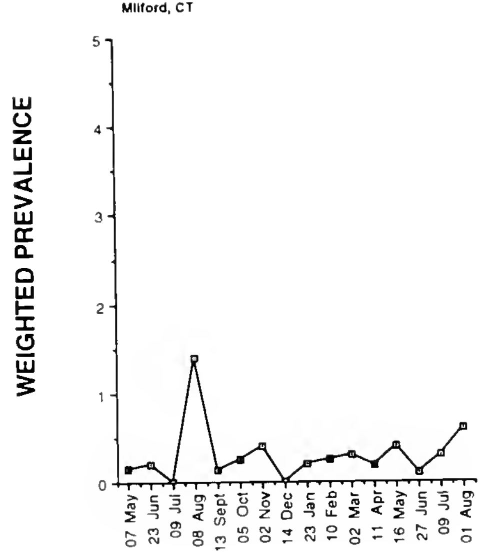
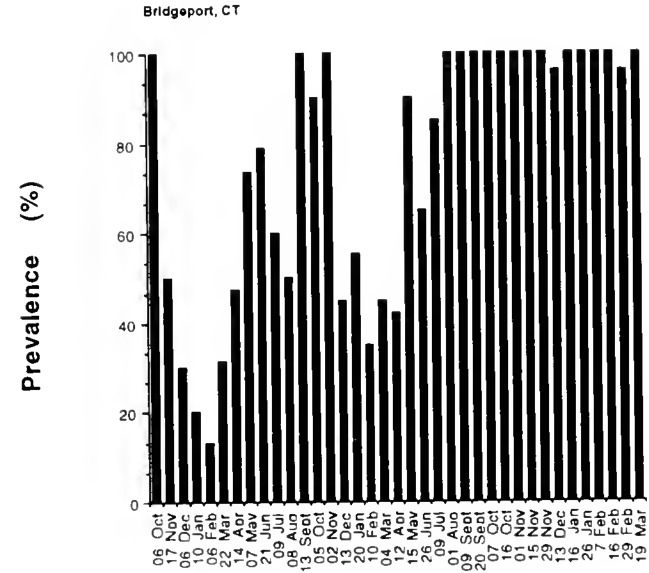
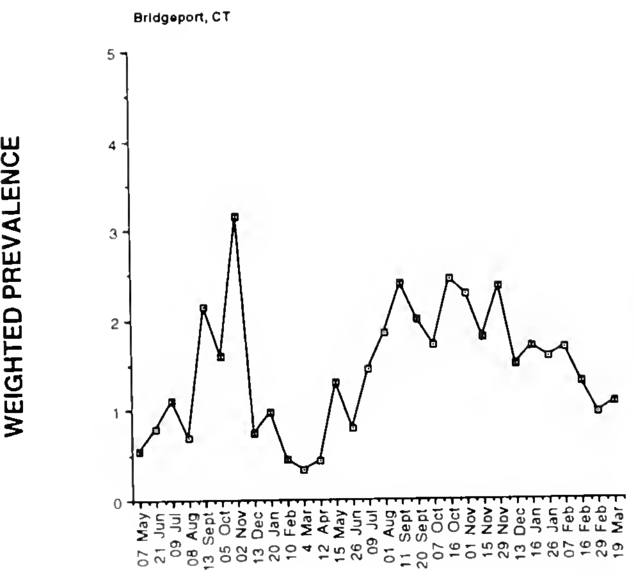


Figure 1. Map of Long Island Sound showing the geographic locations of the three collection sites: (A) Milford Pt., Milford, CT; (B) Black Rock Harbor, Bridgeport, CT; and (C) Saugatuck River, Westport, CT.









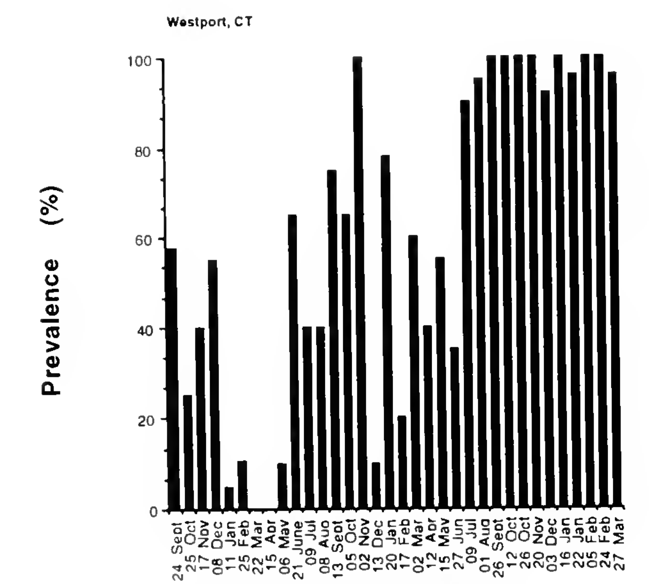


Figure 2. Prevalence of *P. marinus* in oysters from each of the study sites during 1993–1996.

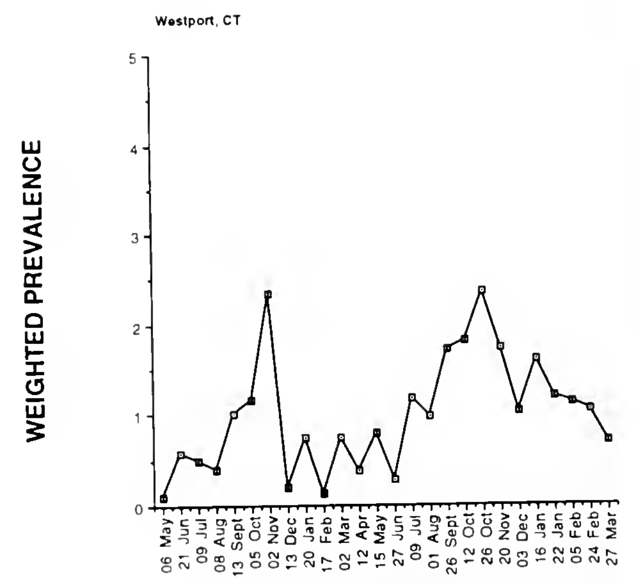


Figure 3. Weighted prevalence of *P. marinus* (Mackin's scale: 0–5) in oysters from each of the study sites.

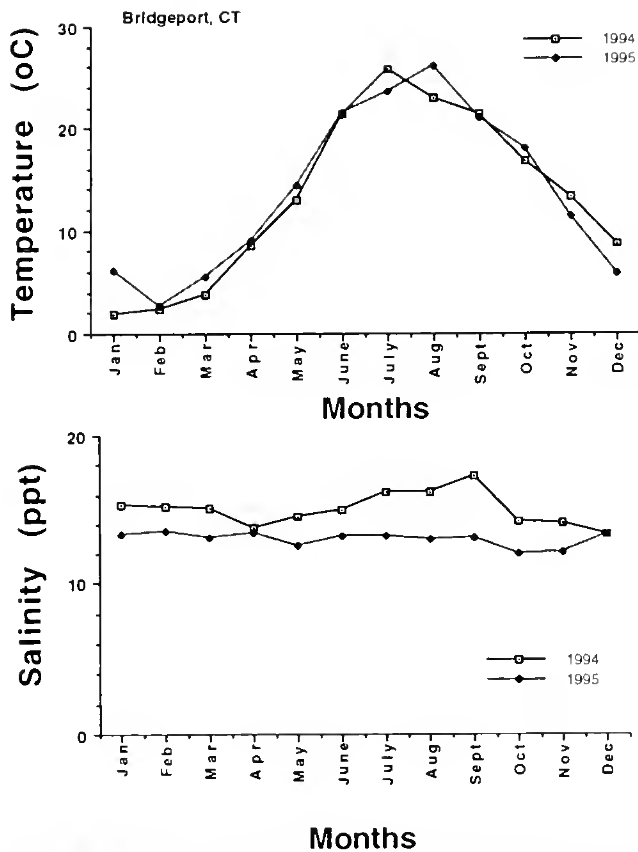


Figure 4. Monthly temperature and salinity patterns at the Black Rock Harbor, Bridgeport, site from January 1994 through December 1995.

monthly and/or bimonthly collection of oysters from the Bridgeport and Westport sites began in September and October 1993, respectively, and ended in March 1996. Sample sizes varied from 20 to 25 oysters. Tissue diagnosis of *P. marinus* was done by culture of rectal and mantle tissue in fluid thioglycollate medium, as described by Ray (1954). Beginning in May 1994, infections were scored for intensity of disease by use of the measure originally described by Mackin (1962) as the weighted incidence and later renamed weighted prevalence (Ragone and Burreson 1994). A total of 419 Milford oysters, 787 Bridgeport oysters, and 725 Westport oysters were diagnosed.

Temperature and salinity readings were taken during high tide at the Black Rock Harbor, Bridgeport, site throughout the study period with a YSI Model 33 SCT meter.

RESULTS

The prevalence of *P. marinus* recorded by site in all oysters examined during the 31-mo study was Bridgeport, 73%; Westport, 56%; and Milford, 26%. The monthly prevalence for each site is given in Figure 2. At all sites, the highest prevalence occurred in late summer and early autumn, when up to 100% of the oysters sampled from Bridgeport and Westport were infected. The lowest prevalence was found in the winter and early spring, with 0% prevalence (uninfected oysters or those with subpatent infections) reported in the months of December, January, February, and March in Milford oysters and in March and April in oysters from Westport. During the winter, the number of infections found in Bridgeport samples fell as well, but the parasite was always detectable in some oysters.

Weighted prevalence varied at the three sites, but followed the typically observed pattern of maximum values in the summer and autumn and minimum levels in the winter (Fig. 3). Overall disease intensity was highest in Bridgeport oysters, followed by those from Westport and Milford. Weighted prevalence peaked during October and November of both 1994 and 1995 at the Bridgeport and Westport sites and in August 1994 at the Milford location. The highest weighted prevalence (3.2) was reported from the Bridgeport population in November 1994. The monthly weighted prevalence for the Westport oysters never climbed above 2.4, and that for the Milford oysters exceeded 1.0 only in August 1994.

The mean monthly water temperature and salinity at the Bridgeport site during 1994 and 1995 are shown in Figure 4. The daily water temperatures recorded for the warm months of the year (June through September) are shown in Figure 5.

DISCUSSION

The seasonal pattern of *P. marinus* prevalence and intensity reported here is similar to that reported for oyster populations along the East Coast north of South Carolina, where the disease increases in late summer and autumn and experiences a dramatic decline during winter and early spring (Andrews and Hewatt 1957, Burrell et al. 1984, Andrews 1988). All of the populations of oysters studied here were intertidal. Previous investigators have shown no significant differences in *P. marinus* prevalence or intensity in comparisons of intertidal and shallow subtidal oyster beds, situations where temperature regimens should be similar (Gibbons and Chu 1989, Burrell et al. 1984, O'Beirn et al. 1994). It seems likely, however, that patterns of prevalence and intensity will be different in the colder subtidal beds commonly used for oyster culture in Long Island Sound. Additional work is needed before our understanding of the details of the seasonal cycle of *P. marinus* in this area is complete.

The extremely cold temperatures during the winter of 1993–1994 did not end the epizootic in Long Island Sound. Overwintering infections were found in both years in the Bridgeport oysters and in 1995 in oysters collected in Westport. The scarcity of overwintering infections in oysters from Milford may be a sampling problem related to the overall lower prevalence of *P. marinus* in this area. The failure of below-average water temperatures to end the epizootic in this area supports predictions of the model developed by Powell et al. (1996) regarding the role of low temperature in terminating an epizootic.

Prevalence data for this region of Long Island Sound indicate that the numbers of infected animals rose markedly between 1994 and 1995. Mean prevalence doubled in the Westport oysters (1994: 36%; 1995: 74%) and increased by a third in the Bridgeport oysters (1994: 60%; 1995: 78%). Data for the Milford oysters in 1995 are incomplete, but the available information suggests a similar trend. The cause of this increase is unknown. Temperature data for Bridgeport (Fig. 4) indicate nearly identical conditions in the spring and early summer of both 1994 and 1995, but the unusually low winter temperatures in 1993–1994 may have helped delay parasite proliferation and spread in the summer of 1994. It is also possible, however, that the higher prevalence of this pathogen during the second year of the study was due in part to the time required for the establishment of a pathogen in a new area. Regardless of the reasons, it is clear from this study that *P. marinus* has become well established in oyster populations in this region.

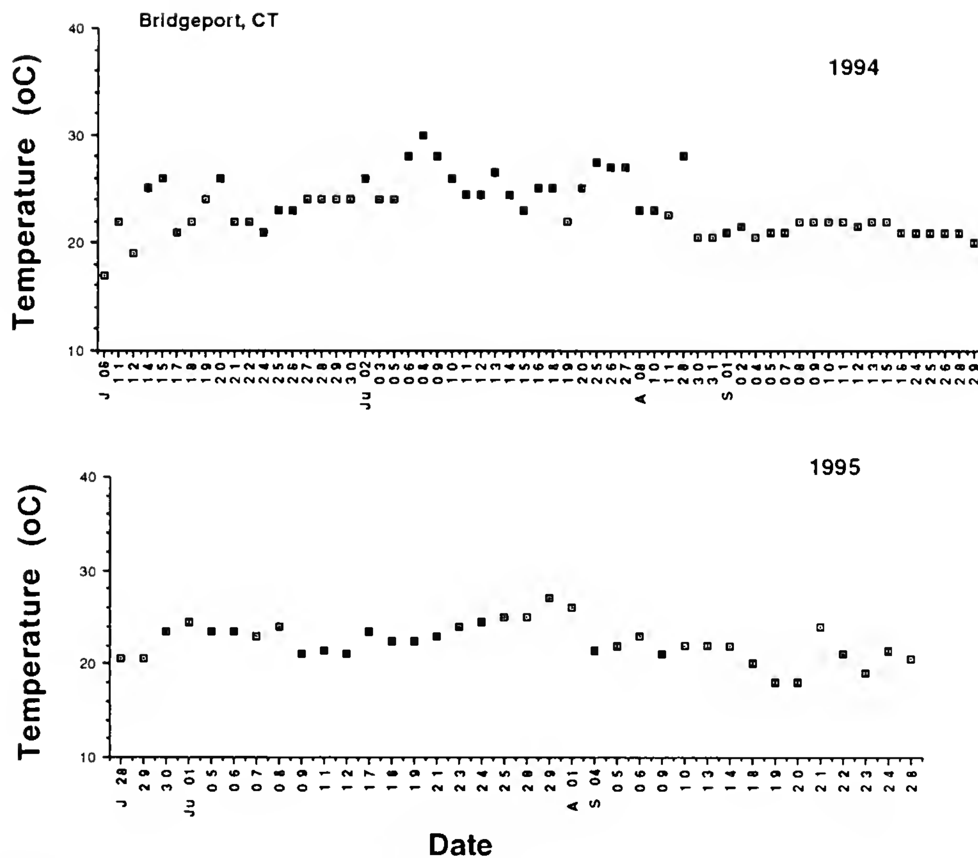


Figure 5. Daily temperature measurements taken at the Black Rock Harbor, Bridgeport, site from June through September of 1994 and 1995.

The level of *P. marinus* infection in an oyster population can be diagnosed by use of the 5-point scale developed by Mackin (1962). Field and laboratory measurements have shown that oyster mortality occurs in individuals showing an infection intensity of 5.0 on this scale (Andrews 1988). Populations with a weighted prevalence of 3.0 or higher can be expected to experience 50–75% annual mortality, and even a rating of 1.0 or more can be expected to cause some deaths in an affected population (Ray and Chandler 1955, Mackin 1961, Mackin and Hopkins 1961). No mortality studies were done as part of this investigation, but on the basis of the findings cited above, the possibility exists that mortality events have occurred, especially in Black Rock Harbor, where weighted prevalence did exceed 3.0 in November 1994. The fact that no reports of unusual oyster mortalities were made may simply be due to a failure to detect such events in an area no longer supporting oystering activity. Clearly, more information is needed before the effect of this parasite on oysters in this region can be assessed.

Numerous studies, both in the field and in the laboratory, have shown that temperature is the dominant environmental factor determining the distribution (geographic) and seasonal activity of *P. marinus*. Infection development in parasitized oysters requires a period of increased water temperatures (Andrews 1988), and the literature contains numerous reports citing temperature as the primary factor controlling the success or failure of the parasite to establish itself in a particular area (Andrews 1988, Ford 1996). The recent establishment of *P. marinus* in waters once thought to be unfavorable for its growth and survival, however, calls for a reevaluation of the conditions governing its distribution and pathogenicity.

The proliferation rates of *P. marinus* increase rapidly above 20°C and reach their maximum between 25 and 30°C (Andrews 1988). In 1994 and 1995, ambient water temperatures at Black Rock Harbor were ≥20°C from June through September but reached 25°C only in July 1994 (Fig. 5); yet, the oysters at this site showed *P. marinus* infection prevalence and intensity levels comparable to those reported in oysters from locations further south. This suggests that the temperature-time course required for infection development may vary significantly from region to region within this parasite's geographic range. In addition, increasing parasite burdens in oysters first appeared in May at this site, fully a month before water temperatures reached 20°C. High infection intensities were sustained well into November, suggesting the possibility that a low temperature–adapted strain of the parasite exists (Bushek and Allen 1996). Further data that examine the relationship between seasonal water temperature cycles and the activity patterns of the parasite at the more northerly limits of its occurrence are needed to fully understand the role of temperature in the distribution of *P. marinus*.

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EFFECTS OF *HYPHOMONAS* PM-1 BIOFILMS ON THE TOXICITY OF COPPER AND ZINC TO *CRASSOSTREA GIGAS* AND *CRASSOSTREA VIRGINICA* LARVAL SET

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ABSTRACT We tested the hypothesis that microbial biofilms bioconcentrate copper (Cu) and zinc (Zn) from ambient water to levels that inhibit oyster set. Cu or Zn concentrations of 0.1 ppm in MBL artificial seawater did not affect oyster larval viability, swimming behavior, DOPA-induced search behavior, or epinephrine-induced metamorphosis; however, they did significantly inhibit larval set in the presence of biofilms as compared with control films which had not been exposed to additions of Cu or Zn. The mechanism is attributed to the bioconcentration of metals by the films. Therefore, complex interactions between toxic metals, bacterial films, and oyster larvae can affect oyster larval set and recruitment in Chesapeake Bay and other aquatic habitats.

KEY WORDS: *Crassostrea virginica, Crassotrea gigas, Hyphomonas* spp., biofilms, metal toxicity, bacteria, biomagnification

INTRODUCTION

Many marine invertebrates have a motile, pelagic larval stage that must find a benthic habitat that is able to support the growth of the sessile adult (Bonar et al. 1990, Pawlik 1990). The process is termed "set" and includes two components, one behavioral (search) and the other physiologic and developmental (cementation and metamorphosis). Marine biofilms provide fertile surfaces and can cue larvae to set (Crisp 1974, Kirchman et al. 1982, Weiner et al. 1989). In fact, for the oysters *Crassostrea virginica* and *Crassostrea gigas*, biofilms can be prerequisites for set, and if a larva fails to locate a suitable filmed substratum, in about 15 days, it fails to metamorphose and dies (Weiner et al. 1988, Weiner et al. 1989).

Prieur et al. (1990) have summarized several ways that bacterial biofilms may influence larval set. Metabolic compounds produced by bacteria, including exopolysaccharides in biofilms, cue larval set (Coon et al. 1986, Fitt et al. 1989, Weiner et al. 1989). Then, the adhesive properties of bacterial films can facilitate larval cementation on substratum (Mitchell and Young 1972). Bacteria and organic particles trapped within the film may subsequently be used as food by metalarvae (Douilet and Langdon 1993, Zobell and Allen 1935). Finally, biofilms may also provide favorable pH conditions for the production of $CaCO_3$ for the shell growth of metalarvae (Mitchell and Young 1972).

However, although microbial biofilms may be conducive or even necessary for invertebrate set, they also contain metal-sequestering domains and may adsorb, concentrate, and biomagnify a variety of inorganic and organic compounds (Geesey et al. 1988). The interactions between metals and microorganisms have recently received much attention (e.g., *J. Ind. Microbiol.*, vol. 14[3] and vol 14[4]), and it is well documented that bacterial capsular polysaccharide binds metals (Geddie and Sutherland 1993, Marques et al. 1990), including copper (Mittleman and Geesey 1985).

There are anthropogenic enrichments of Cu and Zn in marine water, ranging from 0.2 to 500 Cu $\mu g \cdot L^{-1}$ (ppb) (Leland and Kuwabara 1985). Furthermore, in part because of the affinity of some biopolymers and clays for metals, there are more than three orders of magnitude higher concentrations of Cu and six orders of Zn in marine sediments. In other words, total concentrations of Cu and Zn in surface waters positively correlate with concentrations of macronutrients (Boyle et al. 1977, Millero and Sohn 1992). Cu and Zn are removed from these waters by plankton or biologically produced particulate matter; they are regenerated in deep waters when this particulate matter is mineralized by bacteria.

Specific to this report, the biofilms of the marine bacterium Hyphomonas *MHS*-3 were shown to accumulate Zn and Cu 200–10,000×, depending on the metal concentrations and physical conditions (Chang 1995). *Crassostrea* spp. comprise a major group of benthic macrofauna and are classified as stationary epifauna filter feeders of suspended particles. We tested the hypothesis that the close relationship of oysters with the benthic environment (Crisp 1974), in which surfaces may be ubiquitously covered by microbial films, may expose oysters to pollutants there. Toxic metals that are present at "no observed effect levels" in the ambient environment may be concentrated in microbial slime layers to levels that will be harmful to benthic organisms. Here, we show that concentrations of trace metals may be sufficiently high near film surfaces to inhibit the settlement and/or metamorphosis of invertebrate larvae despite harmless levels of metals in the surrounding water.

MATERIALS AND METHODS

Oyster Larvae

Larvae of the Pacific oyster, *C. gigas* (Thunberg), were purchased from the Coast Oyster Company of Quilcene, WA. Larvae of the Eastern oyster, *C. virginica* (Gmelin), were obtained from

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the Virginia Institute of Marine Science oyster hatchery at Gloucester Point, VA. Eyed larvae were shipped by overnight express under moist and cold conditions.

Oyster larvae were maintained in the laboratory according to the procedures described by Coon et al. (1985). After arrival, larvae were slowly warmed to room temperature and transferred to culture vessels at a density of two to three larvae per milliliter. The culture medium was filtered (0.2 μm pole size) artificial seawater (Instant Ocean, Aquarium Systems, Mentor, OH), 30 ppt salinity for *C. gigas* and 20 ppt salinity for *C. virginica*, containing 100 $\mu g \cdot mL^{-1}$ each of penicillin G, streptomycin sulfate, and neomycin sulfate. Larvae were maintained at room temperature and fed with the algae *Isochrysis galbana*, cultured in Alga Gro (Carolina Biological Supply Company, Burlington, NC)–amended Instant Ocean seawater. The water in the culture vessels was changed every other day to eliminate larvae that were dead or spontaneously metamorphosed inside vessels.

The competence of a batch of larvae to metamorphose was based on their responses to 10^{-4} M epinephrine (EPI) induction (Coon et al. 1986). Larvae that metamorphosed within 24 h after EPI induction were considered competent. In addition, larval size (>230 μm for *C. virginica* and >280 μm for *C. gigas*) and fully developed eyespots on both sides of larvae were also used as indications of larval competence (Fitt and Coon 1992).

Metal Toxicity Tests

Toxicity tests were designed to demonstrate how three stages (swimming, searching, and metamorphosis) of larval set were affected by elevated concentrations of metals. Experiments were conducted in plastic tissue culture plates (24-well; Falcon No. 3047) with filtered (0.2 μm pore size) Marine Biological Laboratory (MBL) artificial seawater, which consisted of 423.0 mM NaCl, 9.00 mM KCl, 9.27 mM $CaCl_2$, 22.94 mM $MgCl_2$, 25.50 mM $MgSO_4$, and 2.15 mM $NaHCO_3$. The MBL seawater has a salinity of 37 $g \cdot kg^{-1}$ and was used in all tests of *C. gigas* larvae. Two-thirds-strength MBL seawater has a salinity of 24.7 $g \cdot kg^{-1}$ and was used in all tests of *C. virginica* larvae. These media were adjusted to pH 8.3 with 5N NaOH and were supplemented with 100 $\mu g \cdot mL^{-1}$ of antibiotics as mentioned above. Copper or zinc were introduced as concentrated stock solutions (chloride salts) into the test media in which the larvae were exposed. Total metal concentrations tested were 0, 100, 500 and 1,500 ppb (μg/L), and all treatments were run in triplicate. Metal ion speciation in the test media was simulated through combined functions of the DATAGEN4 software, which compiles all data information, and the WQ4F software, which calculates the activities (and/or concentrations) of major ions and ion pairs; programs were written by the U.S. Geological Survey in 1988.

For larval swimming tests, each well on the culture plates contained a total of 1,500 μL, obtained through the sequential pipette additions of 1,000 μL of seawater, 350 μL of seawater containing 20–30 larvae, and 150 μL of 10× concentrated metal solution prepared in distilled water. For the control, 150 μL of distilled water or seawater was added in place of metal solution. Larval swimming behavior was observed under a dissecting microscope during the 96-h trial. Larval responses to Cu and Zn were compared with the control with respect to the speed of swimming, ciliary activity, and the number of quiescent or inactive larvae.

Larval searching behavior was induced by 10^{-4} M L-3,4-dihydroxyphenylalanine (L-DOPA) (Coon et al. 1986). Induced larvae begin swimming with the foot extended forward, then sink to the bottom, withdraw the velum, thrust the foot anteriorly, and begin crawling. Larvae, in this stage, may resume swimming if the substratum is in some way inappropriate for cementation. Larvae were preexposed (preacclimated) to metal-amended seawater for 96 h before being induced by L-DOPA (10^{-4} M). Behavior was monitored every 5 min for 30 min, and the fraction of searching larvae in each well was recorded.

Oyster larval metamorphosis requires suitable biofilms in nature but can be artificially induced by exposing competent larvae to 10^{-4} M EPI in seawater for 4–12 h (Coon et al. 1986). Larvae respond by immediately sinking to the bottom, without searching behavior, progressively resorbing the velum, and eventually showing significant new shell growth in 24–48 h. Experiments were designed to differentiate metal inhibition periods for larvae exposed to metals: (A) during induction with EPI, (B) after induction with EPI, and (C) during and after induction with EPI. In the first interaction, larvae were exposed to metals with EPI for 4 and/or 12 h before the medium was replaced by clean seawater, in which larval metamorphosis was scored after 96 h. In the second interaction, larvae were exposed to EPI without adding metals; the medium was then replaced by metal-amended seawater. In the third interaction, larvae were exposed to EPI with metals and then were continuously exposed to metal-amended seawater for 96 h and larval metamorphosis was examined.

Larval Set on Biofilms

We used *Hyphomonas* because it is a ubiquitous marine bacterium with a role in biofilm community ecology (viz. invertebrate set (Quintero and Weiner 1995). Bacterial biofilms of *Hyphomonas* spp. strain PM-1 were generated on siliconized glass beakers (Sigmacote, Sigma Chemical Co., St. Louis, MO) as previously described (Chang 1995). All filmed beakers were rinsed first with clean MBL seawater and were individually filled with 50 mL of MBL seawater (salinity, 37 $g \cdot kg^{-1}$) ; then, 100 or so competent *C. gigas* larvae were introduced through a micropipette. Beakers were then rotated gently at 4 rpm for 96 h at 30°C. The water level was maintained at the 50-mL mark by dripping in distilled water on a daily basis.

At the end of the incubation period, vessels were examined individually for set (cemented and/or metamorphosed) larvae under a dissecting microscope. Larvae that did not settle on the surface were collected by Nitex screens (170 μm pore size) and were counted. The total number of larvae applied to each individual beaker was the sum of set and unset larvae.

Effects of Biofilms on Metal Toxicity

For metal toxicity tests, bacterial filmed surfaces were exposed to Cu or Zn and then were presented to competent larvae. Filmed beakers produced from the bacterial cultures first were rinsed by MBL seawater and filled with 50 mL of MBL seawater (salinity, 37 $g \cdot kg^{-1}$ for *C. gigas* and 24.7 $g \cdot kg^{-1}$ for *C. virginica*); Cu or Zn were then spiked to make final total metal concentrations of 0, 100, 500, and 1,500 ppb. After a period of 24 h, during which metals were exposed to the biofilms (Chang 1995), competent larvae were introduced; larvae that cemented and metamorphosed on filmed surfaces were counted after 96 h. For individual metal treatments, five replicates of filmed beakers were prepared.

Chesapeake Bay Samples

Water samples were collected from two sites in the Chesapeake Bay. One site was at the northern bay near the mouth of the Patapsco River in the Baltimore Harbor area (an area of heavy industry), and the other was in the middle bay (Tilghman Island and Chesapeake Beach) (an area that is relatively pristine). Acid-washed and autoclaved sample containers (10 L polyethylene) were totally immersed until fully filled. This estuarine water was stored below 4°C and transported back to the laboratory for *C. virginica* or *C. gigas* larval swimming, searching, metamorphosis, and settling experiments and for metal analyses.

For larval settling experiments, laboratory-produced biofilms were used as the settling surfaces. Collected bay water (50 mL) was then added to individual filmed beakers (five replicates for each water sample). *C. gigas* larvae were introduced, exposed, and examined as described above. Most bay water contained Cu or Zn below the detection limits of flame AAS; therefore, water samples first were digested to release bound metals from organics and then were evaporated to reduce volume to one-tenth of the original. These were then five-fold-concentrated by chelation with ammonium pyrrolidine dithiocarbamate and extraction into methyl isobutyl ketone (Am. Pub. Hlth. Assoc. 1990). Water salinity at the collection sites, measured with a refractometer, was approximately 5–10 $g \cdot kg^{-1}$ at Baltimore Harbor and 11–18 $g \cdot kg^{-1}$ at the middle bay.

RESULTS

Metal Toxicity Tests

The effects of Cu and Zn on larval search behavior and development are shown in Table 1. *C. gigas* larval swimming was not inhibited by total Cu concentrations up to 1,500 ppb (i.e., 24 ppb Cu^{2+} as calculated with WQ4F software) or total Zn concentration up to 500 ppb (i.e., 200 ppb Zn^{2+}). However, larval swimming was significantly reduced by 1,500 ppb Zn (i.e., 610 ppb Zn^{2+}) (*t*-test, $p < 0.01$). *C. gigas* larval searching behavior (induced by L-DOPA) was inhibited by total Cu at 1,500 ppb and more so ($p < 0.01$) by total Zn at 1,500 ppb. *C. gigas* metamorphosis, artificially induced EPI, was inhibited ($p < 0.01$) by 1,500 ppb total Cu and by total Zn ≥500 ppb. As total Zn concentration approached 1,500 ppb, all larvae remained quiescent.

C. virginica larvae were similarly but not identically affected by Cu and Zn (Table 1). The data were influenced by the fact that the ratios of free ion to total metal concentrations of Cu and Zn

TABLE 1.

Effects of copper and zinc on oyster larvae, *C. gigas* and *C. virginica*, including swimming activity, searching behavior, metamorphosis, and set on biofilm.

Oyster (Salinity)	Metal	[M][a] (ppb)	$[M^{2+}]$[b] (ppb)	Swim[c]	Search[d]	Metamorphose[e]	Set on biofilm[f]
C. gigas							
(37 $g \cdot kg^{-1}$	Cu	0	0	+ +[g]	+ +	+ +	+ +
		100	1.6	+ +	+ +	+ +	+
		500	8.0	+ +	+ +	+ +	−
		1,500	24.0	+ +	+ +[h]	+	−
	Zn	0	0	+ +	+ +	+ +	+ +
		100	40	+ +	+ +	+ +	+ +
		500	200	+ +	+ +	+	−
		1,500	610	+	−	−	−
C. virginica							
(24 7 $g \cdot kg^{-1}$)	Cu	0	0	+ +	+ +	+ +	+ +
		100	1.7	+ +	+ +	+ +	+
		500	8.3	+ +	+	+ +	−
		1,500	25.0	+[i]	−	−	−
	Zn	0	0	+ +	+ +	+ +	+ +
		100	50	+ +	+ +	+ +	+
		500	250	+ +	+	−	−
		1,500	750	+ +	+	−	−

[a] Initial dissolved absolute metal concentration in seawater

[b] Estimated concentrations of free Cu^{2+} or Zn^{2+} ions according to metal speciation program (WQ4F) at pH 8 in specific salinity of MBL seawater.

[c] Swimming activity after 96 h.

[d] L-DOPA-induced searching behavior after 96 h of metal exposure.

[e] EPI-induced metamorphosis. Metals were added with EPI for 4 h of exposure; then, the water containing EPI was removed and replaced by water containing only appropriate concentration of metals.

[f] Biofilms of 5-day cultures. Marine broth was replaced with metal-amended MBL seawater 24 h (period of metal-film binding reaction) before the addition of competent oyster larvae.

[g] Activity response: + +, >80% vs. control; +, 35–80% vs. control; −, <35% vs. control. "No" metal controls were done with every individual experiment, so valid comparisons could be made. Control responses varied with larval batch and other variables as follows: Swim, 90–100%; Search, 70–100%; Metamorphose, 50–90%; Film set, 20–70%.

[h] 83% vs. control but statistically significant ($p < 0.01$).

[i] 75% vs. control but statistically insignificant at $\alpha = 0.01$.

were, respectively, 4 and 23% higher in 24.7 g of salts · kg^{-1} seawater (*C. virginica*) than in 37 g of salts · kg^{-1} seawater (*C. gigas*). Swimming and search behavior were affected more by Cu and less by Zn than for *C. gigas*. On the other hand, artificially EPI-induced metamorphosis was affected approximately equally by both metals in both species of *Crassostrea*.

The last column of data, set on biofilms, should be assessed considering at least three additional components that influence the results: (A) set on biofilms must be preceded by search behavior (see introduction) and cementation before metamorphosis, so that the additional factors could make the process more sensitive to potential toxics; (B) the metals are bioconcentrated to some extent; (C) the metals in the biofilms may not be totally available (see Discussion).

The net result was that, in the presence of biofilms, the experimental aqueous metal concentrations became more inhibitory to metamorphosis (Table 1). To compare one treatment with another, the effective concentrations of metals that cause 50% reduction in larval normal responses (EC_{50}) were estimated. The aquatic EC_{50} of initial total dissolved Cu or Zn for EPI-induced larval metamorphosis of *C. gigas* decreased from 1,200 ppb Cu or 500 ppb Zn to <100 ppb Cu or <200 ppb Zn for natural set in the presence of biofilms; that for *C. virginica* decreased from 1,000 ppb Cu and 330 ppb Zn to 500 ppb Cu and 170 ppb Zn, respectively. Therefore, in the presence of biofilms, the detrimental effects of Cu increased more than 10-fold for *C. gigas* and twofold for *C. virginica*, and those for Zn increased threefold and twofold, respectively.

In situ, larvae would be continuously exposed to environmental concentrations of Cu and Zn. However, the following series of experiments asked the question of whether the toxic effects are exerted during or after the induction signal. Both Cu and Zn exerted their most profound effects after metamorphic induction with EPI (Fig. 1). There was significant inhibition in postinduction metamorphic development at 1,500 ppb total dissolved Cu and ≥500 ppb total dissolved Zn; inhibited larvae remained quiescent. Larval metamorphosis was usually not affected when metals were present only during the EPI-induction period. In the presence of 1,500 ppb Cu, a longer EPI-induction period (12 h) was required than in controls; those larvae that did not metamorphose by 4-h EPI induction remained swimming in the water column. Thus, induction time and metal concentration both influence EPI-induced metamorphosis.

As was the case for *C. gigas*, the EPI-induced metamorphosis of *C. virginica* was inhibited more by coincident exposure to Cu than to Zn (≥500 ppb total dissolved Cu and ≥1,500 ppb total dissolved Zn) (Fig. 2). The postinduction metamorphic development of *C. virginica*, like that of *C. gigas*, also was inhibited by 1,500 ppb Cu (i.e., 25 ppb Cu^{2+}) and ≥500 ppb Zn (i.e., ≥250 ppb Zn^{2+}). Only a 4-h EPI exposure period was used, because at longer induction periods, *C. virginica* larvae responded erratically.

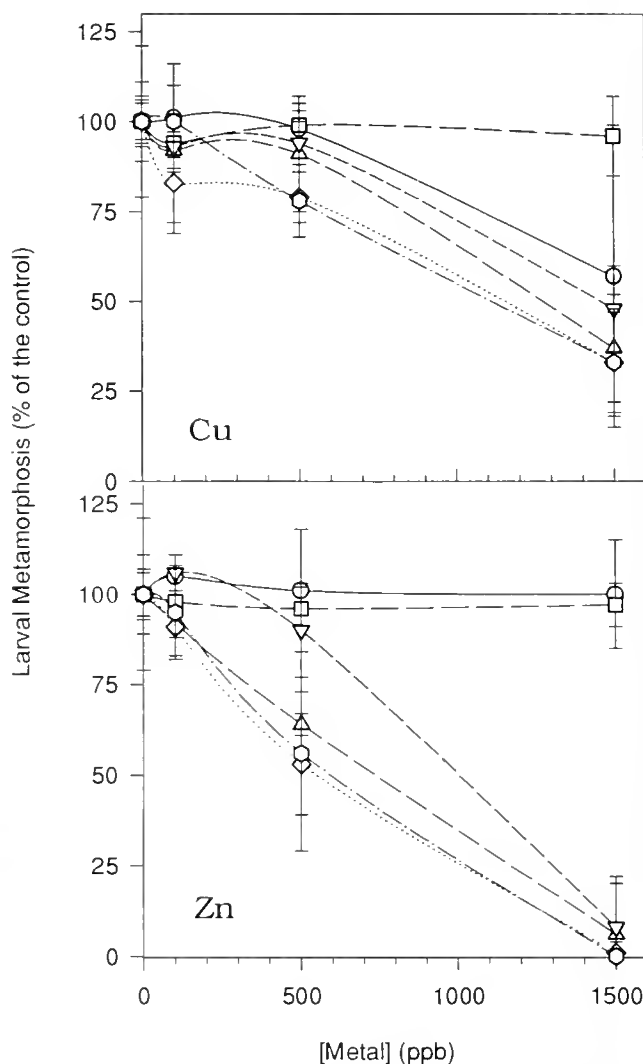

Figure 1. Effects of timed exposure to Cu and Zn on *C. gigas* larval metamorphosis (larval response normalized to control). Experiments were divided into three parts: exposure to EPI with metals for 4 (○) or 12 h (□) and then to clean seawater for 96 h; exposure to EPI without metals for 4 (△) or 12 h (▽) and then to metals for 96 h; exposure to EPI with metals for 4 (◇) or 12 h (⬡) and then to metals for 96 h. Error bar = 95% confidence interval.

Chesapeake Bay Samples

Because experimental models suggested that biofilm bacteria concentrated metals to levels where metamorphosis was inhibited, it remained to use the biofilm model (bioassay) to test natural Chesapeake Bay water, which contains very low concentrations of these metals. Even when concentrated 50-fold by the APDC/MIBK extraction method, total Cu and Zn in water near the Baltimore Harbor and middle bay remained undetected by the methods used here. Because 50 ppb was the preconcentration detection limit, it would mean that even in polluted Baltimore Harbor, the concentration was <1 ppb. Nevertheless, total percent larvae cemented on control biofilms was less in Baltimore Harbor water than in middle bay water or MBL seawater (Table 2). Levels of metamorphosis of set larvae were at least as high in harbor and bay water compared with artificial seawater. This finding makes use of the system as a bioassay, and any one of a number of factors, together or in combination, could have influenced set. Salinity was not an overriding variable, however, because harbor water supported more metamorphosis than did artificial water. Significantly, harbor water did not inhibit *C. virginica* larval swimming, searching, and EPI-induced metamorphosis; it did inhibit biofilm-induced set in the model bioassay organism, *C. gigas*.

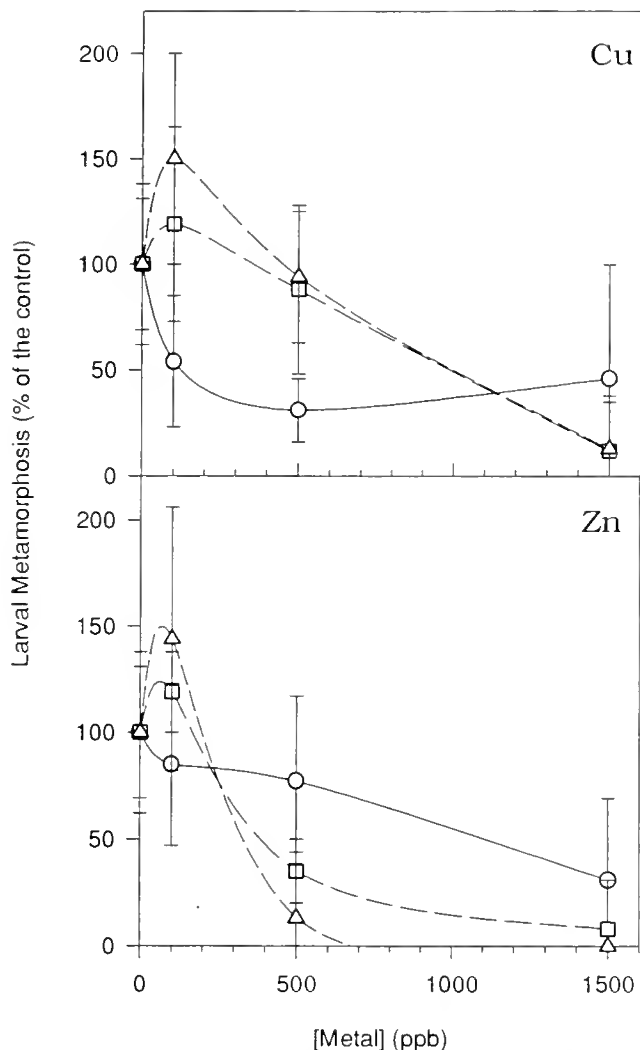

Figure 2. Effects of timed exposure to Cu and Zn on *C. virginica* larval metamorphosis (larval response normalized to control): ○, exposure to EPI with metals for 4 h and then to clean seawater for 96 h; □, exposure to EPI without metals for 4 h and then to metals for 96 h; and △, exposure to EPI with metals for 4 h and then to metals for 96 h. Error bar = 95% confidence interval.

DISCUSSION

Metal speciation in seawater affects bioavailability. Experiments were carried out in environmentally relevant salinities. In MBL seawater with salinity 24.7–37 g · kg^{-1} at pH 8, free available Zn^{2+} ions comprised about 40–50% of the total Zn, but free available Cu^{2+} ions comprised only about 2% of the total Cu. Thus, when total metal concentrations are considered, Zn is more toxic than Cu. However, when concentrations of free Cu^{2+} and Zn^{2+} are compared (Table 1), the opposite is true.

Normally, the length of the EPI-induced period required for competent larvae to metamorphose is 2 h for >80% metamorphosis (Coon et al. 1986). However, a longer period of EPI exposure was required to induce *C. gigas* larvae to metamorphose in 1,500 ppb Cu-amended seawater (37 g · kg^{-1}). Cu may have disturbed EPI induction through its interference with larval EPI receptors, or through its chemical complexation with EPI. Zn coordinates fewer ligands and only inhibited the EPI induction of *C. virginica* in seawater with lower salinity (24.7 g · kg^{-1}).

TABLE 2.

Effects of Chesapeake Bay water and MBL seawater on *C. gigas* larval set on biofilms.

Water[a]	Salinity (g · g^{-1})	% Attached[b]	% Metamorphosed[c]
MBL	37	63 ± 7	18 ± 7
MCB	12	53 ± 15	39 ± 20
BH	6	35 ± 27	26 ± 20

[a] MBL, Marine Biological Laboratory artificial seawater; MCB, middle Chesapeake Bay water; BH, Baltimore Harbor water.
[b] Percentage (mean ± 95% confidence interval) of total cemented larvae/total larvae.
[c] Percentage (mean ± 95% confidence interval) of metamorphosed cemented larvae/total larvae.

Most significantly, Cu or Zn concentrations of 100 ppb in estuarine water did not affect oyster larval viability, swimming, DOPA-induced searching behavior, or EPI-induced metamorphosis; however, they unequivocally inhibited larval set in the presence of biofilms compared with control films, which had not been exposed to additions of Cu or Zn. Although Cu was less toxic than Zn in seawater because less Cu^{2+} than Zn^{2+} was present, Cu became more toxic to larval set on biofilms, implying that Cu was more bioavailable than Zn in the films. Biofilm Cu concentrations at around 15–20 ppm (μg/g of biofilm wet wt.) measured directly in biofilms prevented 50% *C. virginica* larvae from set (Chang 1985). Aqueous concentrations of dissolved total Cu that might lead to this bioconcentration were estimated to be 0.1–0.2 ppm.

Phelps and Mihursky (1986) also found that competent larval oysters (*C. virginica*) had decreased set with increasing Cu concentrations. However, they found an LD_{50} of 534,000 ppb, considerably higher than that reported here and elsewhere (e.g., Mittleman and Geesey 1985, Calabrese et al. 1973). It is possible that the aufwuchs did not readily release detectable metal species in that particular system.

Bound metals can be released from biofilms through highly dynamic biologic activities (e.g., redox and pH gradients in biofilms) (Bender et al. 1995). Hornor (1984) indicated that aquatic heterotrophs can leach and solubilize metallic sulfides at neutral to slightly acidic pH and release heavy metals to the water column under oxygen-limited conditions. The author concluded that two mechanisms were responsible for the leaching: (A) the microbial production of solubilizing agents (i.e., low-molecular-weight organic chelating agent) that diffuse up from sediments and compete with anionic ligands (sulfides) for metallic complex formation; and (B) sulfide oxidation by facultative anaerobes or microaerophiles. Francis (1990) also summarized the mechanisms of metal mobilization mediated by heterotrophic microbes in mixed wastes. These include (A) changes in pH that determine solubility; (B) oxidation-reduction reactions and redox potential, which affect valence states and solubility; (C) chelation, solubilization, and leaching of elements by microbial metabolites and decomposition products; (D) biomethylation and production of volatile and/or toxic alkylated metal compounds; (E) biodegradation of organic complexes of metals; and (F) replacement of Ca^{2+} by heavy metals from anionic ligands (Snoeyink and Jenkins 1980). Biofilm-bound met-

als could also be released through the ingestion of EPS by the low pH in the gut. Metal-contaminated biofilms, fed upon by larvae, would be a route of entry, because some bacteria serve as food sources or growth factors (Douillet and Langdon 1993, Zobell and Allen 1935).

The 12-day LC_{50} of Cu for 2-day-old *C. virginica* D-hinged larvae was reported at 32.8 ppb (Calabrese et al. 1977). Results reported here showed that larvae retained swimming activities (70% of the control) at 1,500 ppb Cu for 4 days. In addition to time of exposure, variations may be attributed to the earlier study's selection of D-hinged larvae versus older competent larvae here; younger larvae generally are more susceptible to pollutants than older larvae. Calabrese et al. (1977) reported that the pH was maintained at 7.0–8.5, which would vary $[Cu^{2+}]$ from 30% to <2% of the total [Cu]; this could result in toxicity variations >15 times. In this study, the pH was maintained around 8.0, at which $[Cu^{2+}]$ comprised about 2% of the total Cu. Last, in the earlier study, larvae were continually fed with algae during the test period; therefore, metal contamination and bioconcentration through algae become a factor. Each of these variables would lower the LC_{50}, making the results more comparable with this report. It is significant that these bioassay systems are very sensitive to specific habitat conditions (Phelps and Mihurksy 1986, Calabrese et al. 1973, Calabrese et al. 1977, this report), underscoring their potential utility in assessing toxicity in situ.

The data with our biofilm/oyster system, as a bioassay for Chesapeake Bay water, were interesting. Seven to 15 ppb Cu^{2+} and 150–230 ppb Zn^{2+} inhibited EPI-induced *Crassostrea* metamorphosis. Moderately polluted portions of the Chesapeake Bay have been shown to contain 1.6×10^{-4} to 5×10^{-3} ppb Cu^{2+} and 3–50 ppb Zn^{2+}; less polluted portions have 1.6×10^{-5} to 3.2×10^{-5} ppb Cu^{2+} and 0.1–0.5 ppb Zn^{2+} (Sunda et al. 1990). Preliminary data of bioconcentration factors of autochthonous films (i.e., ratio of metal concentration in films over that in ambient water) for these metals ranged from 6,000 to 8,000 for Cu and 1,000 to 2,000 for Zn (biofilm dry wt.), depending on the physical parameter and the type of biofilm (Chang 1995). Thus, the predicted concentration in biofilms in 0.96–40 ppb Cu^{2+} and 3×10^3 to 1×10^5 ppb Zn^{2+} in moderately polluted areas and 0.1–0.3 ppb Cu^{2+} and 100–1,000 ppb Zn^{2+} in less polluted areas. If these metals were made available to the larvae, as our biofilm studies suggest, then Cu, in moderately polluted portions of the bay, and Zn in both the polluted and the less polluted portions of the bay, would be concentrated enough to affect set. The results are consistent with that suggestion.

The concentration of Cu in sediments of the Chesapeake Bay may range from <2.0 to 130 ppm and that of Zn may range from <10 to 600 ppm (Wright 1987). If even 0.005–0.8% of Cu or 0.03–2% of Zn partitioned out of the sediments and into set-inducing biofilms, it could affect oyster recruitment. In any case, this study clearly demonstrated that metal toxicity to larval set can be increased by 10-fold in the presence of biofilms. Metamorphosis from the metalarva to the juvenile stage often is used as a sensitive indicator in toxicity tests (Phelps and Warner 1990), because during the transition, there is considerable anatomical reorganization, the larvae are under stress, and toxic ions are more likely to interfere with their metabolic processes. All of this occurs on biofilms (Prieur et al. 1990). Larvae that fail to metamorphose occurs on biofilms (Prieur et al. 1990). Larvae that fail to metamorphose die. Thus, toxic metals may be bioconcentrated where and when oysters are most sensitive, which should be considered in pollutant regulations.

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HAPLOSPORIDIAN INFECTIONS OF THE PACIFIC OYSTER, *CRASSOSTREA GIGAS* (THUNBERG), IN CALIFORNIA AND JAPAN

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ABSTRACT Haplosporidian infections were observed in adult and seed Pacific oysters, *Crassostrea gigas* (Thunberg), in Drakes Estero, CA, and in seed Pacific oysters from Matsushima and Watanoha Bays, Japan. Up to 7% of domestic Pacific oysters and those imported from Japan that were examined from Drakes Estero had mild systemic or localized haplosporidian infections. Plasmodia were observed within the gills and connective tissues surrounding the stomach and intestine or, more commonly, within the epithelium of the heart. An inflammatory response was observed in response to plasmodia within connective tissues; inflammation was not observed in infections within the heart. Multinucleated plasmodia were also observed in the heart of 1–3% of seed oysters examined from Matsushima and Watanoha Bays, Japan. Results from this study suggest that haplosporidia are established at very low levels in Pacific oysters reared in Drakes Estero, CA. These data also suggest that Pacific oysters imported into Drakes Estero from Matsushima Bay, Japan, were a likely source of the introduction of the haplosporidian in California.

KEY WORDS: Haplosporidian, *Crassostrea gigas*, oysters, California, Japan

INTRODUCTION

Haplosporidia that closely resemble *Haplosporidium nelsoni*, also known as MSX, the causative agent of Delaware Bay Disease in the Eastern oyster, *Crassostrea virginica* (Gmelin), were observed in Pacific oysters from Matsushima Bay, Hokkaido, Japan, that were sampled for routine health examination before import into California (Friedman et al. 1991). Pacific oysters had been imported from Matsushima Bay, Hokkaido, Japan, into Drakes Estero, CA (~25 miles north of San Francisco, CA), for many years, ending in 1989 (R. Collins, California Department of Fish & Game, pers. comm.). These facts raised concern that the haplosporidian may have been introduced with Japanese Pacific oysters into domestic oyster populations in California. Domestic and imported oysters in Drakes Estero were examined to assess whether haplosporidia infected Pacific oysters in this embayment. This study describes the first observation of nonsporulated haplosporidian-like protozoans in Pacific oysters spawned and reared domestically in Drakes Estero, CA.

MATERIALS AND METHODS

Oyster Collections

Pacific oysters were collected from four oyster beds in Drakes Estero, CA (Fig. 1), and from Japan, as outlined in Table 1. Domestic oyster stocks had been reared in California, Washington, or Oregon for at least one generation. Imported stocks had been introduced from Japan as seed for growout. The sampling method was designed to detect an infection prevalence of at least 5%, given a population size of at least 2,000 animals (Amos 1985).

Light Microscopy

All oysters were shucked at the Fish Health Laboratory, California Department of Fish and Game. The heart and a 2- to 4-mm cross-section that included digestive gland and gills were excised, placed in invertebrate Davidson's solution (Shaw and Battle 1957) for 24 h, and processed for routine paraffin embedding (Luna 1968). Five-micrometer deparaffinized tissue sections were stained with Harris' hematoxylin and eosin (Luna 1968) and viewed by light microscopy.

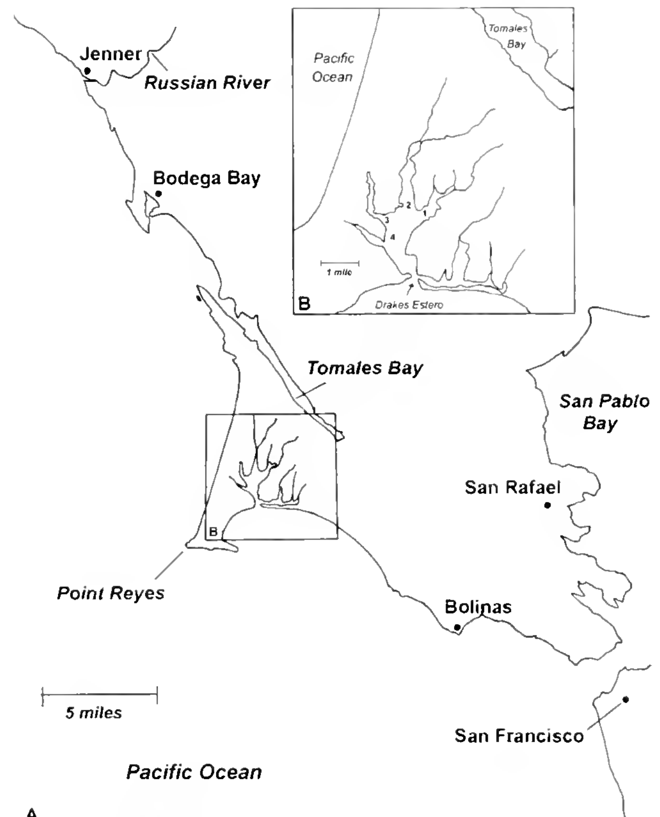

Figure 1. (A) Map illustrating the proximity of Drakes Estero to San Francisco, CA. (B) Inset illustrates the sampling locations within Drakes Estero, CA: (1) Home Bay; (2) Schooner Bay; (3) Creamery Bay; (4) Berries Bar.

TABLE 1.

Sample information of Pacific oysters examined for haplosporidian infections.

Location	Date	Origin	Number	Oyster Size	% Positive
Drakes Estero, CA	2 March, 1990			94–148 mm[a]	
Berries Bar		Domestic	60		5
Creamery Bay		Domestic	60		2
Schooner Bay		Domestic	60		7
Home Bay		Japan[b]	60		0
Drakes Estero, CA	2 May, 1990				
Berries Bar		Domestic	60	56–98 mm	3
Creamery Bay		Domestic	118	20–152 mm	0
Schooner Bay		Domestic	17	52–74 mm	0
		Japan[b]	20	102–127 mm	5
Home Bay		Domestic	60	54–78 mm	0
Drakes Estero, CA	23 January, 1992	Domestic[a]	60[a]	51–76 mm[a]	
Berries Bar					0
Creamery Bay					0
Schooner Bay					5
Home Bay					2
Matsushima Bay, Japan	8 January, 1993	Japan	100	<10 mm	1
	7 July, 1993	Japan[c]	72	<10 mm	3
Watanoha Bay, Japan	8 January, 1993	Japan	100	<10 mm	1

[a] Data apply to all locations.
[b] Matsushima Bay.
[c] Animals were held in sand-filtered seawater at 10–15°C at the Bodega Marine Laboratory for 11 mo before testing.

RESULTS AND DISCUSSION

Haplosporidian infections were observed in up to 7% of the oysters from Drakes Estero and in up to 3% of those from Japan examined in this study (Table 1). Lesions in oysters from California and Japan resembled those previously described by Friedman et al. (1991) and were characterized as focal or diffuse with small, multinucleated plasmodia (6–18 × 11–18 μm) and a mild infiltration of hemocytes into affected tissues surrounding the parasites. Connective tissue necrosis was observed in heavy infections. Plasmodia in the gills were larger than those in connective tissues in systemic infections, and sizes ranged from 12–36 × 14–44 μm. Unlike previous descriptions of haplosporidiosis in Pacific oysters, haplosporidian plasmodia were most frequently observed within cardiac epithelia in this study. In fact, haplosporidian infections in oysters from Japan were limited to the heart. Overall, 67% of the infected oysters had 12–32 × 14–40 μm, multinucleated plasmodia in the cardiac epithelium that resembled those observed in the gills (Fig. 2). However, unlike in the gills, no inflammation was associated with the large plasmodia in cardiac epithelia and these may represent latent infections. This is the first reported observation of haplosporidia in bivalve cardiac tissue, and this novel observation may, in part, be attributed to a frequent lack of examination of this tissue in health examinations and histologic studies (Howard and Smith 1983).

Haplosporidian infections of unknown pathogenicity have been observed in Pacific oysters from many locations worldwide, beginning in the early 1960s. Sporogonic and/or plasmodial stages of haplosporidians that resemble *H. nelsoni* were observed in *Crassostrea gigas* examined from Washington state (Pereyra 1964), Taiwan (Rosenfield et al. 1966), Korea (Kern 1976), and Japan (Friedman et al. 1991). This study is the first documentation of an endemic haplosporidian-like protozoan in domestic Pacific oyster, *C. gigas*, populations in California.

Members of the *Haplosporidiidae* are distinguished on the basis of the nature and origin of spore ornamentation (Perkins 1979; Van Banning 1979; McGovern and Burreson 1990). Only plasmodial stages of the haplosporidian-like protozoan were observed in this study; therefore, neither taxonomic placement nor conclusive elucidation of the source of this parasite was possible using morphological characteristics. However, the morphology and target tissues of the haplosporidia from Japan and California were identical. These observations, in conjunction with the history of importing Japanese Pacific oysters into Drakes Estero, suggest a common ancestry and that the haplosporidian may have originated from Japan.

Catastrophic die-offs of Eastern oysters, *C. virginica*, infected with haplosporidians have been reported (e.g., *H. nelsoni*, Andrews 1976, Ford and Haskin 1982); however, no mortalities of Pacific oysters were observed in oysters infected with the haplosporidian described herein. Thus, the pathogenicity of this protozoan for Pacific oysters and its effect on populations remains to be determined. Genetic analyses are needed to determine the taxonomic relationship between the *Haplosporidium* sp. that infects Pacific oysters in Japan; haplosporidia in Pacific oysters from California, Taiwan, and Korea; and known haplosporidia, such as *H. nelsoni* in the Eastern oyster (*C. virginica*). The expansion of oyster culture and the associated transplantation of commercially successful species (e.g., *C. gigas*) to new locations suggests that further studies are also needed to determine the pathogenicity and epidemiology of haplosporidian-like organisms in Pacific oysters.

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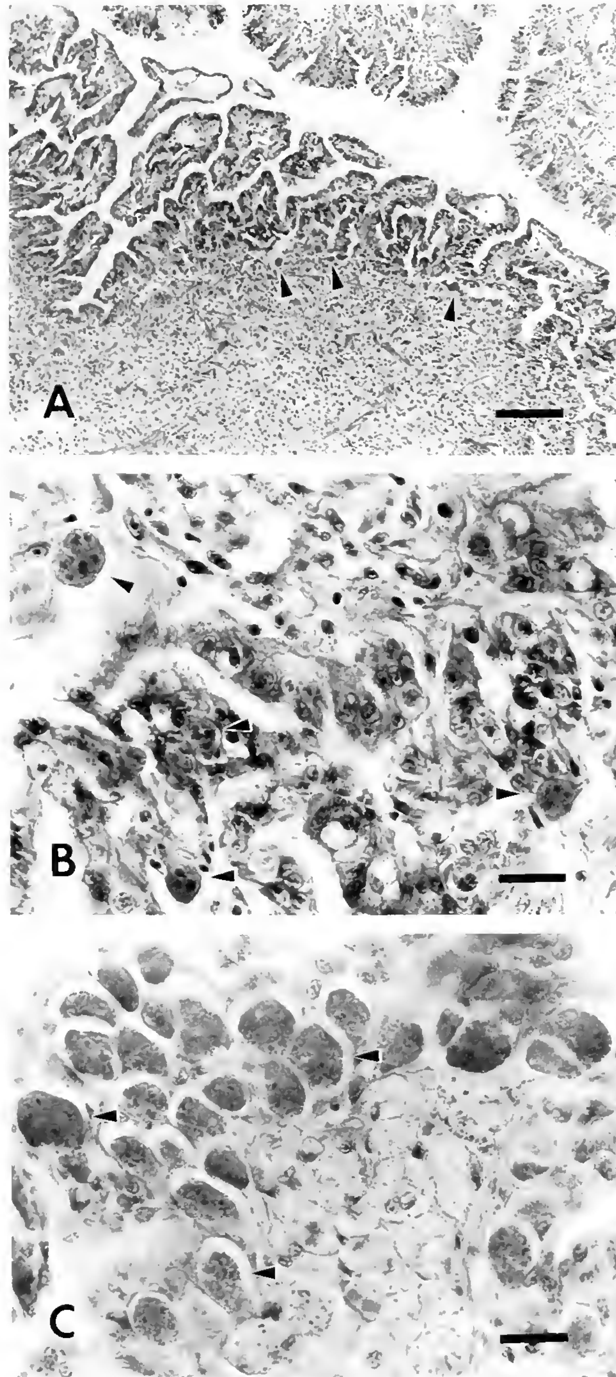

Figure 2. Multinucleated plasmodia within the cardiac epithelium of a domestic Pacific oyster from Drakes Estero, CA. (A) Low-magnification view illustrates plasmodia (arrowheads) within the ventricular epithelia. Note the lack of inflammatory response. Bar = 108 μm. (B) Arrowheads indicate multinucleated plasmodia within the cardiac epithelium. Bar = 18 μm. (C) Plasmodia (arrowheads) within the heart can be quite large and contain numerous nuclei. Bar = 18 μm.

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SECONDARY PRODUCTION, GROWTH AND SURVIVAL OF THE PACIFIC OYSTER *CRASSOSTREA GIGAS* (THUNBERG) IN TROPICAL WATERS, BAHIA DE LA PAZ, MEXICO.

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ABSTRACT Since 1973, when the Japanese oyster was introduced into Mexico, its cultivation has extended into the Northwest Mexican Pacific, yielding the expected profits in only a few areas. The objectives of the project were to determine the mortality, growth, biochemical variables, condition factor, meat yield, and somatic secondary production of *Crassostrea gigas* (Thunberg) in suspended and bottom conditions in Bahia de La Paz and to assess the use of each variable for the cultivation of this resource. Laboratory-reared spat of similar size were introduced with replication into two different areas, both in suspended trays and on the bottom surrounded by a plastic mesh fence. The organisms were checked monthly for almost 2 y. The average growth rates obtained were 6 mm/mo for the first 6 mo and 5.3 mm/mo at 10 mo. The weight gain was 3.6 g/mo for 10 mo. Mortality rates for this period were 35%. The average values of meat yield were 0.35: protein, 50.9%; lipids, 8.2%; carbohydrate, 37.7%; and ash, 3.2%. The somatic secondary production of dry weight was 2.5 g per organism during the first year. The results of the multifactorial analysis of variance estimation with each variable obtained led to the conclusion that only somatic production gave statistical elements for selection and culture decision making among all factors tested.

KEY WORDS: Molluscs, production, Pacific oyster, culture

INTRODUCTION

Molluscs are a dominant component of the invertebrate biomass of coastal, estuarine (Landdon and Newell 1990), and tropical ecosystems. In Mexico, commercial interest in molluscs has gradually increased because of the size and value of its catch, its species diversity, and the prospects for future exploitation. Baqueiro and Castagna (1988) referred to a great abundance and high diversity of molluscs along the Mexican coast. They also noted that molluscs are captured by artisanal fishing and that many species are endangered because of high fishing pressure.

The Pacific or Japanese oyster (*Crassostrea gigas*) was introduced into Mexico in 1973 (Islas 1975). There has been intensive cultivation along the Baja Peninsula and the Gulf of California, extending as far as the tropical waters of southern Mexico. Its cultivation has proceeded even though the practice has not been investigated in any depth nor have the effects on native organisms been studied. In Bahia de La Paz, cultivation attempts have been made since 1975 (Caceres et al. 1986) without the relevant studies being done. Several authors agree that the most accurate estimation of secondary production is one based on cohort studies (Negus 1966, Mathews 1970, Hall 1971, Golikov and Menshutkin 1973, Zaika 1973, Kinne 1978, Warwick 1980, Valiela 1984, Parsons et al. 1984, and Begon and Mortimer 1986). My objective was to study the secondary production, growth, mortality, and nutritional variables of *C gigas* in Bahia de La Paz, analyzing the usefulness of each variable as a tool for optimizing its cultivation.

MATERIALS AND METHODS

Study Area

Bahia de La Paz is located between 110°17′ and 110°25′ W and 24°06′ and 24°16′ N (Fig. 1). Its coast has many coves protected from wind and strong sea surges. The largest cove is Ensenada de La Paz (approx. 5,000 ha), a fertile body of water located at the southern end of Bahia de La Paz (Lechuga 1977). Lechuga described the Ensenada as a water body with high homogeneity in chlorophyll *a*. Bustillos and Olivares (1986), using the oxygen method, reported a gross primary production of 7.3 mg of C/m^3 per hour. Santoyo (1994) reported values from 2 mg of oxygen/m^2 per day in October to 11.5 mg of oxygen/m^2 per day in August. The climate of the area is warm and dry with annual rainfall of 210

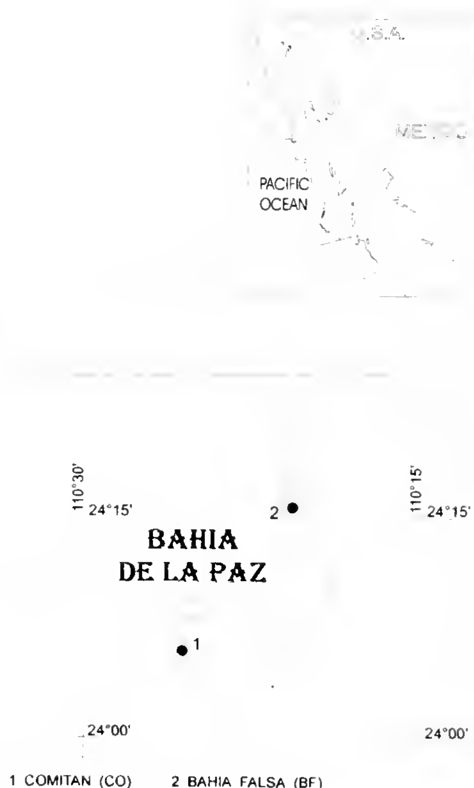

Figure 1. Study Area.

mm, an annual average water temperature of 24.7°C, and an average salinity of 36 with a salinity range from 34 to 37 (Flores 1994).

Villamar (1965) reports strong circulation and hydraulic exchange in Bahia de La Paz. The dominant winds are from the northwest with west southwest winds more common from April to August (Signoret and Santoyo 1980). The average depth of the bay is of 360 m (Flores 1994). The depth at the two study sites was 8 m.

The experimental design was based on previous studies of other bivalves in the area (Arizpe and Félix 1986). Brown and Hartwick (1988) emphasize that to optimize the production of an aquacultural species, it is necessary to select an adequate site. Similar-sized (20 to 25 mm), hatchery-reared spat were introduced with one replication into a protected site at Comitan (CO site 1) and an open bay site at Bahía Falsa (BF site 2). To compare suspended and bottom culture systems, one group was placed in square polypropylene trays, 0.6 m on each side and 0.08 m high ("Nestier" type), suspended at 0.3-m depth on long lines. A second group was placed directly on the bottom at 3 m in conditions and positions similar to those of wild organisms, but surrounded by a square plastic net fence (openings of 12.5 mm with sides of 10 m). The fence and trays were cleaned of macropredators each month. The bivalves were placed at stocking densities of 50, 100, and 150 organisms per square meter in both cultivation systems to determine the possible effects of intraspecific competition. Starting in March 1987, 12 different experimental treatments with replication were established at the two sites (sites 1 and 2), each with two different systems (systems 1 and 2), and each system with the three density levels (densities = 50, 100, and 150).

Each month, shell length (vernier caliper), wet weight, and the number surviving were recorded, in situ, with temperature and salinity measurements (Table 1). One hundred organisms (all approximately the same size) were placed alongside each treatment to obtain the different biometric measurements, to keep from damaging molluscs otherwise subjected to the rigorous monthly recording, and to avoid affecting the experimental design (Crisp 1984). Starting at the eighth month of the experiment (by this time, test animals were approaching commercial size), five individuals were selected randomly, each month, to determine length, total weight, visceral weight, condition factor, and dry weight of soft body parts after drying in an oven at 90°C to constant weight (Gardner and Thomas 1987).

Growth

Length and weight growth functions were estimated using the following methodologies:

(a) applying the Von Bertalanffy growth model, which has a good fit for describing the growth process in molluscs (Zaika 1973, Muhlia et al. 1980, Warwick 1980, Thompson 1984, Berg and Olsen 1989, Munro 1989, Caddy 1989, and Sukhotin and Maximovich 1994). The parameter calculations were derived by the method of Beverton and Holt (1957).

(b) calculating monthly and annual growth rates in weight using the equation, $G = \ln(Mt/Mo)/t$, where G is the instantaneous growth rate, and Mo and Mt are the average dry weight at the start and at the end of sampling period t (Chapman 1978, Crisp 1984, Morin et al 1988, and Fidalgo et al. 1994).

TABLE 1.

Temperature and salinity records in both study sites, Comitan (site 1) and Bahia Falsa (site 2).

	Site 1		Site 2	
Month	Temperature (°C)	Salinity	Temperature (°C)	Salinity
March	24.0	36.1	23.7	35.1
April	25.1	36.6	24.5	35.3
May	25.9	36.9	24.7	35.8
June	25.9	36.3	25.0	35.1
July	26.1	36.6	25.7	35.2
August	27.8	36.8	26.4	35.1
September	28.9	35.9	26.2	34.5
October	27.5	35.5	25.6	34.6
November	26.2	35.4	25.0	34.8
December	25.1	35.9	23.8	35.0
January	23.6	36.1	22.9	35.0
February	23.5	35.8	23.0	34.9
March	23 6	36.1	23.0	35.2
April	24 9	36.5	24.2	35.4
May	25.7	36.7	24.7	35.5
June	26.1	36.9	24.9	35.6
July	26.4	37.2	25.0	35.8
August	27.8	37.0	25.0	36.1
September	28.8	36.3	25.9	36.3
October	27.6	35.8	25.5	35.7
November	26.4	35.6	24.6	35.4
Average	25.8	36.1	24.7	35.0

Mortality

Mortality was estimated by computing monthly and annual instantaneous mortality rates by use of the general negative exponential model, $Z = \ln(Nt/No)/t$, where Z is the instantaneous mortality rate, and Nt and No are the number of individuals at the start and end of period t (Ricker 1975, Chapman 1978, Thompson 1984, Berg and Olsen 1989, Munro 1989).

Meat Yield, Condition Factor, and Biochemical Parameters

To estimate differences in the quality of production among treatments, standard biochemical methods were used to determine protein (by Macro-Kjeldahl), lipid (by Soxhlet extraction), ash (ashing direct method), and carbohydrates, by difference from 100% (A.O.A.C. 1980), with the survivors of all experimental treatments after 21 mo. I estimated several biometric relations, of which the best fitting were: (a) logarithm of dry weight without shell (DW) vs. logarithm of the total wet weight (WW). $DW = aWW.e^{b}$ was used to estimate dry weight for the first 7 mo in each experimental treatment because dry weight measurements only started in the eighth month; and (b) regression relationship between logarithms of total length (TL) and dry weight (DW), the slope of which has been defined as a condition factor (Beukema and De Bruin 1977, Bodoy et al. 1986). I also estimated another condition factor used in molluscs, the meat yield, from the quotient of visceral weight (VW) divided by the total wet weight (VW)/WW).

Secondary Production

Rigler and Downing (1984) examined different methods for estimating secondary somatic production and concluded that all

were simply different ways of obtaining Allen's (1951) weight increase integral per number of organisms over time. I determined the production of natural populations using the growth increment summation method (Crisp 1984, Rigler and Downing 1984, Kimmerer 1987, Morin et al. 1987), standardizing to 1,000 organisms to compare all treatments. Finally, I applied the Kolmogorov-Smirnof normality test, a homocedasticity test (Zar 1996), and then, an analysis of variance (ANOVA) multifactorial test with replication (Statgraphics 1986) to examine the effects of the treatments on the dependent variables.

RESULTS

Growth

The values of the parameters of the Von Bertalanffy growth model and the instantaneous growth rate (G) for each experimental treatment are presented in Table 2. The outer site (BF) in the K length-growth analysis had a maximum value of K = 0.135 compared with the inner site (CO) K = 0.096 for the same density and culture system. The bottom culture system had larger values than did the suspended culture. Monthly and yearly estimates were carried out for each treatment. In Table 2 are the results of the annual growth rates in weight for all of the experimental treatments. The instantaneous growth rate analysis showed results similar to K with respect to site and culture system, but for G, the density effect was clear. We found the largest values of G at the outer side, bottom culture at 50 inds/m^2. G decreases with increasing density. From multifactorial ANOVA applied to treatment means, no significant statistical evidence was found to denote differences between the K parameter means for sites, substrates, and densities, or for their interactions (Table 3). In the analyses of instantaneous growth rates for weight, no statistical evidence was observed for differences between experimental treatments for substrate and density. Site was the only significant factor affecting weight growth ($p < 0.0006$). The largest G values were found at Bahía Falsa, the outer site (Fig. 1).

Mortality

Table 2 also shows annual values for Z for each experimental treatment. There was no significant effect for Z when considering the area or density, but there was ($p < 0.00001$) for the culture system, with far greater mortality on the bottom. In Table 3 are the results of the multifactorial ANOVA test. I found, with a $p < 0.00001$, that there exists statistical evidence denoting that the population means of the mortality rates (Z) are different for the systems tested, with the highest mortality on the bottom. Non-statistically significant differences in the mean of Z as a function

TABLE 2.

Results of parameter K from Von Bertalanffy length growth model, annual weight rate G, and mortality rate Z for all experimental treatments.

Site	System	Density (No. of Organisms/m^2)	K (y^{-1})	G (y^{-1})	Z (y^{-1})
Comitan	Suspended	50	0.120	4.810	0.811
		100	0.070	4.811	0.663
		150	0.089	4.713	0.274
	Bottom	50	0.098	4.921	1.022
		100	0.096	4.834	1.139
		150	0.094	4.811	1.297
Bahia Falsa	Suspended	50	0.108	4.942	0.492
		100	0.105	4.739	0.238
		150	0.102	4.713	0.041
	Bottom	50	0.122	5.057	0.968
		100	0.135	4.878	1.309
		150	0.126	4.763	1.059

of density were found, although evidence exists for an interaction between substrate and density ($p < 0.0025$).

Meat Yield, Condition Factor, and Biochemical Variables

The summary results for meat yield, total length and weight, volumes, and *C. gigas* biochemical components at the end of the experiments are in Table 4. From the multifactorial ANOVA (Table 5), there is no statistical evidence for differences in meat yield, condition factor, lipids, and carbohydrate population means as a function of site, culture type, and density factors tested, or in their interaction. The results of the ANOVA test for proteins show a significant difference ($p < 0.01$) between the two study areas and culture systems. The highest protein values were at the outer site (BF) and in the bottom culture systems.

Secondary Production

Table 6 is an example of the production matrices calculated for the total study period for each experimental treatment. Estimates for monthly increments in weight (total and dry visceral) were integrated with the number of organisms to calculate monthly, annual, and total production for each experimental treatment. The cumulative production for the first year was 2.53 g per individual and, after 21 mo, 2.79 g per individual at site 1 (CO) in suspended system culture at a density of 50/m^2. Figure 2 shows for the first year of life the average monthly production decreases, corresponding to a gradual decrease of growth rate. The production decreases

TABLE 3.

Probability levels and statistic F obtained from values of growth K, weight rate G, and mortality rate Z by the use of a multifactorial ANOVA for the factors: site, system, and density.[a]

ANOVA Factors	Bertalanffy K Length		Weight Growth Rate G		Annual Mortality Rate Z	
	F	p	F	p	F	p
Site	0.091	0.770	19.40	0.001	6.464	0.024
System	0.036	0.854	0.011	0.918	13.950	0.000
Density	0.077	0.927	0.268	0.769	0.681	0.589

[a] No interactions were significant in K and G, but in Z, $p < 0.002$ interaction for culture system and density.

TABLE 4.

Final mean length, total and visceral weight, meat yield, total and visceral volume, proximal composition, and condition factor of cohorts in *Crassostrea* placed in different treatments.

Site	System	Density (No. of Organisms/m^2)	Total Length (mm)	Total Weight (g)	Visceral Weight (g)	Meat Yield	Total Volume (mL)	Visceral Volume (mL)	Dry Weight (%)	Protein (%)	Lipid (%)	COH (%)	Ash (%)	Condition Factor ($\times 10^{-4}$)
Comitan	Suspended	50	65.7	52	17.80	0.342	91.7	44.1	17.22	49.88	9.08	37.79	3.25	4.0
		100	65.0	51	17.28	0.339	91.8	41.4	18.77	50.01	8.62	38.19	3.18	5.0
		150	65.1	47	15.24	0.324	90.8	38.9	18.63	49.97	8.69	38.11	3.23	3.0
	Bottom	50	73.2	59	20.37	0.345	87.8	40.1	17.61	51.33	7.93	37.53	3.21	3.0
		100	73.1	55	19.34	0.352	87.9	39.8	17.89	51.02	8.09	37.70	3.19	3.0
		150	72.7	50	16.85	0.337	87.7	36.3	18.09	51.12	8.10	37.56	3.22	3.0
Bahia Falsa	Suspended	50	71.5	64	23.69	0.370	92.5	38.0	22.88	49.34	8.04	39.27	3.35	1.0
		100	71.4	52	17.81	0.343	90.1	36.2	20.97	50.17	8.13	38.47	3.23	2.0
		150	72.1	48	13.16	0.274	92.0	36.3	21.81	50.19	8.07	38.65	3.09	1.0
	Bottom	50	78.5	72	27.25	0.378	78.7	26.2	21.34	53.01	8.01	35.72	3.26	1.0
		100	78.3	57	20.81	0.365	69.9	25.4	21.09	52.24	7.69	36.80	3.27	1.0
		150	73.3	50	16.85	0.337	73.4	27.3	21.21	52.57	7.80	36.72	3.21	1.0

in the warmer months of summer (August and September) and in the first year of life. The greatest production (Table 5) was in July of the first year, with a value of 6.61 g per individual for total. Even though this was the highest monthly production value, the greatest yearly production was at the second area (Bahia Falsa) under suspended conditions and at a density of 50 organisms/m^2. In all treatments, production decreases from December 1987 to January 1988, in many cases resulting in negative values. A difference between production and biomass is noticeable at this point, because biomass levels vary little compared with production levels. After this production decrease, it began to increase again, but less than in the first year. Production was close to 0 between August and September (summer season) of year 2. The multifactorial (ANOVA) variance analysis test was applied to determine if differences exist in annual somatic production for each of the treatments (Table 7). The means of annual oyster production are different for the areas tested ($p < 0.0003$), with the greatest values at site 2 (BF). There are also greater statistical differences in culture system and density, with the largest values at the bottom and at a density of 50 organisms/m^2.

DISCUSSION

Comparing growth and mortality results obtained for *C. gigas*, Islas et al. (1982) give a monthly length-growth rate for the Japanese oyster of 9.2 mm and mortality rates of 20% over 6 mo for a site on the west coast of Baja California (approximately 28°N). At the same latitude, but in the Gulf of California, Ochoa and Fimbres (1984) found an average monthly length and weight growth of 7 mm and 7.7 g with a mortality of 30% over 10 mo. In Bahia de La Paz, I found slower growth in length, 6 mm/mo the first 6 mo, and at 10 mo, 5.3 mm/mo for length, 3.6 g/mo for weight, and greater mortality (35%). Acosta (1985) obtained average growth of 10 mm/mo and 6 g/mo over a year at an area at 30°N along the west coast of Baja California. In Japan, Fujiya (1970) and Korringa (1976) report growth for the first year of 60 g. Askew (1972) and Hall (1984) in Great Britain cite total weights of 55 and 70 g at 12 and 16 mo, respectively. Berthome et al. (1986) obtained total annual growth of 40 g at 46°N along the Atlantic Coast of France. In the Bay of Arcachon, Robert et al. (1993) achieved an annual mean weight of 35 g. The results of Berthome et al. are similar to the 43 g I obtained in a suspended system at site 1 with 50 organisms/m^2 (CO50), less than the 55 g in the BF50 treatment at the bottom, site 2 with 50 organiams/m^2, and the same as Shafee and Sabatie (1986) for an area at 36°N on the coast of Morocco.

When monitoring proteins, lipids, and carbohydrates as measures of production quality, as many authors call them (Baird 1957, Reinitz and Yu 1981, Boggio et al. 1985, Bodoy et al. 1986, Viola et al. 1988), my objective was not to study changes over time but rather to measure proteins, lipids, and carbohydrates at the end of the study as a comparison of changes in nutritional components over a year. Paez et al. (1993) did this with two species of *Crassostrea* in a study near Bahía de La Paz.

The results of the analysis of the relationship between the ex-

TABLE 5.

Probability levels and statistic F obtained from values of meat yield, condition factor, protein, lipids, and carbohydrates by the use of a multifactorial ANOVA on the factors: site, system, density.[a]

ANOVA Factors	Meat Yield		Condition Factor		Proteins		Lipids		Carbohydrates	
	F	p	F	p	F	p	F	p	F	p
Site	1.307	0.2753	0.963	0.3533	12.76	0.0031	0.261	0.6227	3.492	0.0827
System	0.461	0.5170	0.859	0.3794	9.773	0.0074	0.839	0.3849	2.572	0.1311
Density	0.021	0.9788	0.803	0.4675	0.631	0.5466	0.185	0.8333	0.108	0.8985

[a] No interactions were significant.

TABLE 6.

Production matrix of cohort in site 1 (CO), on the suspended system culture (C), and with a density 50/m².[a]

Month	L (mm)	WW (g)	N	WI (g)	WWP	DMW (g)	DI (g)	DMP	DMB (g)	P/B
1987 M	10.0	0.03	1,000			0.003			2.5	
A	24.4	3	1,000	2.97	2,965.0	0.21	0.21	207.6	210	0.99
M	25.8	7	944	4	3,777.8	0.49	0.28	264.4	462.8	0.57
J	26.7	12	944	5	4,722.2	0.84	0.35	330.6	793.3	0.42
J	30.1	19	944	7	6,611 1	1.33	0.49	462.8	1,256.1	0.37
A	32.2	23	944	4	3,777.8	1.61	0.28	264.4	1,520.6	0.17
S	33.3	26	944	3	2,833.3	1.82	0.21	198.3	1,718.9	0.12
O	34.9	29	833	3	2,500.0	2.03	0.21	175.0	1,691.7	0.10
N	41.7	33	722	4	2,888 9	2.31	0.28	202.2	1,668.3	0.12
D	56.1	39	667	6	4,000.0	2.73	0.42	280.0	1,820.0	0.15
1988 J	61.0	39	667	0	0.0	2.73	0.00	0.0	1,820.0	0.00
F	63.3	41	611	2	1,222.2	2.87	0.14	85.6	1,753.9	0.05
M	63.9	43	444	2	888.9	3.01	0.14	62.2	1,337.8	0.05
A	64.4	45	444	2	888 9	3.15	0.14	62.2	1,400.0	0.04
M	64.8	46	444	1	444.4	3.22	0.07	31.1	1,431.1	0.02
J	65.1	47	389	1	388 9	3.29	0.07	27.2	1,279.4	0.02
J	65.3	48	389	1	388 9	3.36	0.07	27.2	1,306.7	0.02
A	65.5	49	389	1	388 9	3.43	0.07	27.2	1,333.9	0.02
S	65.6	49	389	0	0.0	3.43	0.00	0.0	1,333.9	0.00
O	65 6	50	389	1	388 9	3.5	0.07	27.2	1,361.1	0.02
N	65.7	52	389	2	777.8	3.64	0.14	54.4	1,415.6	0.04
First-year production per 20 m²					36.19 kg			2.53 kg		
First-year monthly production per m²					3,015.6 g			211.1 g		
Total production per 20 m²					39.85 kg			2.79 kg		
Total monthly production per m²					1,992.7			139.5 g		

[a] Units recorded for standardizing to 1,000 organisms (N) were: length (L), individual wet weight (WW), monthly wet weight increment (WI), dry meat weight (DMW) dry meat weight increment (DI), dry meat biomass (DMB), and dry weight production/biomass quotient (PB). Wet weight production (WWP) and dry meat production (DMP) were calculated in grams per square meter by month.

perimental factors affecting growth (in length and weight), mortality, condition state, nutrition, and secondary production were analyzed with a multifactorial ANOVA to observe if differences existed among the conclusions made by considering the parameters separately and for decision making in aquaculture (Table 8). Higher values of growth rate and mortality were obtained at site 1 (CO). At site 2, high production was found. It is difficult to account for site differences because there are few studies of the region. Annual water temperature and salinity averages are higher at the inner site (25.8°C and 36.2 salinity) than at site 2 (24.7°C and salinity of 35.0). Lango (1994) showed the annual mean of other water variables for sites 1 and 2 to be: 5.2 and 5.6 mg/L of dissolved oxygen, 0.47 and 0.44 mg/L of seston, 0.40 and 0.037 mg/L of plankton, and 0.006 and 0.005 mg/L of tripton. There is nothing recorded about hydrodynamic conditions. Depth and sediment composition (fine sand) are also similar at the two sites. However, site 2 had a higher secondary production than site 1. At site 1 (CO), predators of bivalves like the fish *Sphoeroides annulatus* (Jenyns), snails of the genus *Strombus*, and the flatworm

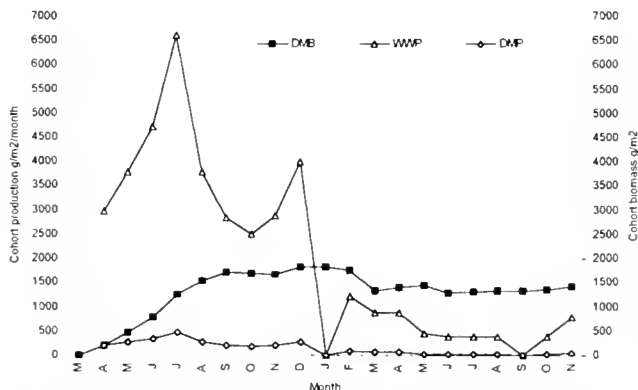

Figure 2. Curves of dry meat biomass (DMB), wet weight production (WWP), and dry meat production (DMP) at site 1, on the suspended culture system, at a density of 50 organisms/m² (Exp. code COC50).

TABLE 7.

Probability levels and statistic F obtained from values of production by the use of a multifactorial ANOVA on the factors: site, system, and density, and their interactions.

Factors	ANOVA—Production F	p
Site	23.042	0.0003
System	64.578	0.0000
Density	21.980	0.0000
Interactions		
Site-system	0.339	0.5759
Site-density	1.567	0.2431
System-density	4.799	0.0259

TABLE 8.

Overall results from multifactorial ANOVA of cohorts in *C. gigas* as a function of meat yield, condition factor, biochemical variables, K of Bertalanffy, weight growth rate, mortality, and the variables of somatical secondary production related to the experimental factors and their interactions.[a]

Parameter	Z Site	S System	D Density	Interactions Z × S	Z × D	S × D
Meat yield	–	–	–	–	–	–
Condition factor	–	–	–	–	–	–
Proteins	**	**	–	–	–	–
Lipids	–	–	–	–	–	–
Carbohydrates	–	–	–	–	–	–
Bertalanffy K	–	–	–	–	–	–
Weight growth rate G	**	–	–	–	–	–
Moratlity rate Z	*	**	–	–	–	**
Production	**	**	**	–	–	*
Meat yield	–	–	–			
Condition factor	–	–	–			
Proteins	BF	F	–			
Lipids	–	–	–			
Carbohydrates	–	–	–			
Bertalanffy K	–	–	–			
Weight growth rate G	CO	–	–			
Mortality rate Z	CO	F	–			
Production	BF	C	50			

[a] The specific factors from which the largest values were obtained are shown in the lower half. [(–) $p > 0.05$, (*) $p \leq 0.05$, (**) $p \leq 0.01$]

Stylochus sp., are common. This caused higher mortality at site 1, not just for *C. gigas* but also for a native bivalve, *Pinna rugosa* (Arizpe 1995). Oysters are better protected against predation in the suspended trays and have lower mortality.

Contrary to tropical bivalves like *P. rugosa*, local *C. gigas* production decreases in summer. The surface water temperature reaches 28–29°C in September. Statistical evidence exists for a density effect on production only (Table 8). The density effect did not appear in growth parameters, mortality, biochemical composition, meat yield, or condition factor. Mortality of growth rate, taken separately, can give contrary results, as in the case of site 1 (CO), which had better weight growth rate (G) but also high mortality. Using only one criterion such as mortality or growth increases the difficulty for a decision with respect to site location. Production showed a higher sensitivity to site location, culture system, and density, than to the other factors, perhaps because its determination includes both mortality and body growth. Maximizing production is directly relevant to the success of aquaculture ventures. Small-scale plantings of similar-sized, hatchery-reared spat and the monitoring of population production are the best approaches for assessing site suitability for the culture of *C. gigas*.

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STUDIES ON TRIPLOID OYSTERS IN AUSTRALIA. VII. ASSESSMENT OF TWO METHODS FOR DETERMINING TRIPLOIDY IN OYSTERS: ADDUCTOR MUSCLE DIAMETER AND NUCLEAR SIZE

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ABSTRACT Two methods for distinguishing triploid Pacific oysters [*Crassostrea gigas* (Thunberg)] from diploid oysters were assessed. Adductor muscle diameter in relation to valve height was significantly ($p < 0.001$) greater in samples of triploid oysters than in diploid samples and was influenced by site. However, variation in this measure was too large to allow individual oysters to be distinguished as either triploid or diploid. A second method was assessed that used differences in the nuclear size of hemocytes and the intensity of staining of hemocyte nuclei to distinguish between diploids and triploids. Histological sections, prepared by standard paraffin histology, were stained for nuclear histones with Gill's hematoxylin. Integrated nuclear optical density and nuclear area were recorded with image analysis. This method was effective in distinguishing individual oysters as diploid or triploid. When histological specimens are required, this method is less expensive than other techniques used to determine triploidy.

KEY WORDS: Triploid, oysters, adductor muscle, nuclei, image analysis

INTRODUCTION

In any attempt to produce triploid bivalves, it is necessary to test to what degree this has been achieved, because generally, some diploid individuals are also produced. The ratio of diploids to triploids is established to save wasted labor rearing batches of larvae when the proportion of triploids is low (Beaumont and Fairbrother 1991), spat before sale, broodstock for the production of tetraploids (Guo and Allen, 1994), and research of triploid performance. Where histologic data are required from these oysters, it would be useful to also establish ploidy by histology.

In an evaluation of the performance of triploid Pacific oysters, *Crassostrea gigas* (Thunberg), at commercial leases in Tasmania (Gardner et al. in prep., Maguire et al. in prep.), it was necessary to establish the ploidy of adult oysters sampled for the histology of gonad development. In concurrent samples, the proportion of diploid oysters in the triploid group was assessed by the use of flow cytometry, although these samples did not include oysters processed for histology. It was found that approximately 25% of the "triploid" group were diploid oysters. Individuals among the putative triploids that developed large gonads may have been diploids; alternatively, they may have been triploids where gametogenesis was less inhibited. Therefore, we attempted to develop another indicator of triploidy that could be used to retrospectively assess samples already processed for histology. Histologic samples could then be used to accurately describe the gametogenesis of triploid oysters and to compare sexes in relation to the suppression of gametogenesis (Gardner et al. in prep.).

Several techniques have been described for the determination of the ploidy of bivalves; the two approaches most frequently used are karyotypic analysis and flow cytometry. Karyotypic analysis involves the preparation, staining, and counting of the chromosomes of separate nuclei (Kilgerman and Bloom 1977, Allen 1983). Although karyotypic analysis is an accurate measure of ploidy, it is very time consuming (Komaru et al. 1988). Flow cytometry is capable of recording the DNA content of cells at a much greater rate, in the order of 10,000–100,000 nuclei per sample. This technique is particularly useful for the estimation of the percentage of triploids in a population, as is required in hatcheries (Chaiton and Allen 1985).

The purpose of this study was to retrospectively verity the ploidy of oysters that had been processed for histology, and also to evaluate techniques that could be used for the assessment of individual oysters in subsequent studies. This was approached in two ways. First, a morphological indicator, adductor muscle diameter in relation to whole body size, was used; it was anticipated that this technique, if effective, could be used on farms. Second, the size and staining intensity of nuclei in histologic sections were assessed.

The first approach, measurement of body structure, was assessed by measuring the size of the adductor muscle in relation to the size of the whole organism. It was hypothesized that the increased DNA content of muscle fiber nuclei may be reflected in the diameter of muscle cells. This in turn may affect the diameter of the entire adductor muscle and an increase in adductor muscle diameter. To some extent, the large nuclei may increase the size of the whole organism (polyploid giantism), so that there would be no increase in the relative size of the adductor muscle. However, an increase in adductor muscle size from triploidy has been reported for Sydney rock oysters *Saccostrea commercialis* (Iredale and Roughley) (Nell et al. 1994). Factors other than nuclear size may influence the observed increase in adductor muscle size, such as increased storage of nutrients in the adductor muscle as a result of reduced gametogenesis (S. K. Allen, Jr., Rutgers University, pers. comm.).

The additional set of chromosomes within the nucleus of triploid cells can be expected to alter the size of the nucleus and/or the density of the DNA contained therein. A simple increase in nuclear size is not always sufficient to distinguish ploidy (Jarvis 1992a); however, Child and Watkins (1994) were able to distinguish triploid Manila clams [*Tapes philippinarum* (Adams and Reeve)] by the larger diameter of the nucleus of gill tissue cells in comparison to diploids. The amount of DNA in the nucleus can be measured by staining the DNA with a specific fluorescent dye, and

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the degree of fluorescence is then quantified by flow cytometry (Coon and Weinstein 1992). Image analysis uses a similar technique to quantify the DNA content of nuclei. Instead of measuring the light emitted by stained nuclei (as in flow cytometry), image analysis can be used to measure the light absorbed by the stained nuclei (Jarvis 1992a). By measuring the light absorbed by nuclei stained by the Feulgen method as a function of their area (as integrated optical density), Gérard et al. (1994) independently used image analysis to distinguish diploid oysters from triploids.

Nuclear size and integrated optical density were calculated for commercial-size oysters (approximately 60 g) from experimental groups of triploid Pacific oysters sampled in the months before and after a spawning recorded at Birch's Bay (southern Tasmania, Australia), December 1992 and January 1993, and comparisons were made with triploid groups at two other sites. This allowed an evaluation of individuals within the triploid group that had gonad morphologies similar to those of diploids. Were these individuals triploids in which gametogenesis was not inhibited, or were they simply diploids?

MATERIALS AND METHODS

Sampling Sites

The diploid and triploid Pacific oysters used in these experiments were from the same groups as those described by Maguire et al. (199-) and were grown at three sites in Tasmania: Little Swanport (east: 148°00′, 42°19′), Pitwater (southeast: 147°30′, 42°50′), and Birch's Bay (south: 147°15′, 43°10′). These groups arose from the same pool of gametes. Triploids were induced by the suppression of polar body 2 with cytochalasin B (Allen et al. 1989) in February 1990, and spat were grown to market size intertidally.

Adductor Muscle

Adductor muscle size index was calculated by dividing adductor diameter (in millimeters) by valve height (in millimeters), with measurements taken from 81 diploid and 86 triploid oysters. The measurement of adductor muscle diameter was taken from the upper valve along the axis of the border between quick and catch (opaque and translucent) muscle. Oysters for adductor muscle measurements were cultured in a replicated trial with three replicates of 10 oysters per ploidy group at Birch's Bay and four replicates at each of the other sites (10 oysters per replicate at Pittwater, 3 per replicate at Little Swanport). Sampling occurred at approximately 90-mm valve height, which was reached in 29 mo at Little Swanport and Pittwater and in 42 mo at Birch's Bay. The effects of ploidy, site, and interaction of ploidy with site on adductor muscle size index were assessed with a two-way fixed-factor analysis of variance after an assessment of the homogeneity of variance (Cochran's test) and normality (Shapiro-Wilk test) (Walpole and Myers 1989).

Nuclear Measurements

Specimens for nuclear measurements were collected from Birch's Bay in December 1992, at the peak of gonad development, and also in January 1993, after spawning had occurred in diploid groups. Standard paraffin sections (5 μm) were prepared from a slice of tissue taken slightly above the junction between the labial palps and the gills (Morales-Alamo and Mann 1989). The proportion of somatic tissue (excluding gills) occupied by gonad, and expressed as percent gonad area, was determined by the use of image analysis from histologic sections, as described by Maguire et al. (199-).

Standard paraffin (7-μm) histologic sections of oysters for image analysis were prepared as described in Gardner et al. (in prep.). The staining of tissue for image analysis must be specific and of high quality (Jarvis 1992b). Jarvis (1992b) suggests that the Feulgen technique is the most appropriate stain for the image analysis of DNA. This was not found to be the case in this study, because Feulgen staining produced a pale stain that provided poor contrast against the surrounding tissue. Variation in staining intensity between slides was also encountered with the Feulgen technique, and this masked variation due to differences of ploidy. The source of variation in Feulgen staining was considered to arise from a critical step of acid hydrolysis. Consequently, an alternative stain was sought that produced strong and specific staining of DNA and that also was very simple so that variation was diminished. Gill's hematoxylin (Gill et al. 1974) was found to be a suitable stain. Sections were stained for precisely 2 min and then were rinsed with tap water for 5 min. Although Feulgen staining was inconsistent in this study, Gérard et al. (1994) used Feulgen staining to accurately distinguish triploids by image analysis.

Nuclear area and densiometric (integrated optical density) measurements were taken with a BH2, Olympus™ compound microscope. Slides were viewed with a 100× planachromat objective with oil immersion to produce a magnification of ×1000. Nuclear area and integrated optical density measurements were recorded for 20 hemocyte cells from each specimen. Measurements were taken with a CUE II™, IBM™-compatible, image analysis system. Hemocyte cells were chosen because they were widely distributed through the tissue and there appeared to be little variation of hemocyte nuclear size within specimens. Also, the nuclei of hemocytes are round, which assists in defining the shape to be measured with image analysis. Child and Watkins (1994) also used hemocytes to facilitate the measurement of cell area.

Illumination between sections was standardized by adjusting the light intensity of the microscope to a standard intensity. This was done when viewing a section of each slide without tissue. The purpose of this step was to remove variation in light intensity due to differences in the slide or cover slip thickness and variation in the optical density of the mounting medium. Densiometric measurements were made with microscope adjustment and illumination filtration and illumination adjustment, as advised by Jarvis (1992b). Basophilic components within the cytoplasm produced pale staining, which compounded nuclear optical density measurements. To correct for this, an optical density reading (from an area the exact size of the nucleus) was taken from the cytoplasm of each hemocyte sampled. By subtracting the integrated optical density of the cytoplasm from the integrated optical density of the nucleus, this component of light absorption was removed. The significance of differences in nuclear measurements between triploids and diploids was tested with a Wilcoxon nonparametric test (Walpole and Myers 1989).

RESULTS AND DISCUSSION

Adductor Muscle

The adductor muscle diameter in relation to valve height (adductor muscle index) was significantly greater ($p < 0.001$) in triploid oysters than in diploids at all sites (Table 1). This index was 9.9% greater in triploids, and Nell et al. (1994) found that the

TABLE 1.

Mean adductor muscle size index (diameter expressed as a percentage of valve height ± SE) for diploid and triploid Pacific oysters (*C. gigas*) from three sites in Tasmania, Australia.

Site	Diploid Group	Triploid Group	Significance of Effect of Ploidy
Little Swanport[a]	20.9 (±0.49)	22.7 (±0.77)	<0.001
Pittwater[b]	17.2 (±0.17)	19.2 (±0.29)	<0.001
Birch's Bay[b]	17.4 (±0.69)	19.2 (±0.35)	<0.001

Note: Replication at Little Swanport and Pittwater = four replicates of 10 individuals and at Birch's Bay = three replicates of 10 individuals. Significant difference between sites (diploids and triploids combined) for adductor muscle index is denoted by a difference in superscript ($p < 0.05$). The triploid samples were from populations that contained triploid and diploid oysters in about a 3:1 ratio.

equivalent value for Sydney rock oysters held subtidally was 5.7%. Although there was a significant difference in adductor muscle index between diploids and triploids, there was extensive overlap of values between ploidy groups at all three sites (Fig. 1). Consequently, it was considered impossible to distinguish diploids from triploids by the use of this index.

The measure of adductor muscle size that was used in this study was adductor muscle diameter, which was measured with callipers and could have been used on farms. More sensitive measures, such as adductor muscle area or overall weight, would require image analysis or precision balances and are unsuitable for farms. Although unsuitable for the objectives of this study, these more sensitive measures may be effective and useful for other situations.

Although ineffective for determining the ploidy of individual oysters, the larger ratio of adductor muscle diameter to valve height in triploids may be beneficial in aquaculture to enhance survival through periods of prolonged exposure. However, this index would be sensitive to any differences in shell shape between diploids and triploids.

Site was also found to influence adductor muscle index ($p < 0.001$), with the largest values recorded from Little Swanport. Oyster racks at this site were exposed for much longer periods than at the other two sites (Maguire et al. in prep.). The interaction between site and ploidy was not significant ($p > 0.05$).

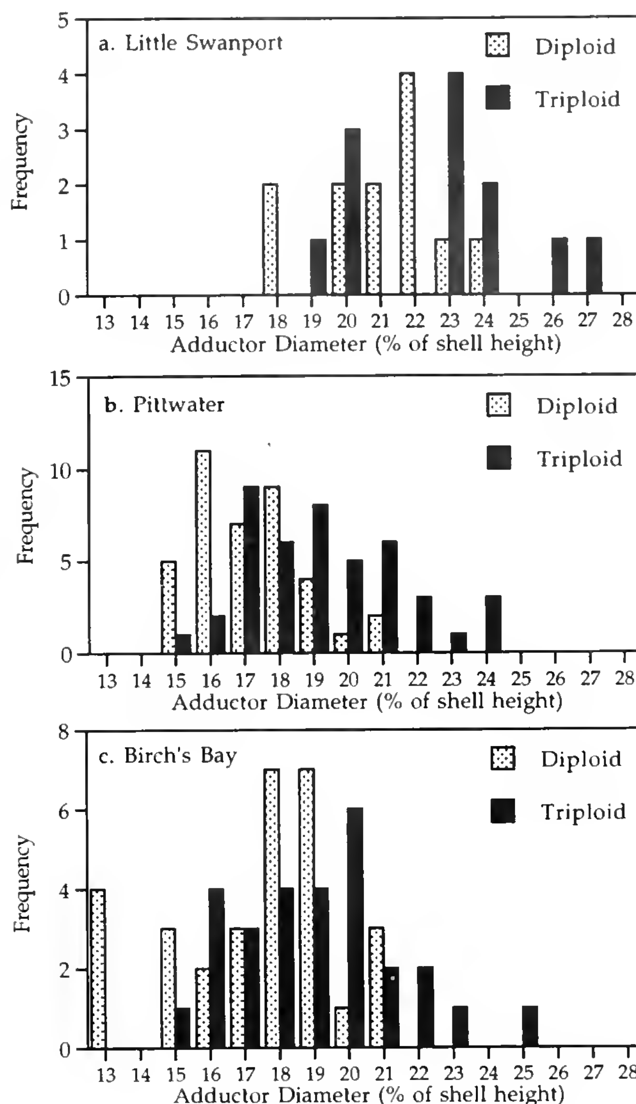

Figure 1. Frequency of adductor muscle index of triploid and diploid Pacific oysters, *C. gigas*, cultured at three sites in Tasmania, Australia. (Triploid samples were from populations that contained triploid and diploid oysters in about a 3:1 ratio.)

Image Analysis

A highly significant difference ($p < 0.001$) was found between diploids and triploids for both nuclear diameter and integrated optical density, as assessed by image analysis (Table 2). This was the case for both December and January samples at Birch's Bay. This demonstrates the potential for hemocyte nuclear area and

TABLE 2.

Comparison of nuclear area and integrated optical density of hemocyte nuclei between triploid and diploid Pacific oysters for December 1992 and January 1993.

Ploidy	Integrated Optical Density: Mean ± SD (n) December	Integrated Optical Density: Mean ± SD (n) January	Nuclear Area (μm^2): Mean ± SD (n) December	Nuclear Area (μm^2): Mean ± SD (n) January
Diploid	0.87 ± 0.06 (22)[a]	0.80 ± 0.09 (21)[a]	5.25 ± 0.40 (22)[a]	4.46 ± 0.25 (21)[a]
Triploid	1.09 ± 0.17 (20)[b]	1.03 ± 0.15 (21)[b]	6.22 ± 0.58 (20)[b]	5.73 ± 0.61 (21)[b]

Note: Triploid group contains triploid and diploid oysters in about a 3:1 ratio. Data are intended to convey differences due to ploidy within monthly samples. Comparison of means between monthly samples may be compounded by error from differences in the set-up of the imaging system between the two samples. Significance of difference between ploidy groups is denoted by a difference in superscript ($p < 0.001$).

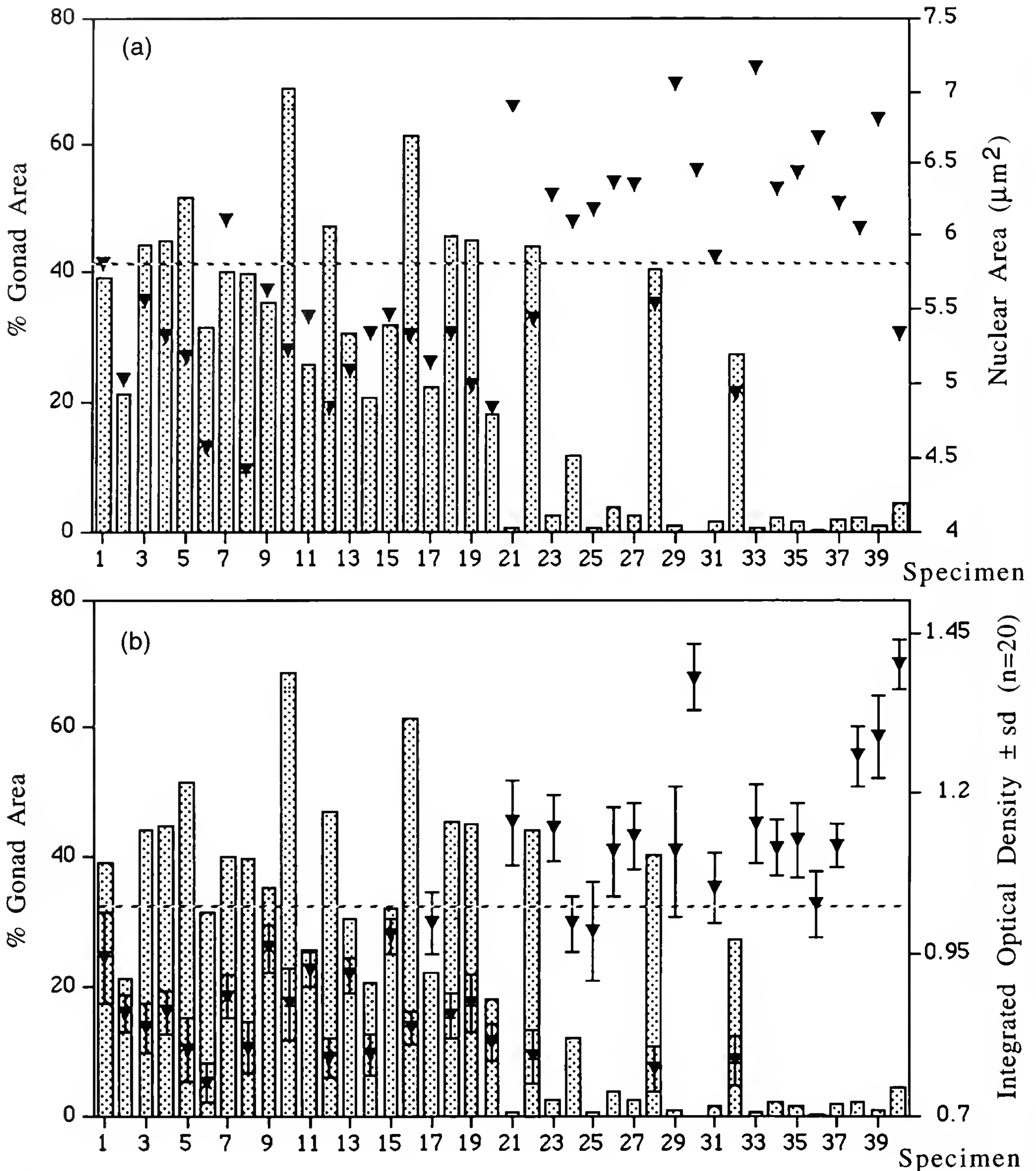

Figure 2. Mean hemocyte nuclear area (a) and integrated optical density (b) in relation to the proportion of somatic tissue occupied by gonad for the December 1992 sample. Gonad area values are represented by columns, and nuclear parameters are represented by solid triangles. Specimens 1–20 are from the diploid group; specimens 21–40 are from the triploid group. No standard deviations were recorded for nuclear area measurements from this sample.

integrated optical density to be effective tools in the determination of ploidy; however, for the purpose of this study, it was necessary to distinguish diploid and triploid oysters on an individual basis rather than by simply separating populations. The degree to which this was achieved was assessed by comparing values obtained by image analysis against the gonad area of individual oysters (Figs. 2 and 3).

Both nuclear area and integrated optical density values ap-

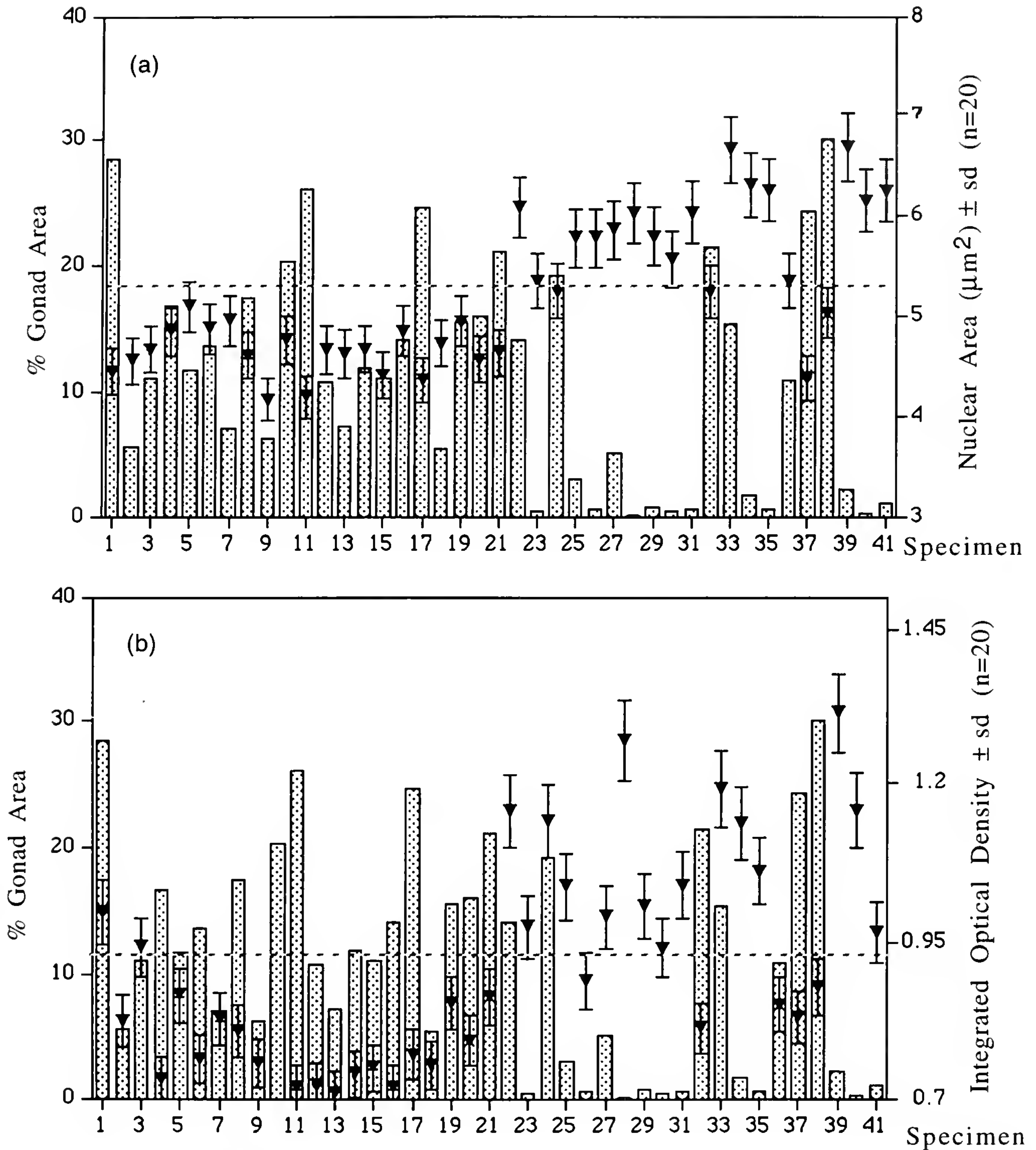

Figure 3. Mean hemocyte nuclear area (a) and integrated optical density (b) in relation to the proportion of somatic tissue occupied by gonad for the January 1993 sample. Gonad area values are represented by columns, and nuclear parameters are represented by solid triangles. Specimens 1–20 are from the diploid group; specimens 21–41 are from the triploid group.

peared to broadly distinguish triploid oysters from diploid oysters in the December 1992 sample. Neither measurement conclusively separated all individuals, but a clear indication of ploidy was apparent when both measures were compared for each oyster. Some triploid oysters had nuclear area measurements that were higher than those of most diploids but that were too low to clearly indicate them as triploid (e.g., specimens 23 and 24, Fig. 3). In these cases, the integrated optical density measurements clearly indicated the oysters to be triploid. Morphometric measurement of nuclear size alone was not sufficient to separate all diploid and triploid Pacific oysters, although this is possible with Manila clams (Child and Watkins 1994). The relationship of each of the

TABLE 3.

Comparison of the variation (by coefficient of variation) between nuclear area and integrated optical density measures for diploid and triploid oysters.

Ploidy	Integrated Optical Density: Coefficient of Variation (n)		Nuclear Area: Coefficient of Variation (n)	
	December	January	December	January
Diploid	7.97 (22)	17.01 (21)	7.20 (22)	6.32 (21)
Triploid	8.69 (20)	11.64 (21)	9.96 (20)	8.40 (21)

Note: Oysters within the triploid group that appeared to be diploids were excluded from analyses. Coefficient of variation = (standard deviation/mean) × 100.

nuclear parameters (integrated optical density and nuclear area) to ploidy was based on both the gonad area measurements of each oyster and the ratio of diploids within the triploid group, as determined by flow cytometry. The nuclear area and integrated optical density values indicated that eight oysters within the triploid group, from both months (a total of 40 triploids), were diploid, which is equal to 20% of the sample (specimens 22, 28, and 32 from the December sample, specimens 21, 32, 36, 37, and 38 from the January sample). Although the sample size is very small, this is in the order of what would be expected on the basis of the original ratio obtained by flow cytometry (approximately 25% diploids within triploid samples).

There tended to be more variation in integrated optical density measurements than in nuclear area data (Table 3). The nuclear staining capacity of hematoxylins has been attributed to the binding of the dye molecule to nuclear histones (Stevens 1982), but other proteins will also bind these dyes. The variation in integrated optical density measurements observed in this study may have been caused by the nonspecific staining of proteins associated with the nucleus, rather than the staining of histones alone. Staining intensity can be expected to vary from day to day, and there appeared to be some difference in intensity between the December and January samples, which were stained separately (Figs. 2b and 3b). To eliminate error caused by changes in the staining of the hematoxylin, diploid controls are necessary for each batch of analyses. Although uniform and intense Feulgen staining could not be achieved in this study, it has been used elsewhere for the determination of ploidy (Jarvis et al. 1992b, Gérard et al. 1994) and it is highly specific to DNA. Variations on the Feulgen technique, used for determining integrated optical density in human pathology (Schieck et al. 1987, Schulte et al. 1988), may improve the staining intensity in oyster sections and further assist in determining ploidy.

The nuclear area and integrated optical density values of triploids from the December 1992 sample confirmed the suppression of gametogenesis as a result of triploidy. Those individuals that did develop extensive gonad were shown to be diploids. In the following sample, January 1993, there were three individuals within the triploid group with large gonad area that appeared to be legitimate triploids on the basis of image analysis (specimens 22, 24, and 33, Fig. 3). All of these triploids were male, and gonad size is less retarded in male triploid Pacific oysters than in female triploids (Allen and Downing 1990). Numerous spermatocytes were present in the follicles of these individuals, yet no spermatozoa were observed, which is a pattern of gametogenesis consistent with that reported for triploid *Crassostrea virginica* (Gmelin) (Barber and Mann 1991).

The technique of image analysis of hemocyte nuclei allowed us to retrospectively determine ploidy and to separate these groups for an analysis of gametogenesis. Where histologic sections are also required, the technique of image analysis provides a useful means of determining ploidy and may also serve as an adjunct to flow cytometry.

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ANNUAL PATTERN OF BROODING IN POPULATIONS OF CHILEAN OYSTERS, *TIOSTREA CHILENSIS*, (PHILIPPI, 1845) FROM NORTHERN NEW ZEALAND

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ABSTRACT The annual pattern of brooding in two populations of adult Chilean oysters, *Tiostrea chilensis*, in northern New Zealand, was examined. Both populations were brooding larvae throughout the year, with less brooding activity in winter and increased larval production around spring and early summer. Despite the extended brooding season, the proportion of individuals brooding during peak periods remained high. Larger broods of larvae appeared to be associated with periods of raised brooding activity and lower water temperatures. In both populations, there were no differences in the size of oysters brooding at different times of the year. Overall, the annual pattern of brooding in both populations was markedly different from previous reports for this species in other regions. These differences tend to confirm the role of water temperature as the most important environmental factor regulating the annual pattern of reproduction in this oyster species. This conclusion has important implications for the hatchery production of larvae for aquaculture.

KEY WORDS: Chilean oyster, *Tiostrea chilensis*, reproductive cycle, brooding, New Zealand, flat oyster, Ostreidae

INTRODUCTION

The Chilean oyster *Tiostrea chilensis* (Philippi, 1845) is a commercially important flat oyster that is native to New Zealand and the Pacific coast of South America (Osorio 1979, Beu and Maxwell 1990, Jeffs and Creese 1996). This oyster, along with all species of the genus *Ostrea*, incubates its eggs after fertilization and releases larvae (Roughley 1929, Millar and Hollis 1963). Like some other species of flat oyster, *Tiostrea* is a protandrous hermaphrodite, maturing first as a male, and later in life, as a female (Hollis 1963, Winter et al. 1984, Chanley and Chanley 1991). However, unlike all other species of oyster, it can brood its larvae through their entire development (Millar and Hollis 1963). Studies of the annual breeding cycle in *Tiostrea*, have generally found a small proportion of adult oysters brooding larvae for only a short period during the spring and/or summer (Tunbridge 1962, Stead 1971, Cranfield and Allen 1977, Westerskov 1980, Winter et al. 1984). Some workers have hypothesized that breeding in *Tiostrea* is regulated by water temperature, and therefore, populations at lower latitudes could be expected to have more extensive brooding seasons (Westerskov 1980, Cranfield and Michael 1989). The aim of this study, therefore, was to investigate the annual pattern of brooding in populations of Chilean oysters from lower latitudes in New Zealand and to compare the results with previous research conducted at higher latitudes, in both New Zealand and Chile.

MATERIALS AND METHODS

Two populations of Chilean oysters in the north of the North Island of New Zealand were used for this study: Te Tau Bank in the Manukau Harbour (37°02′S,174°41′E), a shallow harbour on the northwestern coast, and Moturekareka Island in the Hauraki Gulf (36°28.50′S,174°47.60′E), a large embayment on the northeastern coast (Fig. 1). Around 75 oysters, of a size known to be capable of brooding larvae (Jeffs, unpublished data), were sampled haphazardly from each population at approximately monthly intervals. Sampling began in December 1992 from the Manukau Harbour and in April 1994 from the Hauraki Gulf. For both populations, sampling continued until December 1995. The shell height of the oysters in each monthly sample was measured to the nearest millimeter with vernier callipers before they were opened. Larvae in brooding oysters were removed with a wash bottle and then counted in a manner similar to that described by Walne (1964). An assessment of this method confirmed that it provided an unbiased estimate of the size of broods with a higher degree of accuracy than has been reported previously. A mercury thermometer was used to record water temperatures to the nearest 0.1°C during a series of visits each month to both study sites.

Results

Annual Pattern of Brooding—Manukau Harbour

Thirty-six collections of oysters were made between December 1992 and December 1995. In total, 2,635 adult oysters were examined, of which 176 (6.7%) were found to be brooding. Brooding oysters were found in every month of sampling, with the exception of January 1993, showing that breeding takes place year-round (Chart in Fig. 2). The highest proportions of brooders

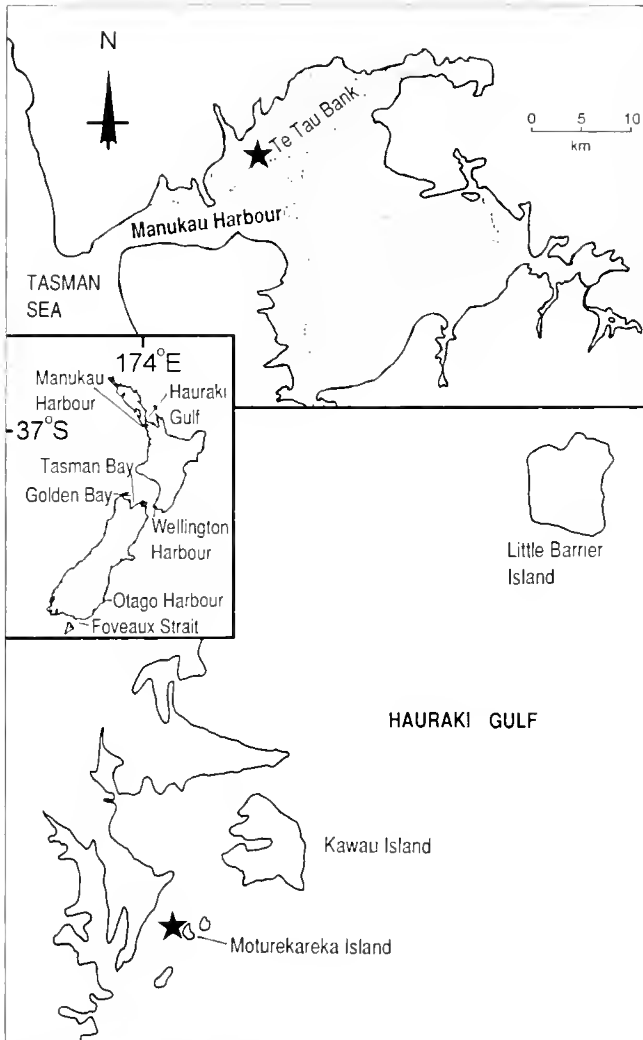

Figure 1. Map showing the location of the two study populations of *T. chilensis* in northern New Zealand and other locations mentioned in this article.

in all of the monthly samples were in September 1993 (18.2%) and in October 1995 (16.9%). In each of the 3 y, lower proportions of oysters tended to brood around January and July and higher proportions tended to brood between September and November. Some samples from within the period of March to May of each year also showed elevated proportions of brooding oysters that perhaps corresponded with a secondary breeding season. The annual pattern of larval production for the population was estimated by dividing the total number of larvae found in each monthly sample by the total number of oysters sampled (Graph in Fig. 2). The annual pattern of larval production followed the same trends as those found for the proportion of the population brooding. A χ^2 goodness-of-fit test was used to further investigate the annual pattern of brooding. The data were pooled for each month, regardless of calendar year, because of the small proportion of oysters found brooding (Sokal and Rohlf 1987). The proportion of brooders varied with the time of year (χ^2_{11} = 26.0, $p < 0.01$). In the months of September and October, considerably more brooding oysters were encountered than were expected, whereas in the months of January and July, there were fewer.

To establish if different-sized animals were brooding at different times of the year, an analysis of variance was used to compare the mean size of brooding oysters for each month of the year (data pooled for 3 y). The analysis of variance showed that there was no difference in the mean size of brooding oysters between months of the year ($F_{11,164}$ = 1.2, $p > 0.05$).

An analysis of variance showed that there were differences in the number of larvae in broods from different months of the year ($F_{11,164}$ = 2.0, $p < 0.05$). The highest mean numbers of larvae per brood were found in the months of October, September, August, and November, whereas the lowest were found in January and May (Fig. 3). The water temperatures recorded at this site followed a general seasonal cycle, but with some variability that was probably due to the tidal nature of the harbour (Fig. 2). The highest temperature recorded was 24.3°C on January 10, 1994, and the lowest was 11.1°C on July 19, 1993.

Annual Pattern of Brooding—Hauraki Gulf

Twenty monthly collections of oysters were made between April 1994 and December 1995. In total, 1,548 adult oysters were sampled, of which 127 (8.2%) were found to be brooding. In addition, 19 brooding oysters were found among oysters remaining after the main monthly sample had been processed. Data on the brood size of these oysters were included in the analyses below, where appropriate, in order to increase the sample size.

Oysters were found to be brooding larvae throughout the period of the study because brooding oysters were encountered in each of the 20 monthly samples (Chart in Fig. 4). The highest proportions of brooders in all of the monthly samples were in December 1995 (16.7%) and September 1994 (14.7%). The lowest proportions of brooders in all of the monthly samples were in April 1994 (1.3%) and May 1995 (2.5%). There tended to be increased brooding activity in spring to early summer (September to December) and generally less brooding activity over the remainder of the year. The annual pattern of larval production for the population was estimated by dividing the total number of larvae found in each monthly sample by the total number of oysters sampled (Graph in Fig. 4). The annual pattern of larval production followed the same trends as those found for the proportion of the population brooding.

A goodness-of-fit test was used to further investigate this annual pattern of brooding. The proportion of brooders encountered varied between the 20 mo sampled (χ^2_{19} = 31.5, $p < 0.05$). In the months of December 1995 and September 1994, considerably more brooding oysters were encountered than were expected, whereas in the months of April 1994 and May 1995, there were fewer. To establish if different-sized animals were brooding at different times of the year, an analysis of variance was used to compare the mean sizes of brooding oysters for each of the monthly samples. The single brooding oyster found in April 1994 was excluded from the analysis. The analysis of variance showed that there was no difference in the mean size of brooding oysters throughout the months sampled ($F_{18,126}$ = 1.68, $p > 0.05$).

An analysis of variance showed that there were differences in the number of larvae per oyster throughout the months sampled ($F_{18,126}$ = 2.5, $p < 0.005$). The highest mean numbers of larvae per brood were found in the months of June 1994 and July 1994, whereas the lowest were found in March 1995 and January 1995 (Fig. 5). The water temperature in the Hauraki Gulf site followed a seasonal cycle similar to that of the Manukau Harbour, but had a slightly smaller range (Fig. 4). The lowest temperature was

Figure 2. A chart showing the proportion of oysters in monthly samples from the Manukau Harbour that were brooding, with corresponding graphs showing water temperature and the monthly estimates of larval production (i.e., the total number of larvae in each monthly sample divided by the total number of oysters sampled).

13.0°C, recorded on both August 1 and August 14, 1995, and the highest was 23.1°C on February 17, 1995.

DISCUSSION

Our two populations of *Tiostrea* from northern New Zealand shared similar annual patterns of larval production. Both populations were brooding larvae throughout the year, with less brooding activity in winter and increased larval production around spring and early summer. Larger broods of larvae appeared to be associated with increasing levels of brooding activity and lower water temperatures. Despite brooding year-round, the proportion of each population brooding during peak periods remained comparable to the highest proportions reported for populations elsewhere with shorter brooding seasons (Table 1). For both study populations, there were no differences in the size of oysters found brooding at different times of the year.

The annual patterns of brooding in both of these populations

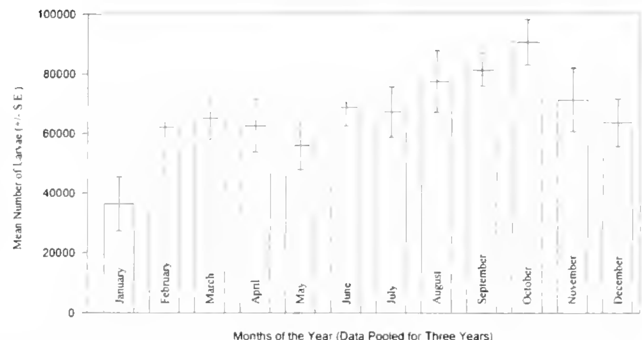

Figure 3. A chart comparing the mean number of larvae for broods encountered in different months of the year in the Manukau Harbour (±standard error [S.E.]).

Figure 4. A chart showing the proportion of oysters in monthly samples from the Hauraki Gulf that were brooding, with corresponding graphs showing water temperature and the monthly estimates of larval production (i.e., the total number of larvae in each monthly sample divided by the total number of oysters sampled).

were very different from those previously reported for this species (Table 1). Earlier studies have found that *Tiostrea* generally produced larvae for at least 2 mo during spring to summer and sometimes into autumn. Other than this study, Westerskov (1980) provides the only account of winter brooding activity in this species. He recorded very low numbers of brooding oysters in Foveaux Strait over winter (0.01–0.1% during April and May and 0.2–0.9% in July). These are much lower than for the corresponding periods during this study (see Figs. 2 and 4).

Differences in the annual reproductive cycles of bivalves from geographically separated populations have been studied extensively, particularly among commercially important species such as oysters, mussels, and scallops (Giese and Pearse 1974, Sastry 1979). Observed differences in breeding between populations have been variously attributed to genetic differences, food availability, latitude, and water temperature, or a combination of these factors (Newell et al. 1982, Barber et al. 1991, Wada et al. 1995).

Cranfield and Michael (1989) observed significant differences

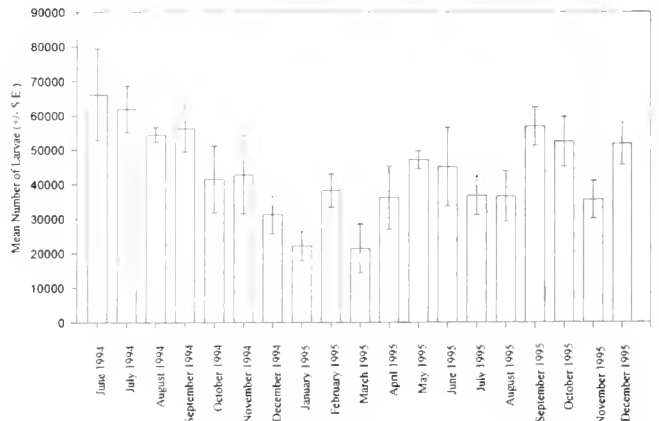

Figure 5. A chart comparing the mean number of larvae for broods encountered in different months of the year in the Hauraki Gulf (±standard error [S.E.]).

TABLE 1.

Annual patterns of brooding recorded in populations of Chilean oysters from a range of locations.

Location	Annual Pattern of Brooding	Comments	Highest Proportion of Brooders Recorded	Author(s)
Auckland, N.Z.	Larvae released at least between January and May	Inferred season from larval settlement on a rocky shore	—	Booth 1983, Luckens 1976
Wellington Harbour, N.Z.	Larvae released from August to March	No samples taken April to July	5.6% in December	Hollis 1963
Tasman Bay & Golden Bay, N.Z.	Brooding at least from October to March	Inferred from limited sampling	4.1% in October	Cranfield & Michael 1989, Tunbridge 1962
Otago Harbour, N.Z.	Brooding September to May with a peak in November	—	15–20% late October to mid-November (in two populations)	Westerskov 1980
Foveaux Strait, N.Z.	Brooding August to March with a peak in November to February; no brooders found April to July	—	8.5% in October	Stead 1971
Foveaux Strait, N.Z.	Brooding October to March with a peak in November	No samples taken April to August	Just over 3% in November	Cranfield & Allen 1977
Pullinque, Chile	Brooding September to February with a peak in December to January	No samples taken May to August	About 15% in December	Solís 1967
Pullinque, Chile	Larvae released November to February	—	—	Inculmar 1982
Bay of Concepción, Chile	Larvae released November to March	—	—	Aracena et al. 1976
Apiao, Chile	Larvae released December to March	—	—	Padilla et al. 1969
Quempillén Estuary, Chile	Larvae released December to January	—	—	Toro 1982
Quempillén Estuary, Chile	Larvae released end of October with a peak at end of December to early January	—	—	Winter et al. 1984

in the breeding of *Tiostrea* populations from southern and central New Zealand and concluded that these were consistent with latitudinal trends identified in many other benthic marine invertebrate species by Thorson (1950). On the basis of these latitudinal trends, they hypothesized that breeding could be expected to start earlier and cease later in populations of *Tiostrea* at lower latitudes. This view is strongly supported by the results presented here from two populations in northern New Zealand. Unfortunately, a similar trend cannot be established from results reported for Chilean populations because of the limited extent of the studies undertaken over a latitudinal range.

Water temperature, which usually varies with latitude in a moderately uniform manner, frequently has been assigned a dominant role in synchronizing reproductive cycles in marine invertebrates (Newell et al. 1982). Indeed, Westerskov (1980) studied the timing of the production and release of larvae in *Tiostrea* in the Otago Harbour over 4 y and concluded that water temperature was critical in triggering the onset of gonad ripening and the subsequent release of larvae. Gonads ripened over winter and into spring, but larvae were not incubated until the water temperature rose above 9–10°C in late August/September. Subsequently, the water temperatures had always reached at least 14.5°C before the first spatfall and 15.9°C before the maximum spatfall was recorded. These findings are consistent with the different water temperatures and annual patterns of brooding found in populations of oysters over a range of New Zealand latitudes. For example, water temperatures at our two northern populations never fell below 10°C, and hence, the incubation of larvae at both of these populations continued uninterrupted throughout the year. Therefore, our results from low-latitude populations in New Zealand tend to confirm the importance of water temperature in regulating the annual cycle of reproduction in *Tiostrea*.

This conclusion has implications for addressing the existing shortage of spat for the aquaculture of this species. In particular, there have been continuing difficulties in developing a hatchery technique for conditioning and synchronizing larval production in *Tiostrea* broodstock (Ramorino 1970, DiSalvo et al. 1983, Wilson et al. 1996). Our findings point toward the manipulation of water temperature as the most fruitful area for research aimed at improving the hatchery production of larvae from broodstock.

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SALINITY TOLERANCE OF THE CATARINA SCALLOP *ARGOPECTEN VENTRICOSUS-CIRCULARIS* (SOWERBY II, 1842)

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ABSTRACT We investigated the salinity tolerance range of the adult catarina scallop (*Argopecten ventricosus-circularis*) by exposing them to increased and decreased salinity in steps of 5 ppt, each step lasting 24 h, from normal salinity (37 ppt) up to 57 ppt and down to 17 ppt at a constant 28°C. Our results indicate that *A. ventricosus-circularis* is a perfect osmoconformer. Hemolymph osmolality followed the same trend as the changing external media throughout the salinity levels tested. Survival records indicate that the salinity tolerance is restricted to 27–47 ppt.

KEY WORDS: Salinity tolerance, osmoregulation, catarina scallop, survival

INTRODUCTION

Scallops are bivalves of great economic importance. The catarina scallop (*Argopecten ventricosus-circularis*) exists in large stocks in the bays of Baja California Sur, Mexico (Tripp 1985, Aurioles-Gamboa 1992), yielding up to 700 tons of adductor muscle in 1988 (Félix-Pico 1991, Félix-Pico et al. 1991) and 5,186 tons during 1989 and 1990 (Maeda-Martínez et al. 1993). Some of these stocks have been overfished (Baqueiro et al. 1981), and others are subject to heavy exploitation. Consequently, the cultivation of this species is gaining importance (Vicencio and Singh 1988, Castro-Ortíz 1993). Spat production in the laboratory (Maeda-Martínez et al. 1989, Maeda-Martinez et al. 1995), field collection, and growout process (Maeda-Martinez and Ormart-Castro, 1995) in the bays of Baja California Sur are well documented (Caceres-Martinez et al. 1993). To determine the cultivation range of the species in other protected areas, we need to know the tolerance of the species to salinity changes, because most of the coastal lagoons along the Pacific coast of Mexico are exposed to heavy rainfall and freshwater runoff. Others in Baja California Sur and Sonora become hypersaline during the year because extremely high summer temperatures cause rapid evaporation and there are bays where water transport from the open ocean is restricted physically or there is limited tidal interchange (Garcia 1988). The bay scallop (*Argopecten irradians*, Lammarck, 1819) is the Atlantic species analogous to the catarina scallop (Winter and Hamilton 1985). This species has been found in salinities as low as 10 ppt and as high as 38 ppt (Castagna and Chanley 1973, Barber 1985). Mercaldo and Rhodes (1982) demonstrated a certain capacity of the bay scallop to withstand reductions in ambient salinity. They found survival >60% could be obtained from scallops exposed to 15 ppt at 24°C for 48 h. Shumway (1977) earlier investigated the effects of lowered salinity on *Chlamys opercularis*, a species closely related to *A. ventricosus-circularis*, finding that the former can withstand rather large decreases in salinity. The effect of hypersaline conditions on molluscs is not well known. Osmoregulation in molluscs has been the subject of many investigations (Robertson 1964, Avens and Sleigh 1965, McAlister and Fisher 1968, Pierce 1970, Bedford 1971, Gilles 1972, Gilles 1974, Gilles 1975, Schoffeniels and Gilles 1972, Pierce and Greenberg 1973, Hoyaux et al. 1976, Shumway 1977, Burton 1983). From these, we can conclude that the hemolymph of most marine molluscs is close to seawater in osmotic pressure and ionic composition. This is achieved by intracellular isosmotic regulation, where free amino acids play a role as solutes. Shell closure in bivalves has complicated investigations into the ability of marine molluscs to hyperosmoregulate actively at low salinities. Studies of osmoregulation in the catarina scallop may provide information on this subject because the shells of *A. ventricosus-circularis* have a byssal notch that does not allow the animal to isolate itself completely from an external medium. Similar to all scallop species, *A. ventricosus-circularis* opens its shells when disturbed or exposed to the air. This behavior is opposite that shown by other bivalves under the same circumstances.

Shell closure in bivalves has also obscured investigations related to the time required by the animal to reach equilibrium with the external medium. Crowe (1981) suggested that the regulation of cell volume by solute extrusion is, in bivalves, a long-term emergency process. In this article, the results of an investigation on osmotic regulation in *A. ventricosus-circularis* as a function of environmental hyposaline and hypersaline changes are presented.

MATERIALS AND METHODS

The osmoregulatory capacity in the catarina scallop was estimated by hemolymph osmotic concentration (HOC) measurements against the external medium osmotic concentration (EOC) and mortality rates. Adults of *A. ventricosus-circularis* (2.4 ± 0.9 cm shell length and 2.32 ± 0.9 cm shell height; n = 300) were collected from cultured populations at Rancho Bueno, Bahia Magdalena, Baja California Sur, México (Fig. 1). The animals were maintained in 70-L plastic tanks in the laboratory, with running filtered (10 μm pore size) seawater at 28 ± 1°C and 37 ppt,

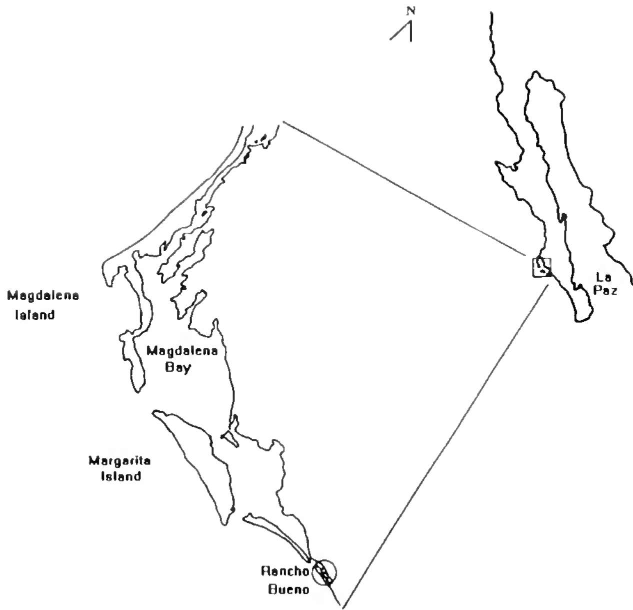

Figure 1. Catarina scallop (*A. ventricosus-circularis*) culture area.

and were fed with 1.5×10^5 cells/mL of a mixture of *Isochrysis galbana*, *Chaetoceros calcitrans*, and *Tetraselmis suecica* (6:3:1). After 3 days, three groups of 50 animals were placed in individual 70-L tanks with filtered seawater at 28°C and 37 ppt to carry out the experiment (Fig. 2). The organisms were subjected to a change of 5 ppt/day above and below 37 ppt (treatments 1 and 2). The test animals were moved from a tank at one salinity to another at a different salinity to subject them to the salinity changes. The hyposaline or hypersaline solutions were made by either diluting seawater with fresh water or by adding a measured amount of sea salt to seawater. One group was kept at 37 ppt and served as the control. All treatments were in duplicate. The osmolality of the media was adjusted with a hand refractometer (Area, Inc.) reading the equivalent salinity in ppt (accuracy ±0.1%). The osmotic pressure of 30-μL samples of hemolymph (HOC) and external medium (EOC) was determined in triplicate for all groups, approximately every 6 h, with a micro-osmometer ("Osmette S": Precision Systems, Inc.).

Hemolymph was obtained by direct puncture of the pericardic

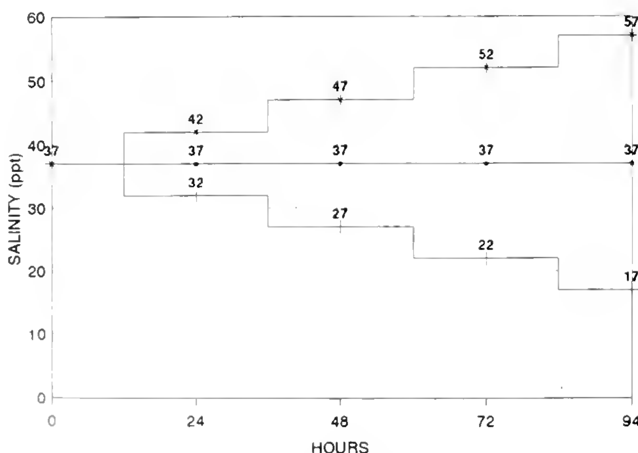

Figure 2. Experimental design.

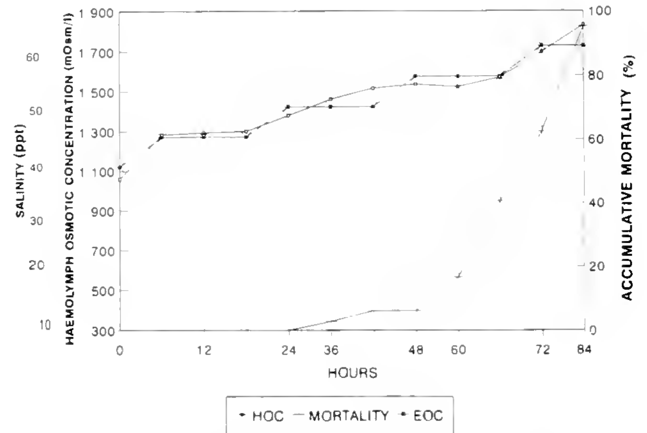

Figure 3. HOC and mortality of *A. ventricosus-circularis* as a function of a hyposaline environment.

cavity with capillary pipettes, after the mantle cavity was blotted dry with a tissue. Simultaneously, the mortality rate was recorded.

RESULTS

Osmotic concentration measurements (Figs. 3 and 4) indicate that scallop hemolymph is isosmotic with the external medium at 28°C under laboratory conditions. This condition was tested statistically by comparing the regression coefficients (Parker 1979) of internal and external osmotic concentration measurements. Results (Table 1) show that the regression coefficient (slope) of the external versus the internal osmotic concentration in all treatments was the same, at the $p > 0.01$ level.

The osmotic hemolymph concentration of the organisms in the control group remained at 1,100 mOsm/L. At salinities of 17 and 57 ppt, the HOC values were 344 and 1,830 mOsm/L, showing a decrease at low salinities (Fig. 3) and an increase at high salinities (Fig. 4). Thus, according to these results, the animals were osmoconforming, because the hemolymph osmotic pressure followed closely that of the external medium.

A. ventricosus-circularis was able to withstand salinity variations from 27 to 47 ppt. Within this range, mortality was low (6 and 8% at 27 and 47 ppt). At salinities of 22 and 52 ppt, mortality

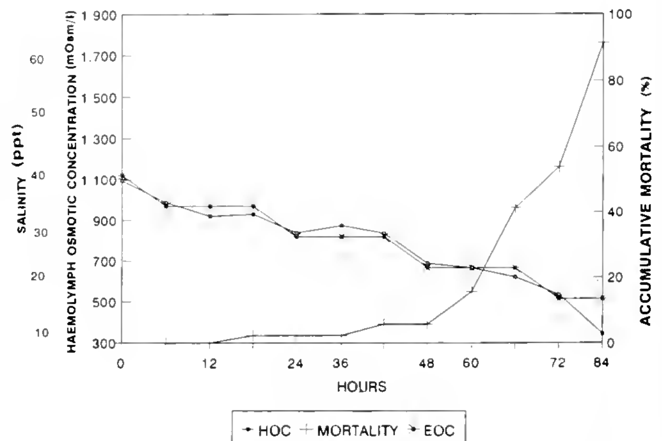

Figure 4. HOC and mortality of *A. ventricasus-circularis* as a function of a hypersaline environment.

TABLE 1.

Regression coefficient (slope) analysis, comparing the equations describing variations of external and hemolymph osmotic pressure against time in *A. ventricosus-circularis* under different osmolality treatments.

	Correlation of Osmotic Pressure and Time		Comparison of External and Internal Regression Coefficients		
	Regression Coefficient		Degrees of Freedom		
Salinity	(Slope)	r	V1	V2	F
Hyposaline					
EXT[a]	−5.58	0.93	1	20	0.66
INT	−4.12	0.93			
Hypersaline					
EXT	6.24	0.91	1	20	1.43
INT	8.00	0.90			

[a] EXT, external, INT, internal

increases to 50–60% over the 24-h holding period. Outside this range, mortality increases drastically, reaching 91 and 95% at 17 and 57 ppt (Figs. 3 and 4). These results show a certain ability of the species to withstand gradual salinity changes in both directions from the salinity level of normal seawater.

DISCUSSION

It has been shown, by many authors, that the mechanisms used by most marine molluscs to cope with external variations in salinity are intracellular isosmotic regulation and shell closing. Shell closing has imposed a limit on the estimation of the response time needed for the animal to adjust to the changing environment (Tettelbach et al. 1985). In this work, the response of the scallop to salinity changes was recorded without interference, because *A. ventricosus-circularis* lacks the shell-closing protective mechanism.

Within certain limits, most marine molluscs maintain the internal medium isosmotic with the external medium. The hemolymph osmolality of *A. ventricosus-circularis* followed the same trend as the external medium. Because in our experiments, the changes in salinity were done in 5-ppt steps, it was possible to see that the adjustment of hemolymph to the external medium was a slow process and that it takes some time to reach the external level.

Regardless of the hemolymph adjustment to the media tested, the salinity tolerance of the species is restricted to the range 27–47 ppt, as shown by mortality records. This is in contrast with the results of Mercaldo and Rhodes (1982), who obtained 100% survival in *A. irradians* transferred directly from seawater to 18 ppt during a 50-h test at 24°C.

Our results suggest, because of the limited salinity tolerance range of the species, that the catarina scallop aquaculture industry is restricted to areas with stable salinity conditions, such as the larger open bays of Baja California Sur. This is in agreement with the natural distribution of the species in Mexico.

ACKNOWLEDGMENTS

The authors express their gratitude to the Centro de Investigaciones Biológicas del Noroeste, La Paz, B.C.S., where this work was done. This work was partially supported by CONACYT (National Council of Science and Technology), CIB, La Paz, and Universidad Autónoma Metropolitana-Xochimilco. Thanks also to Dr. Ellis Glazier, CIBNOR, for the editing of the English language manuscript.

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ONTOGENETIC CHANGES IN OPTIMAL REARING TEMPERATURES FOR THE COMMERCIAL SCALLOP, *PECTEN FUMATUS* REEVE

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ABSTRACT Embryos, larvae, and early juvenile stages of the commercial scallop *Pecten fumatus* Reeve, were held at temperatures ranging from 13 to 27°C. An incubation temperature of 18°C produced the greatest percent development of D-veligers from eggs. The growth rate of larvae increased from 2.5 µm/day at 15°C to a peak of 6.5 µm/day at 24°C but decreased with a further increase in temperature to 27°C. Age-specific larval survival decreased significantly with increasing temperature in the range 15–27°C. However, size-specific survival, which is a more meaningful measure of optimal rearing temperature, exhibited a pronounced peak value at an intermediate temperature of 21°C. On the basis of these results, the maintenance of larval rearing temperatures between 18 and 21°C is likely to provide the maximum yield of pediveligers. The growth of 3-wk-old spat (mean shell height, 1.04 ± 0.26 mm), held in the hatchery, increased from a negligible rate at 13°C to a maximum rate at 24°C. During the fifth and final week of the trial, a constraint to continued exponential growth became evident at all temperatures tested except 13°C. Survival and byssus attachment of spat were highest at temperatures supporting the highest growth rates. The use of byssus attachment as an indicator of favorable spat-growing conditions is discussed. Possible ecological implications of ontologic change in temperature optima are discussed in relation to variability in annual fisheries' catches. Occasions on which optimal temperature regimens for embryo development and larval and spat growth occur are rare in Jervis Bay, NSW. Aspects of El Niño Southern Oscillation events and the oceanography of southeastern Australia are discussed as a possible mechanism by which such regimens might occur.

KEY WORDS: Scallops, embryo, larvae, spat, temperature, survival, ecology

INTRODUCTION

Temperature is not the only environmental factor influencing growth of scallops; however, it is one of the more measurable and controllable parameters. It also has a profound influence on the survival and distribution of scallops (Nakanishi 1977, Ventilla 1982), including the commercial scallop *Pecten fumatus* Reeve (Young and Martin 1989). Temperature directly influences the metabolic rate and survival of scallops (Nakanishi 1977, Ventilla 1982) and indirectly influences the nutritional environment (Wallace and Reinses 1985, Ito 1991). Accordingly, seasonal temperature regimens, critically influence the siting and seasonal timing of hatchery, farming, and stock enhancement programs.

The geographical range of the Australian commercial scallop *P. fumatus* extends south from central New South Wales (NSW) to Victoria, Bass Strait, and southern Tasmania and west to the Gulf of St. Vincent in South Australia. It is found at depths ranging from 7 to 60 m on substrates varying from muddy sand to course sand (Young and Martin 1989). Mean monthly sea temperatures over the geographical range vary from minimum winter values of 9–11°C in southern Tasmania, to maximum summer values usually in the range of 23–25°C on the central coast of NSW.

As part of a research project to develop optimal hatchery and nursery rearing protocols for *P. fumatus*, embryos, larvae, and spat were exposed to a broad array of temperatures falling within the above-natural range and (presumably) within the tolerance limits of the species. This study complements research on the effects of temperature on reproductive conditioning, gamete storage, and fertilization in *P. fumatus* (Heasman et al. 1996a, Heasman et al. 1996b).

MATERIALS AND METHODS

The embryos, larvae, and spat used in this study were obtained from broodstock collected by divers in Jervis Bay, NSW, and road-freighted to the hatchery within 12 h of capture. Reproductive conditioning and induced spawnings were conducted by the use of methods described by Heasman et al. (1996 a and b). In all cases, sperm and eggs, each from a minimum of five scallops, were used to reduce the effects of variable gamete viability.

The seawater (35 g/kg salinity) used in all experiments was filtered to 1 µm (pore size) and contained 1 mg/kg Na_2EDTA (disodium ethylenediaminetetraacetic acid) as a precaution against metal contamination (Utting and Helm 1985). During Experiments 2 and 3, larvae and spat were fed mixed microalgal diets of *Pavlova lutheri* (Droop) Green, Tahitian *Isochrysis* aff. *galbana* Green (clone T. ISO), and *Chaetoceros calcitrans* (Paulsen) Takano, originally shown to be suitable for rearing larval Sydney rock oyster (*Saccostrea commercialis*) larvae (Nell and O'Connor 1991). Within this study, growth is defined as an increase in shell length for larvae or height for spat, whereas survival is the number of live scallop larvae or spat at a particular time expressed as a percentage of the original stocking density.

Experiment 1. The Effect of Temperature on Embryo Development to D-Veliger Stage

Eggs from five scallops were pooled in a 5-L glass beaker filled with seawater (21 ± 0.5°C, 35 g/L) and thoroughly mixed with a perforated polyvinyl chloride plunger. The concentration of eggs with in the beaker was estimated from the mean count of four

replicate 1-mL aliquots sampled while mixing and examined at 40× magnification on a Sedgewick rafter slide. Within an hour of spawning, sufficient sperm was then added to ensure that between one and five sperm were visible at the periphery of each egg (Heasman et al. 1996b). Eggs were again thoroughly mixed within 15 min of fertilization, and samples of a volume that was calculated to contain 5,000 fertilized eggs were collected with an adjustable automatic pipette and transferred to 1-L beakers filled with filtered seawater.

Replicate sets of four 1-L beakers, stocked at five fertilized eggs/ml, were maintained at each of five temperatures—16, 18, 21, 24, or 27°C (±0.5°C)—with waterbaths fitted with thermostatically controlled immersion heaters. The water baths were housed in a coolroom held at a constant air temperature of 14 ± 1°C. After 48 h, the seawater in each beaker was thoroughly mixed as described above. A 20-mL sample, made up of four replicate 5.0-mL samples, was taken from each beaker, and the number of fully developed D-veligers was determined by dispersing the samples on petri dishes and counting larvae with the aid of a dissecting microscope (40× magnification). The total number of D-veligers in 20 ml was expressed as a percentage of the original number of eggs stocked in that volume (100 eggs).

Experiment 2. Effect of Temperature on Larval Growth and Survival

Fertilized eggs were stocked at approximately 50/mL into 90-L aerated polyethylene cylindroconical rearing vessels filled with filtered (1 μm pore size) seawater at 21°C. After 48 h, sufficient D-veliger larvae were collected to stock 20 90-L aerated cylindroconical polyethylene tanks at 5/mL. Four replicate tanks were held at each of five temperatures (15, 18, 21, 24, or 27 [±0.5]°C) by supporting the tanks in 1,000-L temperature-controlled water baths (Fig. 1).

Every 48 h, the entire contents of each 90-L tank was siphoned onto a 45-μm-pore-size nylon mesh screen that retained the larvae. Larvae from each 90-L tank were resuspended in 4 L of seawater, and a 1-mL sample was collected. From this sample, mean larval size (greatest shell length parallel to the hinge) and survival were determined with a dissecting microscope (40× magnification) fitted with an eyepiece micrometer. The seawater in each 90-L tank was replaced with fresh, temperature-equilibrated, filtered (1 μm pore size) seawater, and the larvae were returned to the tank.

P. fumatus pediveligers normally begin to settlement behavior at shell lengths of 220 μm or more; however, some larvae as small as 185 μm had previously been found attached to the surface of the rearing tanks, thereby making samples taken from the water column potentially unrepresentative. Consequently, survival data were recorded at the first appearance of 185-μm larvae in each replicate. The effect of temperature on survival was calculated in terms of both age and size, with size-specific survival being considered the most appropriate means for selecting optimal rearing temperature.

Experiment 3. Effect of Temperature on Growth, Survival, and Byssal Attachment of Spat

Hatchery-reared *P. fumatus* spat were maintained for 20 days after settlement on 150-μm-pore-size polyester mesh screens at 21°C in a downweller system (Bayes 1981). Forty spat (1.04 ± 0.26 mm, mean ± standard error) were randomly allocated to each of 20 miniature downweller systems (Fig. 1). Previously described, 90-L cylindroconical rearing vessels fitted with lids were used to house individual miniature upwellers. These vessels were suspended in 1,000-L water baths maintained within ±0.5°C of prescribed temperatures of 13, 17, 21, 24, or 27°C.

Seawater was replaced with fresh, temperature-equilibrated seawater every 48 h. Salinities were monitored daily throughout the experiment to ensure that they did not vary outside the range of 35 ± 1.0 g/L. The number of spat byssally attached to the internal walls and bottom mesh of each downweller screen was monitored. Whether or not scallops were byssally attached was determined by gently directing a stream of seawater from a squeeze bottle at individual spat 5, 20, and 40 min and 12 h after the initial stocking of the experiment and subsequently at weekly intervals. Each week, the miniature downwellers were placed in a 45 g/L hypersaline solution prepared by the addition of artificial sea salt (Instant Ocean; Aquarium Systems, Sarrebourg, France) to seawater. This procedure induced the rapid release of byssally attached scallops without imposing the traumatic injury and stress associated with mechanical methods of detaching spat (Heasman et al. 1994a). Detached spat were transferred to petri dishes, and the shell heights of live scallops were measured at 25× with a dissecting microscope fitted with a calibrated eyepiece micrometer. The total number of dead spat detected in each miniature upweller was recorded on each sampling occasion, their shells were removed, and the shell height was measured and recorded.

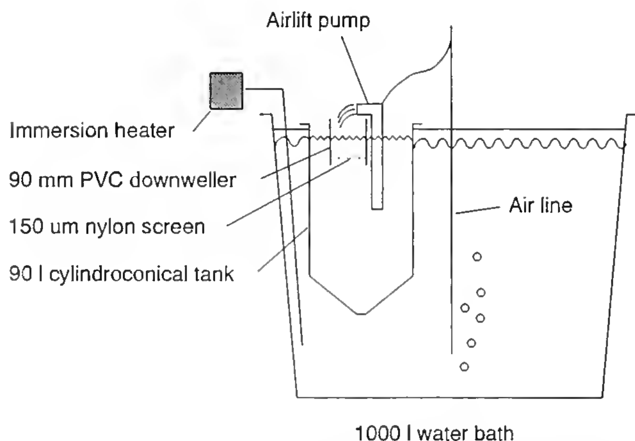


Figure 1. Mini downweller system used for growing *P. fumatus* spat. PVC, polyvinyl chloride.

RESULTS

Experiment 1. Effect of Incubation Temperature on Embryo Development to D-Veliger Stage

The greatest mean yield of normal D-veliger larvae, 51 and 54%, respectively, occurred at the lowest test temperatures of 15 and 18°C (Fig. 2). The yield of D-veligers decreased sharply with increasing temperature above 18°C, falling to a mere 10% at 24°C and to 0% at 27°C.

Experiment 2. Effect of Temperature on Larval Growth and Survival

Larval growth (Fig. 3A), as indicated by mean growth increment after 9 days (Fig. 3B), increased markedly with increasing temperature from 15 to 24°C but decreased with a further increase in temperature to 27°C. The age-specific survival of larvae (Fig. 4) was inversely related to rearing temperature, being highest at 15°C and lowest at 27°C. However, scrutiny of size-specific survival

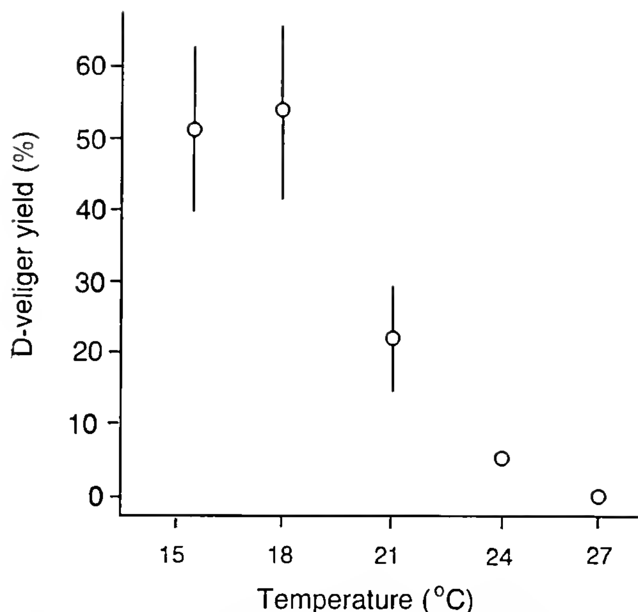


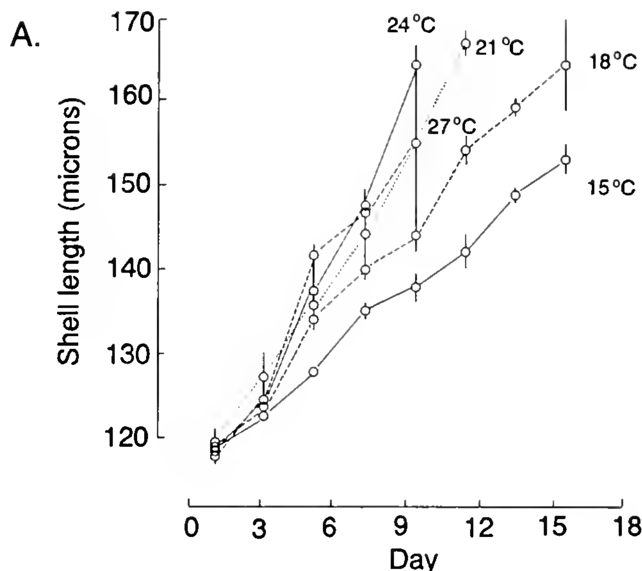
Figure 2. Percent development of *P. fumatus* D-veligers after the incubation of embryos at 15, 18, 21, 24, or 27°C. Values are means ± standard error (SE).

data (Fig. 5) revealed a different pattern. Larvae survived to reach a shell height of 150 μm at all temperatures. At this time, size-specific survival was highest at higher temperatures, reaching a maximum value at 21°C (40% survival). It then decreased sharply and progressively with further increases in temperature to 24 and 27°C. Larvae in one replicate tank held at 24°C survived to a mean shell length of 166 μm, but all died before the next water change. The experiment ceased when the largest larvae in a replicate commenced settlement. Larvae only survived to metamorphosis in treatments held at 18 and 21°C.

Experiment 3. Effect of Temperature on Growth, Survival, and Byssal Attachment of Spat

P. fumatus spat with an initial shell height of 1.04 ± 0.26 mm (mean ± standard deviation) grew exponentially at each of the five temperatures tested, i.e., 13, 17, 21, 24, and 27°C ± 0.5°C, during the first 4 wk of the trial (Fig. 6). Exponential equations were fitted to the first 4 wk of growth data (Table 1) ahead of linear and other polynomial equations on the basis of higher r^2 goodness of fit values (83.08–99.30%). This experiment was, however, terminated a week later when constraints to continued exponential growth became evident at all test temperatures other than 13°C. This growth "stalling" in spat retained in the hatchery and fed diets selected for larval growth and survival was not unexpected, having occurred in all previous batches of *P. fumatus* spat reared at our hatchery. In all cases, spat growth ceased when they had either been retained in the hatchery beyond about 6 wk postsettlement or to a mean shell height in the range of 2–4 mm and fed a standard bivalve larval diet (Heasman et al. 1994b).

Age-specific survival was lowest at the two lowest test temperatures, with survival at the end of the fifth week falling to 67% at 17°C and 60% at 13°C. By contrast, age-specific survival exceeded 92% at the three higher test temperatures of 21, 24, and 27°C after 5 wk. Size-specific survival shows the advantage of maintaining spat above temperatures of 17°C (Fig. 7).



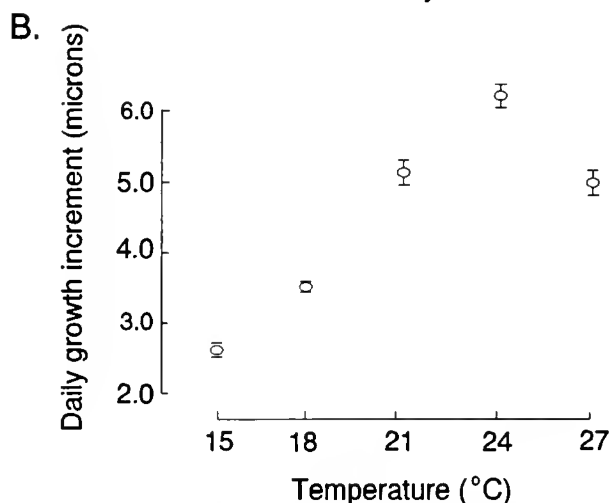


Figure 3. (A) Mean size of *P. fumatus* larvae held at 15, 18, 21, 24, or 27°C. (B) Mean daily growth increment (±SE) after 9 days of larval rearing.

As indicated in Fig. 8, after stocking, most spat attached rapidly to the bottom mesh or side walls of miniature downwellers screen. Peak numbers of spat attached were always attained within 12 h, and most were attained within 40 min at the temperatures tested. Short-term percentages attached nevertheless increased with temperature, from a peak of about 77% at 13°C to over 95% at 24 and 27°C. Much greater differences in percentages of byssal attachment, however, developed over the 5-wk course of the experiment. The effects of rearing temperature on percentages of spat attached were thus consistent with its previously described effects on growth and survival.

DISCUSSION

Temperature has a marked effect on incubation and larval development in pectinids, beginning with the rate of cell division during early cleavage stages. For example, cell division rate in embryos is distinctly higher at 20°C than at 15 or 10°C (Zavarzeva 1981, cited in Cragg and Crisp 1991). Although we made no detailed observations of the effect of temperature on the incubation

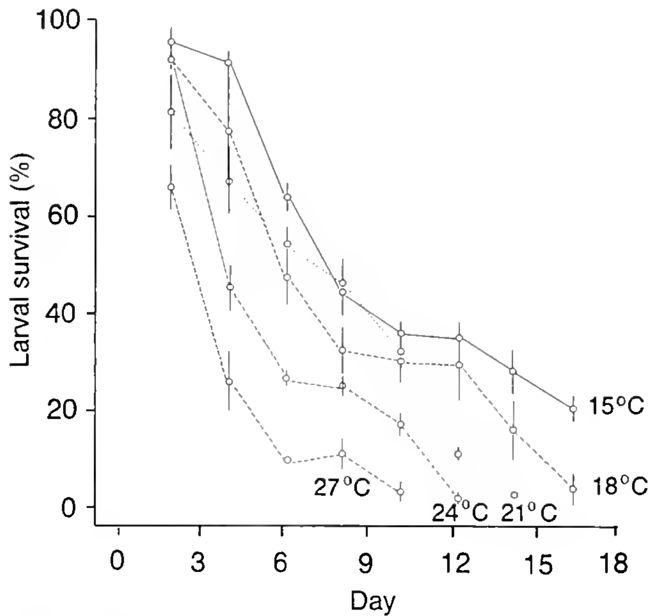


Figure 4. Age-specific survival of *P. fumatus* larvae held at 15, 18, 21, 24, or 27°C.

rate of *P. fumatus*, it was noted that development to the straight-hinge (D-veliger) first-feeding stage always occurred within 48 h when temperature was maintained at or above 18°C. The possibility that poor percent development to D-veliger stage was the product of increased bacterial levels was discounted on the basis of previous studies. The inclusion of several antibiotics, including erythromycin, oxolinic acid, and chloramphenicol, to experimental batches of embryos failed to increase percentages developing to D-veliger at elevated temperatures (Heasman, O'Connor, and Frazer unpub. data).

In a review, Cragg and Crisp (1991) found that time to metamorphosis in pectinids is related to temperature and, when ex-

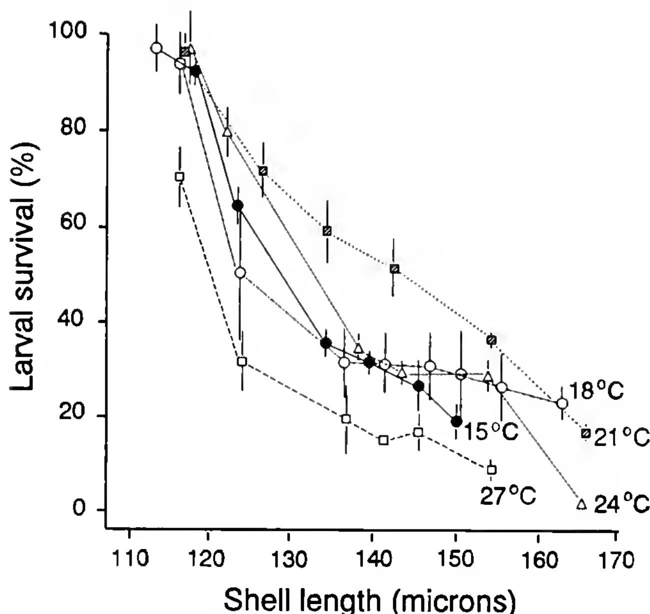


Figure 5. Size-specific survival of *P. fumatus* larvae when held at a temperature of 15, 18, 21, 24, or 27°C.

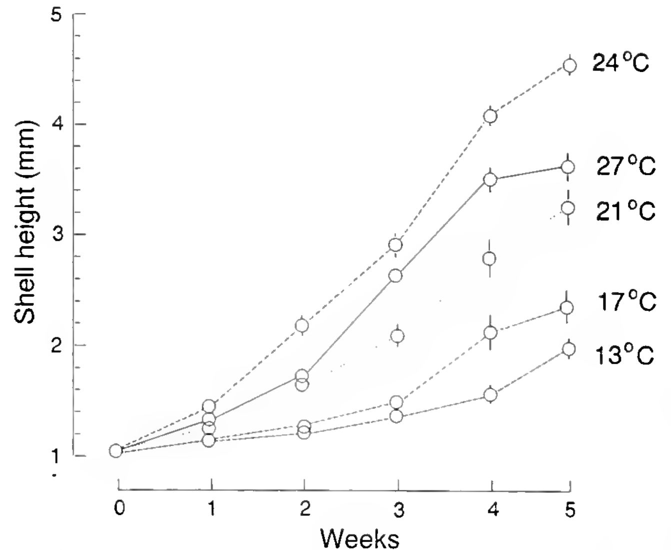


Figure 6. Growth of *P. fumatus* spat held in the hatchery at 13, 17, 21, 24, or 27°C.

pressed as an Arrhenius plot, is described by a single regression line. Figure 9 shows that equivalent data of *P. fumatus*, which ranges from 15 to 16 days at 18–19°C (Frankish et al. 1990) and 31 days at 13–15°C (Dix and Sjardin 1975), conform to this relationship. These results for *P. fumatus* suggest possible benefits in further increases in rearing temperature, although larval growth rates have been shown to increase with increasing temperature to a maximum value and then to decline with a further rise in temperature. Ursin (1963) described the relationship between temperature and the time to complete a specified amount of growth as a symmetrical caternary curve:

$$y = y_o \cosh p(x - x_o)$$

where y is time, x is the temperature at which development is most rapid, y_o is the development at x_o, and p is a temperature coefficient. By the use of this relationship, the larval growth data of *P. fumatus* from this study were compared in Figure 10 with equivalent data for four other marine bivalves and a gastropod species compiled by Bayne (1983). Apices of the curves for each species coincide with their respective maximum growth rates. Maximum growth rate (up until a shell length of 150 μm) occurs about 24°C in the case of *P. fumatus*. This temperature corresponds with the highest sea temperatures that occur in late summer on the central coast of NSW (Wolf and Collins 1979), the northern extent of the

TABLE 1.

Exponential equations describing increases in shell height over 4 wk of *P. fumatus* spat reared at temperatures of 13, 17, 21, 24, or 27°C (n = 4).

Temperature (°C)	Exponential Equation	r^2
13	$Y = e^{(0\ 0394 + 0.0559X)}$	83.86
17	$Y = e^{(-0\ 0234 + 0\ 1647X)}$	83.08
21	$Y = e^{(0\ 0320 + 0.2231X)}$	96.70
24	$Y = e^{(0.0348 + 0.3447X)}$	99.30
27	$Y = e^{(-0.0394 + 0.3317X)}$	97.73

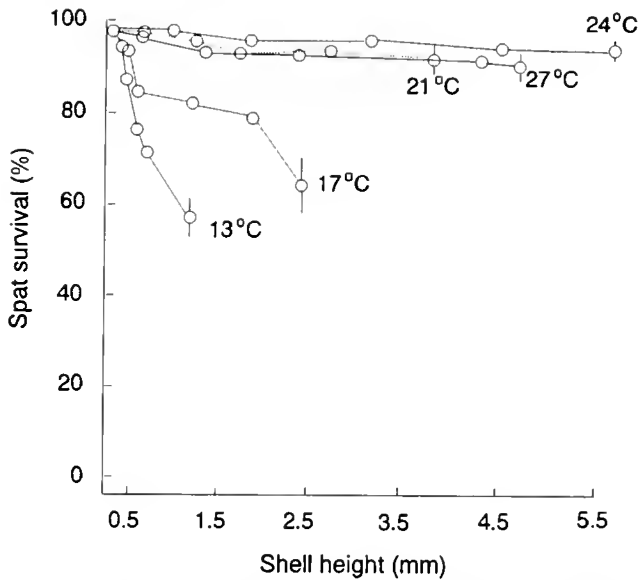


Figure 7. Spat survival with shell height increase over 5 wk for *P. fumatus* spat held at 13, 17, 21, 24, or 27°C. Points indicate mean shell height and cumulative survival after each week.

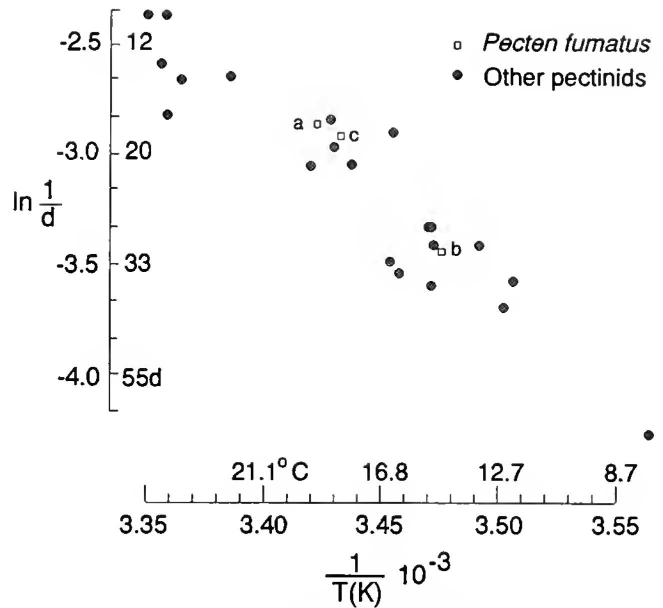


Figure 9. Arrhenius plot of the relationship between time from fertilization to metamorphosis and water temperature for various pectinids reared at close to the optimal temperature for each species (redrawn from Cragg and Crisp 1991). Datum points represented by squares are for *P. fumatus* using data from (a) this study and those of (b) Dix and Sjardin (1975) and (c) Frankish et al. (1990). Time is in days (d), temperature (T) is in degrees Celsius, and K is a rate constant.

species range. However, size-specific survival data indicate that the maintenance of larvae in the hatchery at about 21°C is likely to provide the maximum yield of larvae for settlement (Fig. 5). Although larvae initially grew rapidly at 24°C, they did not survive to metamorphosis. The reasons for this are unclear, but could involve the failure of larvae to maintain a net positive energy balance as a product of increased metabolic rates at higher temperatures. Overall larval survival in this experiment was poor, but was thought to reflect a general trend for poor yields from small experiment vessels in our hatchery.

Unlike larvae, both the growth and the survival of *P. fumatus* spat were high at temperatures in the range of 21–27°C and greatest at 24°C, up until the fifth week of the experiment. Comparisons of the growth of spat retained within a hatchery and fed diets of cultured algae with those placed in the wild or maintained in flow-through systems with natural phytoplankton have found the latter to be superior for both pearl oysters (Alagarswami et al. 1989) and scallops (Bourne and Hodgson 1991). This was also the

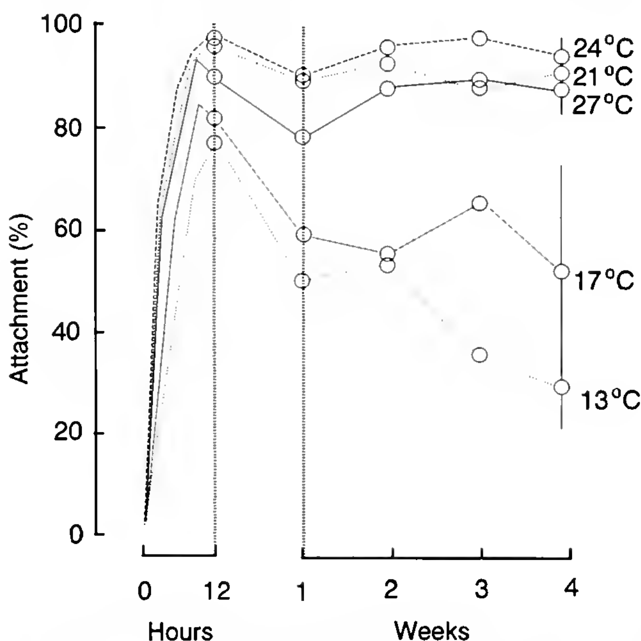


Figure 8. Reattachment of *P. fumatus* spat in the first 12 h and in the succeeding weeks when held at 13, 17, 21, 24, or 27°C.

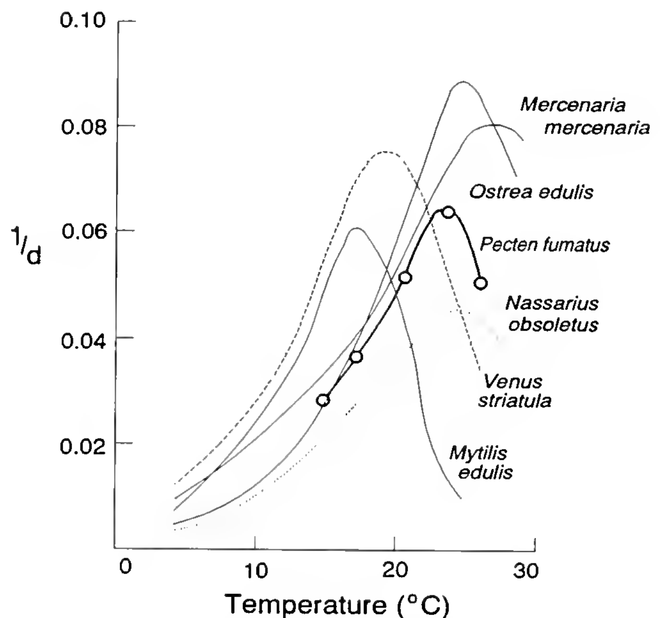


Figure 10. Growth rates of veliger larvae, calculated as the reciprocal of time in days from fertilization to pediveliger stage, related to temperature. Graphs are redrawn from Bayne (1983), with data for *P. fumatus* included.

case for *P. fumatus* spat. The apparent barrier to continued growth, evident toward the end of this experiment, has been investigated in recent studies (M. Heasman unpub. data 1995). The causative factor appears to be nutritional, with a shift in preferred diet from the larval diet used in this study toward diatoms at some point after metamorphosis. Regardless, the high cost of hatchery algal production encourages the deployment of spat to field nursery systems, thereby avoiding growth retardation due to dietary factors. The preference exhibited by spat for elevated temperatures, then, raises the prospects of greenhoused nursery systems or perhaps farming this species as far north as Queensland, i.e., up to 1,000 km north of its natural geographic range, at mean monthly sea temperatures as high as 27°C. However, field farming trials are needed to test this assertion.

One means to quickly assess the suitability of more northern growout sites may be byssogenesis. In scallops, it has been proposed as a useful bioassay for potentially toxic substances (Roberts 1973, cited in Paul 1980a) and has been suggested to be a useful observation for the establishment of temperature optima in the scallop *Chlamys opercularis* (Paul 1980a). Byssogenesis by *P. fumatus* spat within 1–2 h of their transfer to new environments consistently reflected subsequent growth and survival under the particular set of physiochemical conditions involved. Short-term byssal attachment percentages may additionally serve as a useful quick indicator of suboptimal nursery and farming sites such as those subject to pollution, excessive turbidity, or suboptimal water chemistry due to coastal runoff.

Most studies on the influence of temperature on scallops have focused on the tolerance limits of adult scallops (e.g., Paul 1980a, Paul 1980b), with particular reference to ecological implications. However, sublethal temperature effects can also limit geographic range. For example, where metabolic rate increases with increasing temperature, greater energy acquisition via increased phytoplankton clearance is also required to maintain a positive energy balance. Barber and Blake (1985) suggest that, in this way, elevated metabolic rate limits the southerly distribution of *Argopecten irradians irradians* in the United States. On the other hand, interactions between temperature and other environmental variables such as dissolved oxygen may restrict the northerly range of the same species (Voyer 1992).

The large disparities between optimal temperatures of approximately 15°C for gonadal development, 15–18°C for fertilization and incubation, 21°C for larval growth and survival, and 24°C for growth and survival of juvenile *P. fumatus* found in this and related studies (Heasman et al. 1994b, Heasman et al. in press b) are of ecological interest. The significance of these findings needs to be considered in relation to the life cycle of wild populations of *P. fumatus*. Studies by Jacobs (1983), Fuentes (1994), and Heasman and O'Connor (unpub. data) have revealed multiple (three or four) annual peaks in gonadosomatic index in Jervis Bay *P. fumatus*. These peaks can occur at approximately 1- to 2-mo intervals over an 8- to 9-mo breeding season, beginning in midautumn (April) and ending in late spring (November). This period coincides with mean monthly seawater temperatures in Jervis Bay in the range of 14–17°C (May et al. 1978, CSIRO 1994). These temperatures are similar to those experimentally determined to be suitable for gonad conditioning (15°C; Heasman et al. 1996a).

The relationship between spawnings in *P. fumatus* and subsequent settlement and recruitment is variable and is thought to be greatly influenced by environmental conditions (Zacharin 1994). The incidences of such variation are common in the literature. Between October 1989 and October 1990 in Jervis Bay, Fuentes (1994) recorded four peaks in reproductive activity, indicative of spawning events, yet only two pulses of spat settlement were detected. Further, as also observed in Tasmania and Victoria (Hortle and Cropp 1987, Sause et al. 1987), recorded spat settlements sometimes produced negligible subsequent recruitment to the fishery. Throughout its range, major spat settlements of *P. fumatus* have emanated largely from spawnings in spring and early summer, when larvae encounter warm and rising sea temperatures. Although this observation is in general agreement with the findings of this study, the probability of *P. fumatus* larvae generated from spring or even early summer spawnings encountering optimal temperatures around 21°C would seem very low. However, it is conceivable that intermittent "boom" catches, which occur every 10 y or so in the NSW *P. fumatus* fishery (Hamer and Jacobs 1987) and comparable widely fluctuating catches reported from Tasmania (Zacharin 1989, Young and Martin 1989) and Victoria (Gwyther 1989), may coincide with unusually large and rapid increases in sea temperature in spring or early summer. Such events could result in a mass synchronized spawning, especially if preceded by extended (3- to 6-wk) periods of low and stable sea temperatures and a high abundance of phytokplanktonic food, favorable to rapid synchronous growth and the development of gonads. Mass spawning at a time most conducive to subsequent high growth and the survival of larvae would in turn enhance the probability of spat settlement sufficient to overwhelm natural predation and hence to generate high-level recruitment to the fishery.

Some support for the above hypothesis that booms in *P. fumatus* fisheries are linked with favorable but unusual thermal sequences is provided by oceanographic studies of southeastern Australia. The southeast coast of Australia, including Jervis Bay, is subject to two major interrelated influences, the warm "East Australian Current" (EAC), originating in the coral Sea (Fig. 11) and flowing south along the eastern seaboard, and a deeper, cooler, nutrient-rich upwelling comprising an Ekman boundary layer. The latter is generated by the overlying EAC and is driven tangentially from the continental slope toward the coast. Flow patterns of the EAC are often intense and highly variable. Between latitude 27°C (Tweed heads) and 32°S (Tuncurry/Forster) (Fig. 11), the flow often consists of strong southward currents near the edge of the shelf and equally strong northward currents further offshore. South of 32°S, the current degenerates into large, counterclockwise eddies. Each year on average, four to six of these warm meandering eddies progress as far south as southern Tasmania. They may affect any given section of the coast, especially during spring and summer (September to February), for periods of 4 wk to 4 mo (Boland 1979).

Seawater exchange in Jervis Bay (Fig. 11) occurs mainly as a near-surface inflow on the southern side of the entrance in phase with a deeper outflow on the northern side. Flushing times for the bay were estimated by Holloway et al. (1992) to vary from 10 to 74 days, with a median of 21 days. Those authors also detected large pulses of cold (14–16°C) shelf water at a depth of 30 m beneath a surface layer of warm (20–24°C) seawater at the bay entrance during summer 1989/90. These cold, nutrient-rich seawater intrusions persisted for periods of up to 3 wk at the entrance. One such dramatic intrusion of cold (15°C), nutrient-rich continental slope water was driven into Jervis Bay by a near-shore, warm-core eddy of the EAC in late November 1992. This intrusion is thought to have caused a dramatic algal bloom (*Gephyrocapsa oceanica*) that turned the whole bay milky for a month (Blackburn

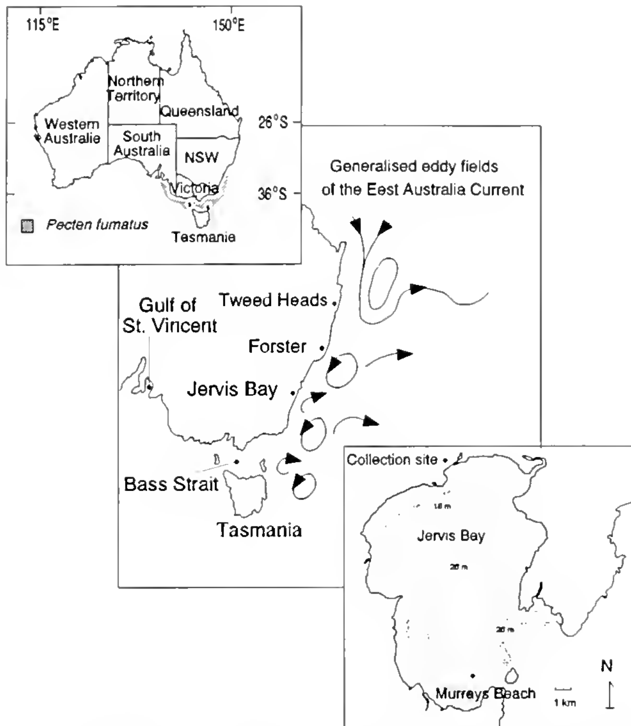


Figure 11. Maps of Australia, southeast Australia, and Jervis Bay, depicting the distribution of *P. fumatus*, position and conformation of the EAC, places referred to in the test, and the broodstock collection site.

and Cresswell 1993). This event lowered bottom temperatures within the bay from 18 to 16°C, but only fleetingly. Bottom temperature subsequently returned to 18°C by mid-January and rose to a peak of only 21°C by mid-February 1993 (M. Heasman et al. unpub. data). This was considerably lower than peak February temperatures of 23°C in 1989 (CSIRO 1994) and 1994 (M. Heasman unpubl. data) and of 25°C in 1991 (CSIRO 1994). The warm-water eddies of the EAC and associated cold, nutrient-rich upwellings regularly cause coastal phytoplankton blooms along the NSW (Tranter et al. 1986) and Tasmanian coasts (Harris et al. 1987) in spring and summer. These eddies and upwellings therefore exhibit the potential to occasionally create ideal conditions for mass spawning, high subsequent spatfall, and thence, high-level recruitment of scallops. Harris et al. (1988), in an analysis of Tasmanian scallop catches from the 1940s to the 1960s, found that years of high incidence of "Zonal Westerly Winds" (ZWW), linked to an El Niño Southern Oscillation cycle with a mean periodicity of 11 y, "appear to favour good recruitment perhaps by a link between high productivity in high ZWW years, and increase in spawning and high larval survivorship." Similarly, high recruitment in the Shark Bay *Amusium balloti* fishery was usually associated with weak Leeuwin current activity in the winter months (Joll 1994, Joll and Caputi 1995). It was thought that the strong current activity may flush *A. balloti* larvae from embayments along the coast or may expose larvae to less favorable, high-temperature, low-nutrient-level seawater.

ACKNOWLEDGMENTS

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OPTIMUM CONCENTRATIONS OF *ISOCHRYSIS GALBANA* FOR GROWTH OF LARVAL AND JUVENILE BAY SCALLOPS, *ARGOPECTEN IRRADIANS CONCENTRICUS* (SAY)

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ABSTRACT Bay scallops from Homosassa, FL, were spawned at the Department of Marine Science, University of South Florida at St. Petersburg, FL. Total dry weight (DW) and ash free dry weight (AFDW) were determined for larvae and juveniles. DW and AFDW (in milligrams) increased with increasing shell length (L, in millimeters, for larvae) or shell height (H, in millimeters, for juveniles), according to the following allometric equations. $DW = 0.0693L^{2.715}$ and $AFDW = 0.0459L^{2.984}$ in larvae and $DW = 0.0715H^{3.069}$ and $AFDW = 0.0138H^{2.664}$ in juveniles. Growth was determined for larvae and juveniles at 25°C under six algal concentrations of *Isochrysis galbana* (1–30 cells/μL for larvae and 1–50 cells/μL for juveniles). Growth rates increased as algal concentration increased. The maximum larval growth rate ranged from 7–23 μm/day. The development of eyespots and metamorphosis started earlier at higher algal concentrations. The optimal algal concentration for growth was 20 cells/μL for larvae and 10 cells/μL for juveniles. In juveniles, the growth rate was higher and increased with increasing body size. The relative growth rate (daily percent increase in AFDW) was higher in larvae than in juveniles and decreased with increasing body size in juveniles.

KEY WORDS: Bay scallop, *Argopecten irradians concentricus*, larvae, juveniles, growth.

INTRODUCTION

Growth is the most integrated response of organisms to changes in their surrounding environmental conditions (MacDonald and Thompson 1985), among which food availability is one of the most influential, and thus best studied, factors. Growth occurs when an animal is in positive energy balance, i.e., when assimilated energy exceeds the maintenance needs of the animal, and additional energy is used to increase body weight. The relationship between growth and food availability has been an important research subject of physiologic energetics for a variety of bivalves, especially some commercially important species, e.g., mussels (Widdows 1978, Bayne and Worrall 1980), oysters (Abdel-Hamid et al. 1992, Beiras and Camacho 1994), and scallops (MacDonald 1988, Hollett and Dabinett 1989), but such information on bay scallops is limited (Cahalan et al. 1989).

The early life history of the bay scallop is characterized by a planktonic larval stage lasting 10–19 days, depending on environmental conditions (Sastry 1965, Castagna and Duggan 1971). At the end of the planktonic stage, larvae settle onto a substrate, typically seagrass blades, where they metamorphose to juveniles (Belding 1910, Gutsell 1930). Juveniles remain attached and grow to a shell height of about 20 mm before they detach and settle to the bottom to begin adult life. Existing data suggest that the highest mortality occurs during the larval and juvenile stages and mortality declines with increasing animal size (Castagna and Duggan 1971).

The growth and survival of larvae and young juveniles in animals with planktonic stages, like the bay scallop, determine the success of recruitment into adult populations. Yamamoto (1964) found that only 5–10% of newly settled *Patinopecten yessoensis* juveniles survive their first 2 mo of life in Mutsu Bay. Paynter et al. (1993) reported that survival rates of *Crassostrea virginica* from pediveliger larvae to 5-mm spat averaged less than 5% at the Horn Point Hatchery of the University of Maryland. Considerable fluctuations in the abundance of bay scallop populations occur along the Florida Gulf Coast, and although the causes of such fluctuations are not fully clear, they may be related to reductions in early embryonic development and larval survival. Mortality during larval and juvenile stages can be lowered by fast growth. Unfortunately, detailed information is lacking on the physiology of the early growth of this species in relation to environmental conditions, particularly food availability.

This study was designed to examine the growth of larval and juvenile bay scallops, *Argopecten irradians concentricus*, under various concentrations of the unicellular algae (*Isochrysis galbana* strain TISO. *I. galbana* has been shown to be an adequate food species for bivalve culture and has been used in many studies as food for bivalve larvae (e.g., Castagna 1975, Peirson 1983, Sprung 1984, MacDonald 1988, Lu 1989, Zhang et al. 1991).

MATERIALS AND METHODS

Bay scallops collected from Homosassa, FL, were spawned at the Department of Marine Science, University of South Florida. Mature scallops were allowed to spawn in a 500-L fiberglass tank at 24–26°C and 25–28‰. Fertilized eggs were allowed to develop for 20–30 h, at which time they became D-shaped larvae. The larvae were then filtered onto a 35-μm-pore-size screen and released into fresh seawater. Larvae were cultured at a density of 4–8/mL and were fed daily with 10,000–30,000 cells/mL of *I. galbana*, which was grown in f/2 median in 10-L plastic bags. Seawater was replaced every day in the amount of one-third of the total volume. As soon as larvae started to develop eyespots, black plastic *Thalassia* mimics were added to the larval tank as substrate for larval settlement. The daily food ration for juveniles was increased gradually from 30,000 to 100,000 cells/mL of *I. galbana*.

Weight Determinatians

Every 2–3 days during their planktonic life, larvae were sampled from a stocking tank, filtered onto a 35-μm-pore-size screen, and resuspended to 200 mL of filtered seawater. Larval density was determined by counting five subsamples of 2 mL each under a microscope. About 1,000–4,000 larvae were filtered onto a precombusted (475°C) and preweighed Whatman GF/C filter (punched to 7-mm diameter), rinsed with a 3% ammonium formate solution, and dried at 60°C for 48 h. Filters with larvae were weighed on an electronic microbalance to ±1 μg. They were then

combusted in a muffle furnace at 475°C for 5 h and reweighed for ash weight. Total ash free dry weight (AFDW) was obtained by subtracting total ash weight from total dry weight (DW).

The weights of juveniles were measured in groups (for juveniles <2 mm in shell height) or individually (for juveniles >2 mm in shell height). They were rinsed with a 3% ammonium formate solution and transferred to a precombusted and preweighed Whatman GF/C filter (cut to one-eighth of its original size) with a pipette for small individuals or forceps for larger ones. Total DW, total ash weight, and total AFDW of juveniles were determined by use of the same procedure as described for larvae.

To remove the soft parts for shell-weight determinations, larvae and small juveniles were killed in fresh water, and later, the animals were placed in beakers with seawater. The seawater in the beaker was changed every couple of days in the course of 1–2 wk until the soft tissue could be removed by microorganisms. For larger juveniles, shells were obtained by the removal of the soft body tissue with forceps.

Growth Rates

All experiments were carried out at 25°C, a good approximation of the temperature in late September and early October in Homosassa (Barber and Blake 1983), where the scallop stock was collected. Larvae (48 h old) from a stocking tank were placed in 2-L plastic beakers containing filtered (0.5 μm pore size) seawater at an initial density of 1 individual/mL. *I. galbana* was added to the beakers to make a series of cultures, each containing one of the six algal concentrations: 1, 2, 5, 10, 20, and 30 cells/μL. Larval cultures with no *I. galbana* were used as controls. All larval cultures were duplicated and were placed in a 25°C water bath. Gentle aeration was provided to the beakers to supply oxygen and to keep food particles in suspension. Experimental media were changed every day by filtering larvae onto a 35-μm-pore-size screen and resuspending them in freshly prepared media. In addition, algal concentrations were monitored daily with a Coulter Counter and were adjusted if variation occurred by >10% from the original levels (MacDonald 1988). Each day, a sample was taken from each culture with a pipette, and the shell lengths of 20 larvae were measured with a microscope fitted with an ocular micrometer. Experiments lasted until larval settlement occurred.

Two-liter plastic beakers were used for experiments with juveniles 0.5- and 1.0-mm shell height, whereas 16-L glass tanks were used for juveniles 2–5.7 mm. Twenty animals were placed in each beaker or tank, except for the 5.7-mm size class, which was held at 10 animals per tank. Growth was determined at 1, 5, 10, 20, 30, and 50 cells/μL of *I. galbana*. For juveniles larger than 2 mm, *I. galbana* stocks from 1,000-mL beakers were pumped with a Manostat cassette pump unit into the tanks continuously at a rate of 1,000 mL/day, to keep up with the reduction of algal concentrations caused by juvenile feeding. These algal stock solutions were prepared freshly every day at concentrations ranging from 5 to 400 cells/μL, which were determined by calculations from juvenile ingestion rate (Lu 1996). Algal concentrations in juvenile culture media were also monitored daily with a Coulter Counter and adjusted as needed. Experiments with juveniles of all sizes were terminated on the 5th day, when juveniles were gently removed from experimental containers, collected in a Petri dish, and measured under a microscope.

RESULTS

Weight Determinations

Body weight increased with increasing shell length (for larvae) or height (for juveniles), and the relationship can be described by the allometric equation $W = aH^b$, where W is body weight and H is shell length or height. The fitted parameters a and b for various weight-size relationships are listed in Table 1. Measured data and fitted curves are shown in Figure 1.

Total AFDW of larvae as a percentage of total DW was high, averaging about 37–43%, and it increased slightly as larvae grew (Fig. 2a); however, juveniles showed a dramatic drop in the percentage of AFDW from about 30% for 0.4-mm juveniles to about 9% for 5-mm juveniles. The percentage of AFDW becomes constant at approximately 8% for juveniles >5 mm (Fig. 2b).

Shell AFDW increased in relation to shell height. When expressed as a percentage of shell weight, shell AFDW decreases from a mean value of 12.5% in larvae to 2% in 5-mm juveniles. It further decreased to about 1.2% in larger juveniles (Fig. 3).

Growth of Larvae and Metamorphosis

The shell growth of larvae at various algal concentrations is illustrated in Figure 4. Mean growth rate increased to its maximum at shell lengths between 140 and 150 μm and then decreased as shell length further increased. The maximum growth rate showed a strong positive correlation with algal concentration, being 7 μm/

TABLE 1.

A. i. concentricus: **fitted parameters (a and b) for allometric relationships between body weight (mg) and shell length (larvae, mm) or shell height (juveniles, mm) ($W = aH^b$).**

Stage	Parameter	Total DW	Total Ash Wt	Total AFDW	Shell Wt	Shell Ash Wt	Shell AFDW
Larvae	a	0.0693	0.0345	0.0315	0.1574	0.1644	0.0024
	b	2.715	2.622	2.755	3.583	3.680	2.531
	r^2	0.910	0.859	0.870	0.950	0.974	
	n[a]	33	33	33	10	10	10
Juveniles	a	0.0715	0.0571	0.0138	0.0592	0.0567	0.0023
	b	3.069	3.140	2.664	3.105	3.119	2.648
	r^2	0.987	0.983	0.985	0.987	0.984	0.891
	n	81	81	81	47	47	47

[a] n, number of datum points evaluated.

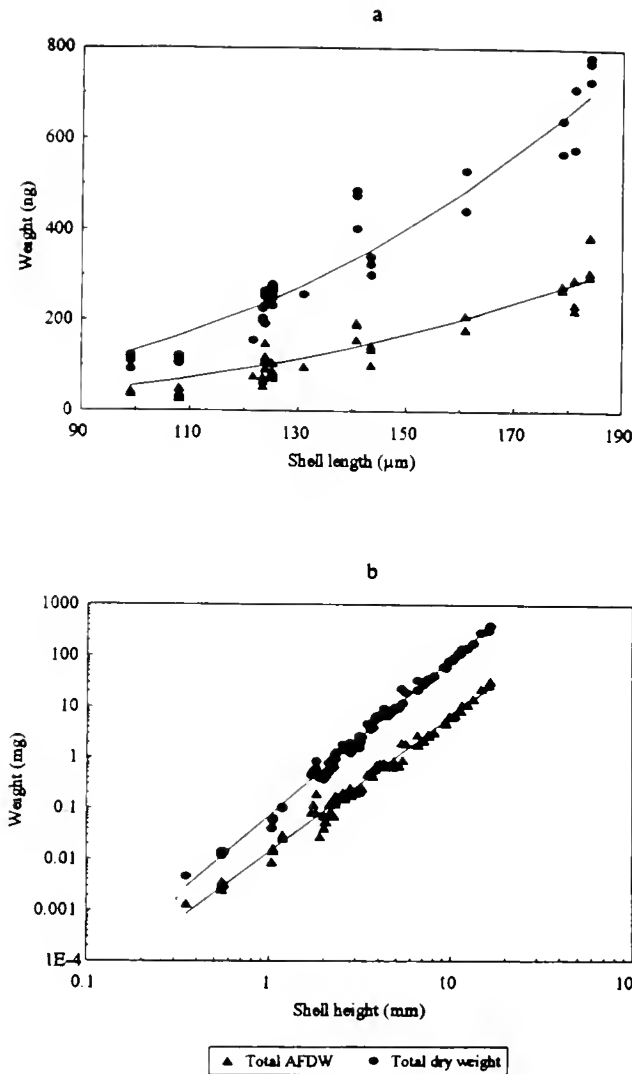

Figure 1. ***A. i. concentricus.*** **Allometric relationship between body weight and shell length of larvae (a) and shell height of juveniles (b). Fitted parameters for the curves are listed in Table 1.**

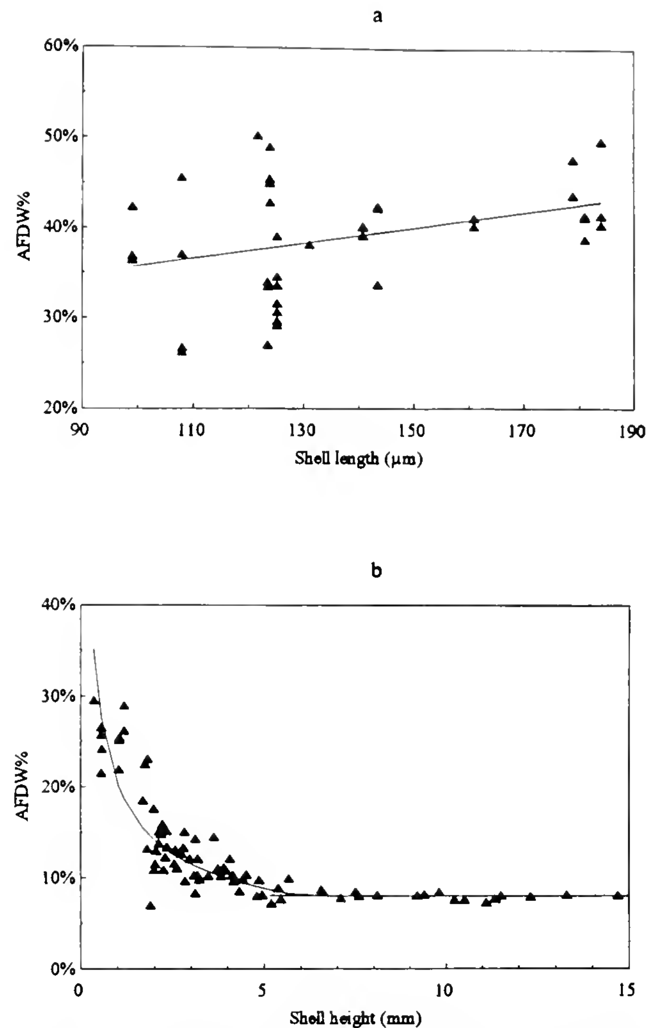

Figure 2. ***A. i. concentricus.*** **Total AFDW as a percentage of total DW in larvae (a) and juveniles (b). Fitted line in panel a: AFDW% = 0.271 + 0.000865L(μm), r^2 = 0.13. Fitted curve in panel b: AFDW% = $0.204H(mm)^{-0.45}$ at H < 5 mm; at H > 5 mm, AFDW% becomes constant at around 8%.**

day at 1 cell/μL and increasing to 23 μm/day at 30 cells/μL. The following logistic growth function was fitted to the measured data:

$$L_t = \frac{a}{1 + \frac{(a-b)}{b} e^{-r(t-5)}} + c$$

where L_t is shell length at time t; r is growth rate; and a, b, and c are constants. The fitted parameters are shown in Table 2, and the fitted curves are shown in Figure 4.

Two-way analysis of variance (ANOVA) demonstrate that the growth of larvae was significantly influenced by algal concentration and larval age (day) (Table 3). Multiple range analysis on least significant differences between means showed that the growth of larvae was significantly different between any algal concentrations from 0 (control) to 20 cells/μL, whereas there was no difference statistically between 20 and 30 cells/μL (Table 4).

The development of eyespots was highly synchronized in the cultures of 20 and 30 cells/μL (Fig. 5). Eyespots were observed on the 10th day after fertilization, and over 95% of larvae developed eyespots a day later. In contrast, no eyed larvae were found in the 1 cell/μL cultures on the 11th day and eyed larvae composed only 18% of the larval population in the 2 cells/μL cultures. At 1 cell/μL, the lowest algal concentration tested, larvae could develop eyespots and eventually metamorphose, but there was a 2-day delay in development compared with those larvae cultured at 10, 20, and 30 cells/μL. The average shell length of eyed larvae was also a function of algal concentration, with higher average shell length found at higher algal concentrations.

The metamorphosis of larvae was first recorded 11 days after fertilization at the three higher algal concentrations, 10, 20, and 30 cells/μL; 13 days after fertilization at 5 and 2 cells/μL; and 15 days after fertilization at 1 cell/μL, demonstrating a strong correlation between the rate of development and algal concentration. The same trend was observed in the number of successful metamorphoses. A higher percentage of metamorphosis was observed as algal concentrations increased, although the rate of successful

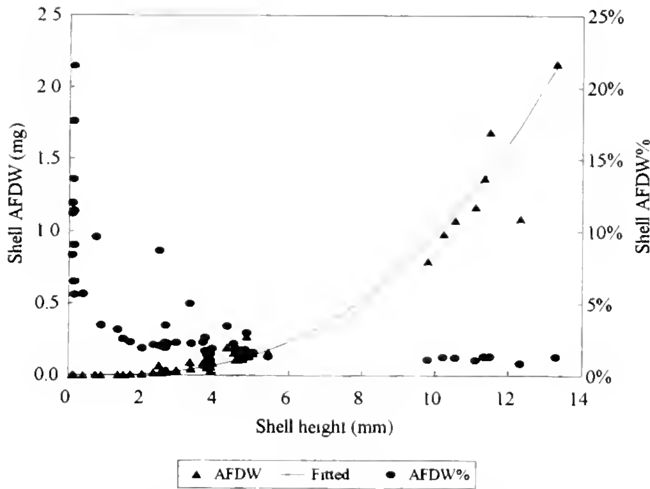

Figure 3. ***A. i. concentricus*. Shell AFDW of larvae and juveniles and its relative content (as a percentage of shell weight) versus shell height. Fitted curve: AFDW(mg) = 0.00158H(mm)$^{2.786}$.**

metamorphosis was not documented quantitatively. Little metamorphosis was observed at algal concentrations of 1 cell/μL.

Growth of Juveniles

The growth rate of juveniles as a function of algal concentration is shown in Figure 6. The growth rate increased as algal concentrations increased to 10 cells/μL and then became less dependent on algal concentrations. The growth rate increased as juveniles increased in size up to 3 mm in shell height, after which, growth rate showed little change with body size. Two-way ANOVA showed that the growth rate of juveniles was significantly affected by both algal concentration and body size (Table 3). Multiple range analysis shows that there was a significant difference in growth between algal concentrations of 1, 5, and 10 cells/μL (Table 4), whereas at >10 cells/μL, growth was not significantly different. Growth at a concentration of 1 cell/μL was not significantly different from that of controls.

The above growth rates in shell size were converted to growth rates in AFDW, and the results are shown in Figure 7. Parameters fitted for the allometric curves of growth rate in AFDW against shell height are listed in Table 5. All graphs shown in Figure 7 are plotted on the same scale for easy comparison. It is obvious that growth rates in AFDW at 1 and 5 cells/μL were significantly slower than that at >10 cells/μL. At all algal concentrations, larger juveniles showed higher growth rates.

The relative growth rate (daily percent increase in AFDW) was generally higher in larvae than in juveniles (Fig. 8) and decreased with increased shell height in juveniles. It was 21–44%/day in 150-μm larvae, 20–25%/day in 0.5-mm juveniles, and about 10%/day in juveniles larger than 5 mm.

DISCUSSION

The growth curve of shell length against time for bivalve larvae is generally sigmoidal in shape (Loosanoff et al. 1951, Bayne 1965). Sigmoidal growth was observed for the larvae of *Argopecten irradians irradians* (Lu 1989) and for the larvae of *A. i. concentricus* in this study. The growth of the shell is characterized by an initial and a final phase of slow increment and a middle, rapid phase. During early larval development, either the digestive system is not fully developed or the velum does not reach its full capacity to capture food. As a result, larvae are unable to take full advantage of the external food resources available, and they may still need to partially use energy reserves from eggs. The combination of low energy acquisition and high demand (Lu 1996) may lead to a negative energy balance at this stage.

As larvae further develop, their ability to filter food particles increases (Lu 1996). Positive energy balance at this stage leads to increased growth rates. Shell growth slows down just before metamorphosis, when larvae are at a critical stage to leave the plankton and become benthic. It is during the planktonic stage that larvae accumulate energy reserves for metamorphosis.

The growth rate of larvae increased as *I. galbana* concentration increased. The optimal *I. galbana* concentration corresponding to optimal growth of bay scallop larvae was 20 cells/μL. This value is comparable to the 30 cells/μL reported for the optimal growth of the larvae of the Japanese scallop *Patinopecten yessoensis* (MacDonald 1988). In contrast, the algal concentrations required for the optimal growth by larvae of other bivalve species were 10–100 cells/μL for *Mytilus edulis* (Bayne 1965, Jespersen and Olsen 1982, Sprung 1984), 20–400 cells/μL for *Ostrea edulis* (Davis and Guillard 1958, Walne 1956, Wilson 1979, Beiras and Camacho 1994), 100 cells/μL for *Crassostrea gigas* (Abdel-Hamid et al. 1992), 25–325 for *C. virginica* (Rhodes and Landers 1973), and 50–400 for *Mercenaria mercenaria* (Davis and Guillard 1958).

The *I. galbana* concentration of 1 cell/μl is close to the lower limit for normal scallop larval growth and development. At this concentration, only a few larvae reached metamorphosis and completed larval development successfully. However, growth and development was considerably slower. Energy acquisition through feeding at such a low algal concentration was no more than respiration loss (Lu 1996). Low energy reserves resulting from low food levels and high metabolic demand may be the key to the failure of metamorphosis of the majority of larvae at this low food concentration. In another study, Sprung (1984) found that *M. edulis* larvae failed to reach the pediveliger stage at 1 cell/μL of *I. galbana*, although larvae started growing.

Once metamorphosis is complete, the juveniles grow much faster, possibly because of the high capacity of their gills to catch food particles. The optimal *I. galbana* concentration for growth was 10 cells/μL for juveniles, lower than the 20 cells/μL found for larvae. This difference could be the result of the high weight-specific metabolic rates of larvae (Lu 1996) because of energy used for swimming, and hence, larvae require a denser food concentration. Another possibility is that juveniles had an increased ability to obtain food particles so as to saturate gut capacity at lower algal concentrations.

No previous information is available on the weight-shell size relationships of larvae and juveniles of the bay scallop. The exponents determined for allometric relationships between AFDW and size in this study are comparable with those found for two other bivalve species: the mussel *M. edulis* and the oyster *O. edulis*. Jespersen and Olsen (1982) reported values of b to be 3.49 and 2.42 for larvae and young postmetamorphic mussels, respectively (soft tissue DW-shell length), whereas Sprung (1984) gave a b value of 3.02 for mussel larvae (total AFDW-shell length). Slightly lower values of 2.50 (AFDW-shell length) and 2.64 (DW-shell length) were found for *O. edulis* larvae (Beiras and Camacho 1994).

AFDW of bay scallop larvae was found to be 26.2–50.3% (mean, 38.6%) of the total DW (including shells). A high AFDW

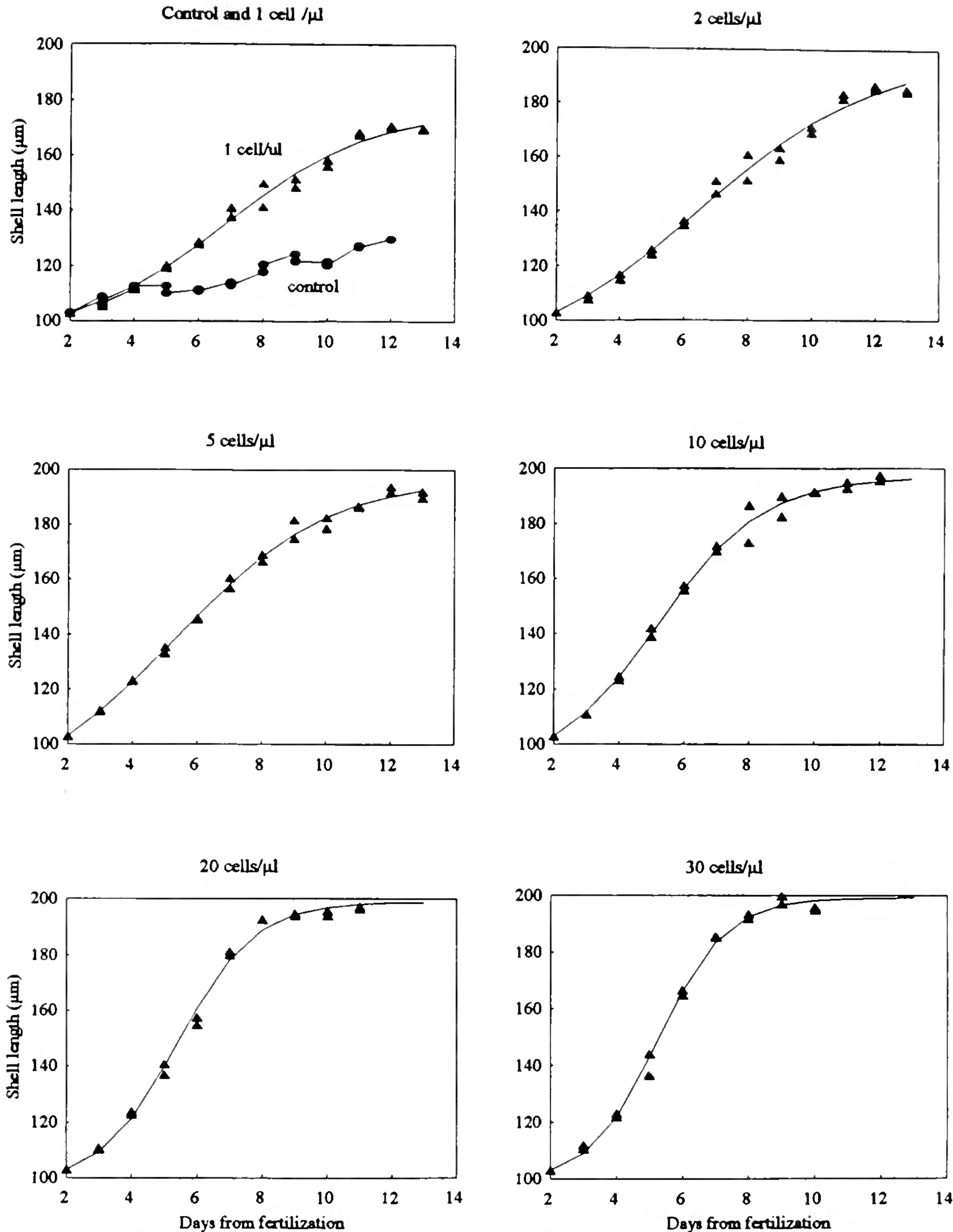

Figure 4. *A. i. concentricus*. **Shell growth of larvae at various** *I. galbana* **concentrations. Fitted parameters for the logistic functions are listed in Table 2.**

content was also found in the larvae of *M. edulis*, composing 18.3–48.2% (mean, 32.6%) of total DW (calculated from data of Sprung 1984). Such a high AFDW content indicates that larvae are well adapted to the planktonic lifestyle, because high organic content increases the buoyancy of larvae and hence reduces the energy needed to maintain their position in the water column. High organic content also represents a substantial energy reserve that can be used later for metamorphosis (Holland and Spencer 1973, Bartlett 1979, Rodriguez et al. 1990, Lu 1996).

Survival during metamorphosis and early benthic stages is often low. Juvenile bay scallops have a 50–80% mortality before reaching 2-mm shell height (Castagna and Duggan 1971). Juvenile scallops are vulnerable to predation by crabs, starfish, gastropods, and bottom-feeding fish (e.g., Medcof and Bourne 1964, Elner and Jamieson 1979, Lake et al. 1987). The high mortality of young juveniles can be offset by fast growing during this developmental stage. The daily growth of 0.5-mm juveniles can be as high as 24% of their body weight in terms of AFDW. This is in

TABLE 2.

A. i. concentricus: fitted parameters (a, b, and c) for logistic growth functions of larvae at various *I. galbana* concentrations.

Parameters	1 cell/μL	2 cells/μL	5 cells/μL	10 cells/μL	20 cells/μL	30 cells/μL
a	82.47	112.34	118.92	106.74	100.49	102.02
b	25.64	40.43	56.17	49.80	42.80	45.97
c	94	85.3	78.5	90.2	97.7	98.0
r[a]	0.434	0.366	0.412	0.621	0.876	0.922

[a] r, growth rate.

TABLE 3.

A. i. concentricus: ANOVA for growth of larvae and juveniles.

Stage	Source of Variation	Sum of Squares	d.f.	Mean Sq.	F Ratio	Significance Level
	Main effects					
Larvae	Age (day)	1,434,487.0	9	159,387.4	1,265.0	0.0000
	Cell Concn.	380,780.4	6	63,463.4	503.7	0.0000
	Residual	264,209.5	2097	126.0		
	Total (corrected)	2,040,696.9	2112			
	Main effects					
Juveniles	Shell size	3.1566E9	7	4.5095E8	9,659.4	0.0000
	Cell concn.	3.0061E7	6	5.0102E6	107.3	0.0000
	Residual	47152149	1010	46685.3		
	Total (corrected)	3.2453E9	1023			

TABLE 4.

A. i. concentricus: multiple range analysis (95% Tukey HSD) for growth of larvae and juveniles by *I. galbana* concentrations.

Level (Cells/μL)	Larvae Count	Larvae Least-squares Mean	Larvae Homogeneous Groups	Juveniles Count	Juveniles Least-squares Mean	Juveniles Homogeneous Groups
0	225	114.2	X	99	2,712.4	X
1	319	141.1	X	148	2,786.9	X
2	328	143.8	X	—	—	—
5	330	149.4	X	152	2,993.7	X
10	332	156.2	X	152	3,122.0	X
20	310	158.9	X	161	3,132.8	X
30	269	161.3	X	154	3,205.5	X
50	—	—	—	158	3,180.0	XX

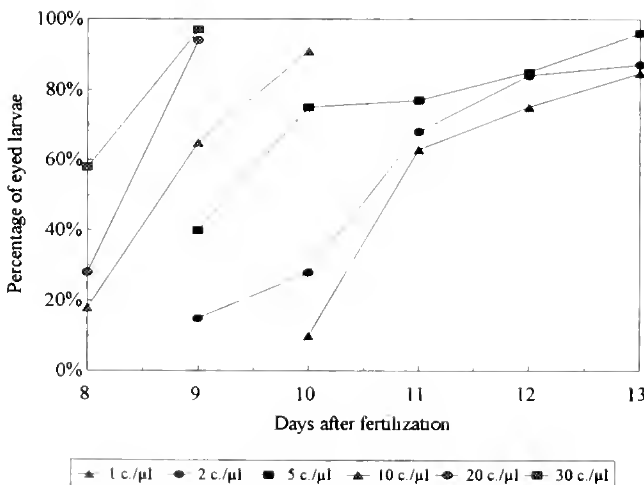

Figure 5. *A. i. concentricus.* Development of eyespot in larvae at various *I. galbana* concentrations. c., cell.

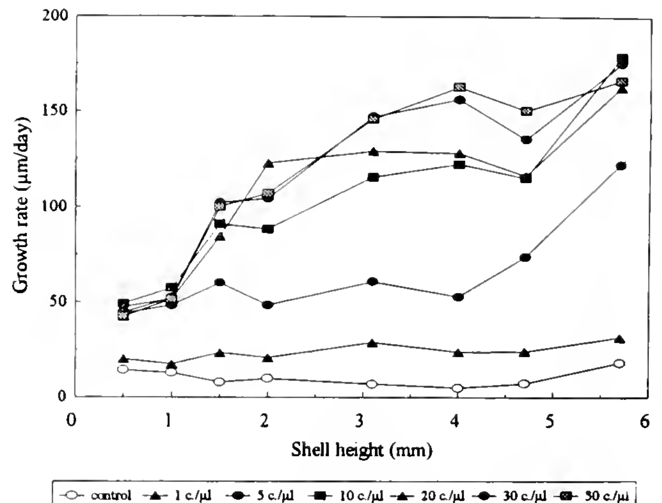

Figure 6. *A. i. concentricus.* Shell growth rate of juveniles in relation to *I. galbana* concentration. c., cell.

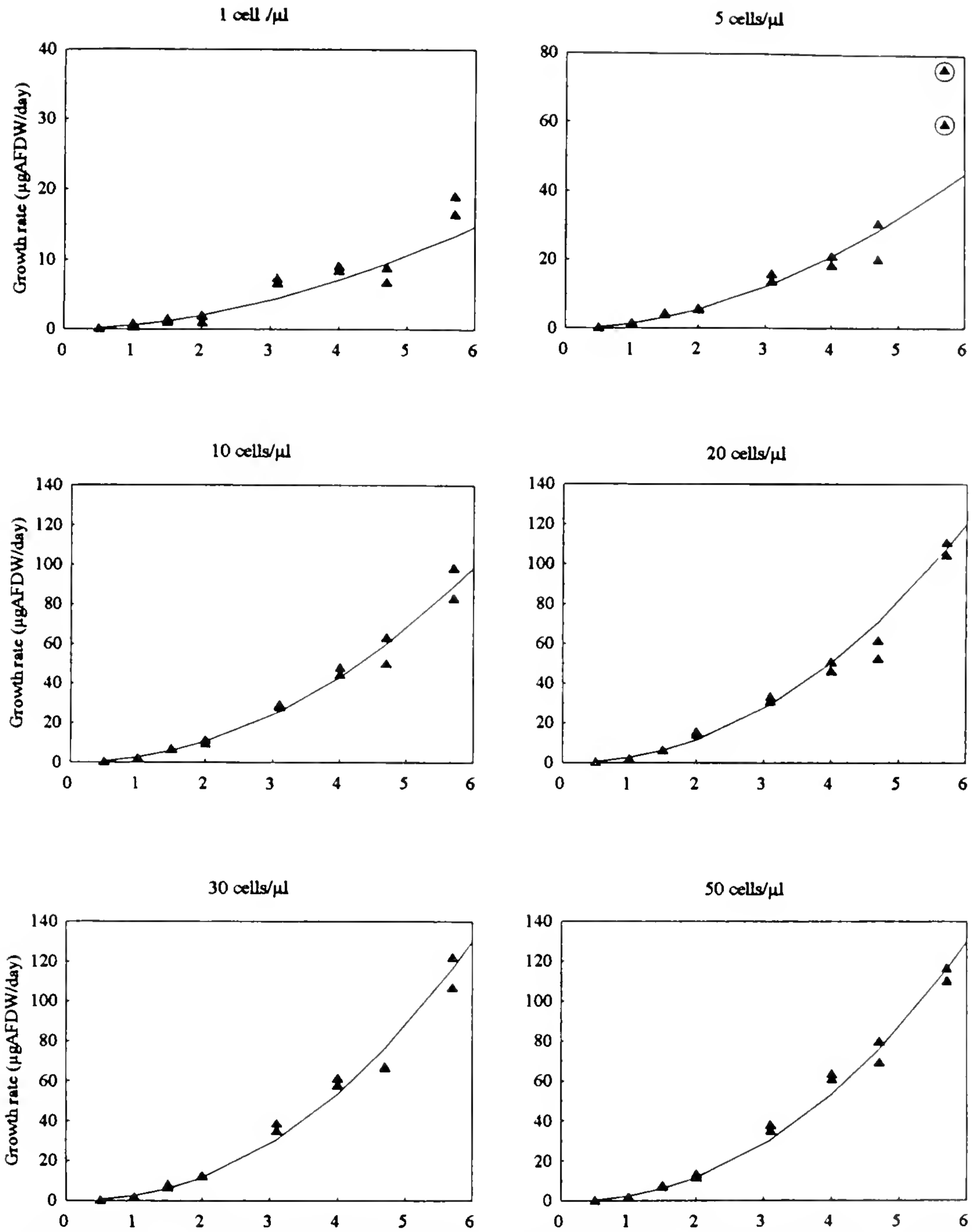

Figure 7. *A. i. concentricus*. Growth rate (in AFDW) of juveniles in relation to shell height at various *I. galbana* concentrations. Fitted parameters for the curves are listed in Table 5. Datum points in circles are excluded from regression.

TABLE 5.

A. i. concentricus: Fitted parameters (a and b) for allometric relationship between growth rate (mg of AFDW/day) and shell height (mm) of juveniles at various *I. galbana* concentrations.

Parameter	1 cell/µL	5 cells/µL	10 cells/µL	20 cells/µL	30 cells/µL	50 cells/µL
a	0.583	1.503	2.547	2.560	2.546	2.519
b	1.802	1.901	2.040	2.146	2.196	2.201
r^2	0.922	0.944	0.934	0.950	0.958	0.981

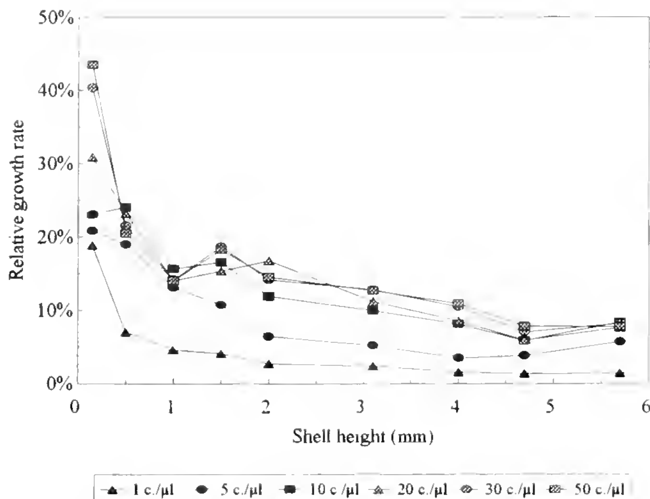

Figure 8. *A. i. concentricus.* **Relative growth rate of larvae and juveniles in relation to shell size at various** *I. galbana* **concentrations.**

contrast to the 8% found for larger juveniles of >5 mm in shell height. The faster growth of shell relative to soft body parts in young juveniles represents another protective strategy to reduce predation. Total ash weight as a percentage of total DW increased from 71% in 0.5-mm juveniles to 92% in 5-mm juveniles. The organic content of the shell also decreased with increasing animal size. This agrees with observations that shell composition undergoes a transformation from low-calcium to high-calcium content when larvae metamorphose to juveniles (Merrill 1961) and that later juvenile shells are strengthened and thickened.

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GENOTYPE-DEPENDENT SPAWNING: EVIDENCE FROM A WILD POPULATION OF *PECTEN JACOBAEUS* (L.) (BIVALVIA: PECTINIDAE)

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ABSTRACT In order to investigate whether there was a genetic basis for spawning asychrony in a wild population of the Mediterranean scallop, *Pecten jacobaeus* (L.), we scored 15 protein loci during a 6-mo period in which maturation and spawning were expected to occur. Multilocus heterozygosity was chosen as the variable to be related to the timing of spawning. Our results indicate that the more heterozygous loci in individuals, the earlier they tend to spawn. Possible ways to reach asynchrony during the spawning period, either by an earlier start of maturation or by a shorter maturation time in heterozygotes, are proposed. In addition, the implications of this genotype-dependent spawning time for the genetic structure of the population are also discussed. As others have postulated, we propose that this dynamic could be, in addition to other acting forces, one of the possible explanations for the heterozygote deficiency often recorded in marine bivalves.

KEY WORDS: *Pecten jacobaeus*, heterozygosity, spawning asynchrony, heterozygote deficiency, western Mediterranean, Spain

INTRODUCTION

In recent years, experimental studies have demonstrated the existence of positive relationships between multiple-locus heterozygosity at electrophoretically detectable enzyme loci and fitness traits in many natural populations of marine bivalve species: oysters (Singh and Zouros 1981, Fujio 1982, Koehn and Shumway 1992, Zouros et al. 1983, Rodhouse and Gaffney 1984), mussels (Koehn and Gaffney 1984, Diehl and Koehn 1985, Rodhouse et al. 1986), clams (Green et al. 1983, Garton et al. 1984, Koehn et al. 1988), and scallops (Foltz and Zouros 1984). However, most fitness-related characteristics refer to growth and viability, and with a few exceptions (Rodhouse et al. 1986, Hilbish and Zimmerman 1988, Barber et al. 1991, Bricelj and Krause 1992), there is relatively little information about the genetic background of reproductive features.

The scallop *Pecten jacobaeus* (L.) is a pectinid of Mediterranean distribution. In spite of the commercial interest, there are only a few studies concerning their reproductive features (Valli and Dovier 1977, Valli 1979, Castagnolo 1991, Mestre 1992) and genetic structure (Huelvan 1985, Peña et al. 1994, Ríos et al. 1995) in some natural populations of this species. As is true of other members of the family, it is a functional hermaphrodite, having gonads with distinct male and female portions. The gametes are produced simultaneously; however, spawning is most protandric. Fertilization occurs externally, and larva is planktonic for 3–4 wk before settlement occurs. In this species, self-fertilization has not been investigated to date. However, in the congeneric *Pecten maximus* (L.) (Mason 1958, Gruffydd and Beaumont 1972, Beaumont and Budd 1983) and other hermaphroditic pectinids (Castagna 1974, Ibarra et al. 1995), some laboratory studies have recorded self-fertilization, although the importance of this process in the field remains unknown.

During a 2-y period (October 1989 to April 1991), we studied the gametogenic cycle of a wild population of the scallop *P. jacobaeus* off of the coast of Castellón (E. Spain, W. Mediterranean). After a preliminary analysis of data from the first season (Mestre et al. 1990), several reproductive dynamics were noted: (1) a strong seasonal nature was observed; (2) the spawning period for the whole population was between late winter and early spring; (3) during the period before gamete release, a slight degree of asynchrony was observed among individuals with regard to gonadal maturation; (4) this asynchrony became very evident during the spawning period, where a fraction of individuals of the population had either spent, partially spent, or ripe gonads.

In order to investigate whether there was a genetic basis underlying these differences, an experimental program was designed for the second season. According to our hypothesis that the genetic structure of individuals could affect the timing of reproduction, one should expect individuals showing different reproductive behavior to display differences in some genetic traits in the period of maximal asynchrony. In earlier phases, where asynchrony is far from being evident, the genetic differences perhaps will not be as clear.

We have chosen to observe multilocus heterozygosity, an integrating measure, as a genetic trait. As discussed above, several studies have related this parameter to fitness (for a review, see Zouros 1987).

MATERIALS AND METHODS

One hundred twenty scallops of the species *P. jacobaeus* were collected between November 1990 and April 1991 from a natural population, located in front of the Oropesa coast (Castellón, E. Spain) (Fig. 1) at a depth of 65–75 m. Investigation of the eventual genetic influence on reproductive timing was made on the basis of pooling samples in three periods: (1) November to December, at the beginning of gonad maturation; (2) January to February, at the maximal gonad maturation; (3) March to April, at the spawning time.

All individuals collected were adults of the 3-y age class, determined by the presence of the annual growth ring on the shell according to Gibson (1956). Each individual was measured, weighed, and dissected. As a maturation index, macroscopic examination and microscopic examination of the gonads were made. The gonads were classified into three stages of maturity according to the following—a scheme modified from a scale proposed by Mason (1958) for *P. maximus*—(1) **recovering** (stages III and IV); (2) **ripe** (stages V, half full, and VI, full); (3) **spent** (stage VII, partially spent and spent).

Sections of the adductor muscle were homogenized in an equal volume of homogenization buffer (Tris, 10 mM; EDTA, 1.27

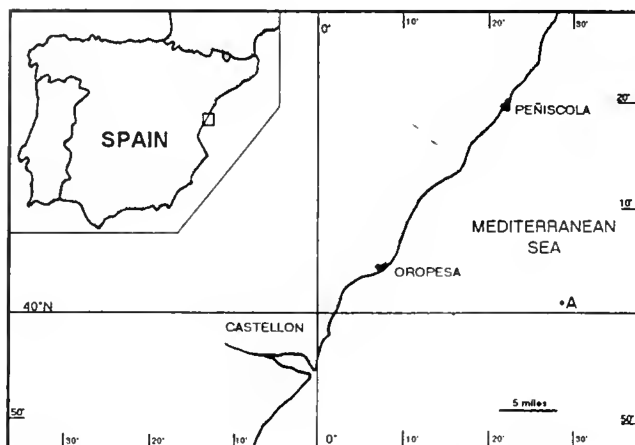

Figure 1. Location of the sampling area.

mM; NADP, 0.03 mM; pH 6.8) and centrifuged at 4°C for 10 min at 5,000 g. The supernatant was used as the enzyme source. Enzymatic electrophoresis was performed on horizontal starch gels according to standard protocols detailed in Pasteur et al. (1987). For each individual, genotypes at 15 loci coding for 12 enzymes were determined. These enzymes included aspartate aminotransferase (AAT; E.C. 2.6.1.1), α-glycerophosphate dehydrogenase (α-GPD; E.C. 1.1.1.8), glucose phosphate isomerase (GPI; E.C. 5.3.1.9), isocitrate dehydrogenase (IDH; E.C. 1.1.1.42), leucine aminopeptidase (LAP; E.C. 3.4.11.1), malate dehydrogenase (MDH; E.C. 1.1.1.37), NADP-malate dehydrogenase (ME; E.C. 1.1.1.40), mannose phosphate isomerase (MPI; E.C. 5.3.1.8), octopine dehydrogenase (ODH; E.C. 1.5.1.11), 6-phosphogluconate dehydrogenase (6-PGD; E.C. 1.1.1.43), phosphoglucomutase (PGM; E.C. 2.7.5.1), and superoxide dismutase (SOD; E.C. 1.15.1.1). All enzymes were resolved with a Tris-citrate (pH 8.0) buffer system, except α-GPD and LAP, which were run with a Tris-citrate discontinuous buffer system (pH 6.7 gel buffer; pH 6.3 electrode buffer).

For enzymes with multiple isozymic forms, loci were numbered sequentially starting with the most anodally migrating system. The different alleles at each polymorphic locus were assigned values that indicate their mobility relative to the most common allele, which was designated as "100."

The loci Aat-2, Idh-1, Idh-2, and Mdh-1, which were completely monomorphic, were discarded in further analyses. The locus Aat-1 showed poor resolution, and therefore, it was not interpretable.

At each period sampled, allelic frequencies and deviation of heterozygosity (D) between heterozygosity expected under Hardy-Weinberg equilibrium (He) and observed (Ho) heterozygosity (D = [Ho-He]/He, where negative D values indicate a deficiency of heterozygous genotypes) (Selander 1970), were calculated for each locus. Conformity to Hardy-Weinberg equilibrium of distribution of genotype frequencies (genotype classes not being pooled) was tested by a Markov chain method that provides unbiased estimations of the exact p value (Guo and Thompson 1992) by the use of the computer program GENEPOP (Raymond and Rousset 1994).

Between-periods differentiation was quantified by means of Wright's (1965) F_{ST} statistic, modified by Nei (1977) and Wright (1978) for multiple alleles, with the program BIOSYS-1 (Swofford and Selander 1989). Values of this statistic close to zero indicate no subpopulation differentiation. The deviation from zero was tested for each locus by computing $\chi^2 = 2N \cdot F_{ST} (k - 1)$, a statistic that follows a χ^2 distribution with $(k - 1) \cdot (s - 1)$ degrees of freedom, where N is the total sample size, k is the number of alleles for the locus, and s is the number of subsamples (Workman and Niswander 1970). The statistical significance of mean F_{ST} for overall loci was tested by $\chi^2 = 2N \cdot F_{ST}$ with $(n - 1)$ degrees of freedom, where N is the total sample size and n is the number of subsamples (Workman and Niswander 1970).

The number of heterozygous loci per individual (multilocus heterozygosity) was used as a measure of heterozygosity. Each individual was heterozygous at zero to four loci; no individual was heterozygous at more than four loci.

The effects of heterozygosity on asynchrony were tested for March to April data and for November to December data by linear-by-linear association exact tests with Monte Carlo accuracy to estimate the exact p value (Mehta and Patel 1995). Comparison of heterozygosity within the group of ripe individuals at three time periods was tested by a Kruskal-Wallis one-way analysis of variance (Sokal and Rohlf 1981) and by a linear-by-linear association exact test with Monte Carlo accuracy to estimate the exact p value (Mehta and Patel 1995).

RESULTS

The number of scallops at each stage of maturity within the heterozygosity level, referred to each period, is summarized in Table 1. Allelic frequencies, deviation of heterozygosity (D), and conformity to Hardy-Weinberg equilibrium at each locus, within scallop samples from the three different periods and pooling samples, are shown in Table 2.

The F_{ST} values estimated individually for the 11 loci, and combined with the set, are presented in Table 3. The results show low F_{ST} values for most loci. The null hypothesis of homogeneity

TABLE 1.

Number of individuals at each gonad stage for a given heterozygosity class within each period.

Period	Hetero-zygosity Class	Gonadal Stage		
		Recovering (III–IV)	Ripe (V–VI)	Spent (VII)
November to December	0	1	2	—
	1	3	8	—
	2	1	10	—
	3	0	4	—
	4	0	0	—
	N[a]	5	24	—
January to February	0	—	5	—
	1	—	12	—
	2	—	4	—
	3	—	5	—
	4	—	0	—
	N	—	26	—
March to April	0	—	8	11
	1	—	12	12
	2	—	2	13
	3	—	2	4
	4	—	0	1
	N	—	24	41

[a] N, sample size.

TABLE 2.

Allelic frequencies (p_i), heterozygote deficiency (D), and conformity to Hardy-Weinberg equilibrium by exact p value, at the 11 polymorphic loci in each period and pooling samples from the three periods.

		November to December			January to February			March to April			Total Period Sampled		
Locus	Alleles	p_i	D	p	p_i	D	p	p_i	D	p	p_i	D	p
α-Gpd	105	0.000	−0.785	0.0048	0.019	0.000	NT	0.015	−0.657	0.0006	0.013	−0.654	0.0000
	100	0.914			0.981			0.954			0.950		
	95	0.086			0.000			0.031			0.038		
Gpi	110	0.103	0.068	0.1319	0.115	−0.225	0.0025	0.092	−0.078	0.0525	0.100	−0.092	0.0000
	105	0.069			0.038			0.038			0.046		
	100	0.517			0.558			0.754			0.654		
	95	0.121			0.135			0.023			0.071		
	90	0.190			0.154			0.085			0.125		
	85	0.000			0.000			0.008			0.004		
Lap	103	0.017	−0.281	0.2489	0.058	0.074	1.0000	0.000	−0.579	0.0002	0.017	−0.346	0.0018
	100	0.897			0.885			0.900			0.896		
	97	0.086			0.058			0.092			0.083		
	94	0.000			0.000			0.008			0.004		
Mdh-2	110	0.000	—	NT[b]	0.000	—	NT	0.023	0.016	1.0000	0.013	0.008	1.0000
	100	1.000			1.000			0.977			0.998		
Me-1	100	1.000	—	NT	0.981	0.000	NT	0.985	0.008	1.0000	0.988	0.006	1.0000
	95	0.000			0.019			0.000			0.004		
	90	0.000			0.000			0.015			0.008		
Me-2	105	0.115	−0.926	0.0000	0.058	−0.563	0.0001	0.069	−0.751	0.0000	0.087	−0.757	0.0000
	100	0.707			0.731			0.823			0.775		
	95	0.138			0.173			0.108			0.129		
	90	0.000			0.038			0.000			0.008		
Mpi	105	0.000	—	NT	0.038	−1.000	0.0196	0.023	−0.557	0.0020	0.021	−0.658	0.0000
	100	1.000			0.962			0.946			0.962		
	95	0.000			0.000			0.031			0.017		
Odh	105	0.017	−0.094	0.6946	0.000	0.109	1.0000	0.000	−0.392	0.0046	0.004	−0.228	0.0290
	100	0.759			0.885			0.815			0.817		
	95	0.224			0.115			0.185			0.179		
6-Pgd	105	0.017	0.009	1.0000	0.000	0.000	NT	0.000	0.024	1.0000	0.004	0.022	1.0000
	100	0.966			0.981			0.969			0.971		
	95	0.017			0.019			0.031			0.025		
Pgm	105	0.000	−0.369	0.0214	0.038	0.020	1.0000	0.038	0.048	1.0000	0.029	−0.077	0.1162
	100	0.914			0.962			0.923			0.929		
	95	0.052			0.000			0.023			0.025		
	90	0.034			0.000			0.015			0.017		
Sod	100	0.914	−0.355	0.1707	0.981	0.000	NT	0.954	−0.306	0.1135	0.950	−0.301	0.0242
	90	0.086			0.019			0.046			0.050		
		N[a] = 29			N = 26			N = 65			N = 120		

[a] N, number of individuals scored for each locus.
[b] NT, not tested.

in the allelic frequencies from the three periods considered was accepted in all loci examined except in the Gpi and α-Gpd loci, which were found to show significant differences. However, the mean of F_{ST} for overall loci shows a value not significantly different from zero ($F_{ST} = 0.017$, $\chi^2 = 4.08$, d.f. = 2, $p > 0.1$). It could be concluded that all scallops taken within each period were members of the same breeding population, regardless of the period sampled.

During the spawning period (March to April), spent and ripe individuals showed differences in multilocus heterozygosity (Fig. 2), but these were not significant (linear-by-linear association; p = 0.0791, one-tail). According to these results, although the more homozygous individuals tend to correspond with the delayed group (ripe individuals), whereas the more heterozygous individuals were largely confined to the spent group, because the test for linearity gave a not significant value, the causal relationship between the variable multilocus heterozygosity vs. gonadal stage remains somehow unclear. Therefore, these results should be taken with caution. In the same way, during the November to December period, the recovering and ripe individuals show slight differences (Fig. 2), although these were not significant (linear-by-linear association; p = 0.0993, one-tail).

Nevertheless, the average heterozygosity of ripe individuals from the three periods considered decreased with time (November to December, $\bar{x} = 1.667$; January to February, $\bar{x} = 1.346$; March to April, $\bar{x} = 0.917$; Kruskal-Wallis $\chi^2 = 8.368$, d.f. = 2, p = 0.0152). Moreover, the linear association of the variables, multilocus heterozygosity vs. time period, was highly significant (linear-by-linear association; p = 0.0048, one-tail) (see Fig. 3). Therefore, these results confirm the hypothesis that animals rip-

TABLE 3.

Single-locus and multilocus values of F_{ST} among scallops sampled within each period.

Locus	F_{ST}	χ^2	d.f.
α-Gpd	0.022	10.56[a]	4
Gpi	0.026	31.20[b]	10
Lap	0.004	2.88 ns[c]	6
Mdh-2	0.016	3.84 ns	2
Me-1	0.009	4.32 ns	4
Me-2	0.013	9.36 ns	6
Mpi	0.016	7.68 ns	4
Odh	0.016	7.68 ns	4
6-Pgd	0.003	1.44 ns	4
Pgm	0.011	7.92 ns	6
Sod	0.016	3.84 ns	2
Multilocus	0.017	4.08 ns	2

[a] $p < 0.05$.
[b] $p < 0.005$.
[c] ns, not significant.

ening earlier tend to be more heterozygous. This may represent a genetic control over spawning asynchrony.

DISCUSSION

As we postulated, individuals in different stages of gonad development during a given period show differences in genetic traits. The results indicate that the more heterozygous loci present in individuals, the earlier they tend to spawn. This became evident during the spawning period (see Fig. 2). In order for individuals with more heterozygous loci to spawn earlier, we hypothesize that three events could occur: (1) heterozygous individuals start to mature before; (2) even if a population was synchronous at the beginning of maturation, less time would be spent on gonad maturation in heterozygotes; (3) a combination of the two mechanisms could be involved, i.e., heterozygous individuals tend to start maturation earlier, but at the same time, they spend less time to reach spawning. These three hypotheses and their implications are summarized in Figure 4.

Because under the three hypotheses, the final result is the same, our results from the third period (Fig. 2) do not provide evidence enabling a choice among these three possibilities. However, the analysis of November to December data among recovering and ripe individuals and their degree of heterozygosity show slight, although not significant, differences. Our results on this last point agree with the third model: a combination of the two mechanisms, earlier start and shorter time of maturation, seems to lead heterozygotes to an earlier spawn. This could also explain the results shown in Figure 3.

It could be concluded that, even though a tendency for an earlier start time of maturation in heterozygotes seems to exist, our results do not provide clear evidence to support the idea that a nonsynchronous start of maturation is the only reason for the evident asynchrony reached at spawning time. In this sense, these results serve as a pilot data set that might aid in the design of future, more definitive experiments.

At present, there is no consensus on the genetic mechanism underlying the correlation of heterozygosity with phenotypic traits related to fitness (growth, viability, fecundity, etc.), and researchers are divided into two principal points of view: (1) one school of thought supports the idea that the enzymes recorded by electrophoresis are the causal agents of the correlation, i.e., heterozygosity for these enzymes directly affects those traits (Koehn et al. 1988); (2) another school (Zouros et al. 1988) advances the view that the enzyme variants act as mere markers of genetic abnormalities that are responsible for the genetic variation in traits related to fitness, but cannot be detected by the electrophoretic assay involved. Thus, an individual that is multiply heterozygous for the marker genes will have a much lower probability of carrying one or more of these abnormalities compared with an individual that is multiply homozygous for the marker genes. On the other hand, researchers appear to be in agreement concerning the physiologic basis of this correlation. Some studies demonstrate that the effect of increasing heterozygosity is to endow an individual with a lower energetic cost of maintenance metabolism (Koehn and Shumway 1982, Garton et al. 1984, Hawkins et al. 1986, 1989, Koehn et al. 1988). The emerging physiologic interpretation of the correlation is that the increased level of heterozygosity enables an individual to sustain its basal metabolism with lower expenditures of ATP. Therefore, according to Koehn (1990), this would allow a higher allocation of energy for growth and reproduction. As a result, time of maturation would be shorter. However, it is imperative to note that the eventual effect of heterozygosity would be to increase production and not necessarily to induce changes in the allocation of energy. In this context, Rodhouse et al. (1986) concluded that, in the case of *Mytilus edulis*, it is in older individuals that the majority of energy production is allocated for reproduction instead of growth. Therefore, it is later in the life of the organism when a higher production rate hypothetically associated with multilocus heterozygosity could be translated into increased gamete production. On the other hand, in younger individuals, the majority of energy production would be allocated for growth, and subse-

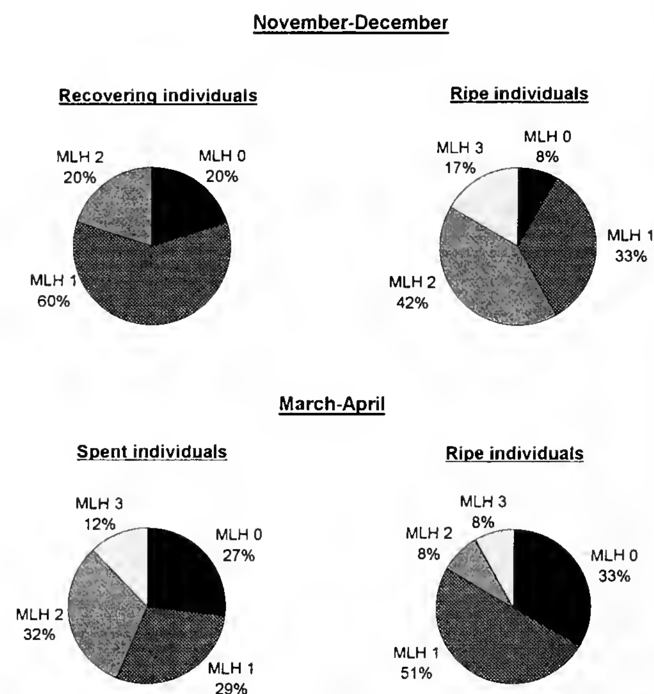

Figure 2. Proportion of individuals at multilocus heterozygosity (MLH) within each gonad stage during the November to December and March to April periods.

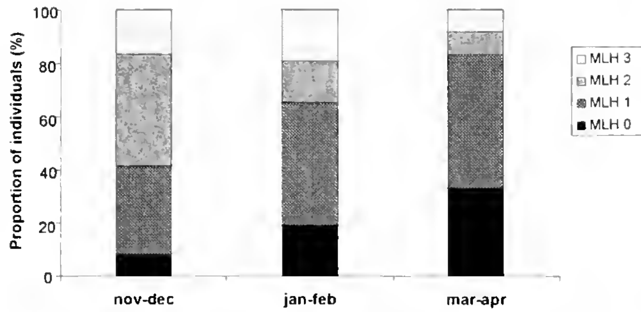

Figure 3. Proportion of ripe individuals at each multilocus heterozygosity (MLH) within each of the three time periods.

quently, the effect of heterozygosity on reproduction would not be detected.

As far as we know, this is the first work that provides empirical evidence for a relationship between multilocus heterozygosity and the timing of reproduction in marine bivalves. Although Zouros et al. (1988) attempted to prove this relationship in a natural population of mussels, the authors failed to provide experimental evidence. This could be due to several reasons. In this sense, Koehn (1990) argued that the reproductive cycle of that population of bivalves had not been previously studied, and consequently, the period when spawning was expected to occur was unknown. In addition, the period considered (1 mo) was too short for a degree of asynchrony to become evident. On the other hand, the animals were originating from a 2-y-old cohort, and as discussed above, the allocation of energy for reproduction appears to be age dependent (Rodhouse et al. 1986). According to Koehn (1990), this would be a critical age for the energetic balance. We tried to overcome these difficulties both by performing a preliminary study on the gametogenic cycle (Mestre et al. 1990) and by choosing animals old enough so that energy would be mostly allocated for reproduction.

CONSEQUENCES OF THE SPAWNING PATTERN FOR THE GENETIC STRUCTURE OF THE POPULATION

Until now, we have been dealing with the phenomenon of genotype-dependent spawning itself and the mechanisms that could be involved in its maintenance. However, at the same time, and because this dynamic leads to a nonpanmictic mating, it becomes evident that it could have consequences on the genetic structure of the population.

Thorough analysis of these consequences is far beyond the limits of this work. Nevertheless, we would like to add some notes on this matter, because genotype-dependent spawning has been regarded as one of the possible explanations for the most characteristic genetic trend among marine bivalve populations: the heterozygote deficiency (Zouros and Foltz 1984, Alvarez et al. 1989). As proved by many experimental studies, this phenomenon seems to be a general trend among populations of marine bivalves. This is apparent for hermaphroditic and gonochoric species, for wild and cultivated populations, and also for different families of bivalves (see Zouros and Foltz 1984 for a review). Neither pectinids (Wilkins 1978, Beaumont and Beveridge 1984; Foltz and Zouros 1984; Gosling and Burnell 1988) nor other populations of *P. jacobaeus* (Huelvan 1985), including this particular population (Ríos et al. 1995), appear to be an exception to this rule.

Several studies attempted to determine the cause of this heterozygote deficiency in marine bivalve populations (Zouros and Foltz 1984, Gaffney et al. 1990, Beaumont 1991). Within this context, the plausibility of the genotype-dependent spawning models and subsequent nonpanmictic mating was first invoked by Zouros and Foltz (1984). Those authors considered a theoretical model under which a population spawns in two discrete periods of time, the union of gametes being random within each period. The probability of an individual to spawn in one of the two periods (or alternatively, the fraction of gametes released in each period) was made to be a function of its genotype. One of the one-locus diallelic models that the authors built and thoroughly explored was an "overdominance" model. It appears that under this model: (1) the heterozygote deficiency (D) generated is a function of allelic frequencies (p); (2) it also depends on k, the fraction of a given genetic class that spawns in a different period; (3) for some combinations of these parameters, high D values can be generated. Because this model assumes no changes on allelic frequencies over generations but changes on genotype frequencies, theoretically at least, it appears to give rise to a stable situation.

Although the discussed models were one locus, its eventual prolongation to a multilocus situation could be supported on the context of the hypothesis termed "associative overdominance" (see Zouros and Mallet 1989), which relies heavily on the genetic structure of the population as well as that of the individual. According to this, the fitness of individuals scored homozygous for several loci would be reduced compared with multiple-locus heterozygotes as a result of homozygosity of deleterious genes at loci in linkage disequilibrium (Zouros and Mallet 1989). Moreover, the negative effects of deleterious loci "will be amplified by any process that causes an excess of homozygosity in the population" (Zouros and Mallet 1989, p. 319), such as inbreeding. Nevertheless, that effects will be present to some degree when sampling multiple-locus homozygotes in outbred populations (Beaumont 1991).

However, a multilocular extension of the model of Zouros has been believed to be unlikely (Gaffney et al. 1990) to generate large, single-locus, heterozygote deficiencies at several loci simultaneously. Those authors argued the difficulty in separating homozygotes and heterozygotes at all loci, at the same time, "with-

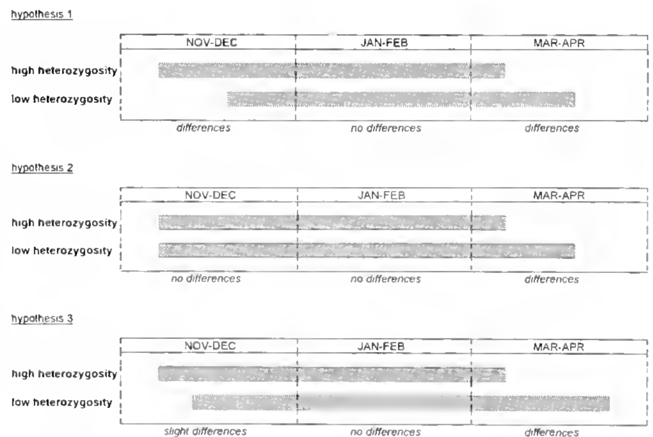

Figure 4. Three possible explanations to the situation reached during spawning time, *viz*, earlier spawning of the individuals being more heterozygous (see Fig. 3). Bars indicate maturation (gonad stages V and VI) time. The expected differences eventually found in within-period comparisons are illustrated. See text for details.

out creating numerous breeding groups" (Gaffney et al. 1990, p. 696).

Nevertheless, we should not exclude the plausibility that the heterozygote deficiencies reported in this work could have been originated, to a certain extent, by the genotype-dependent spawning concurrently described. However, it is worth noting that the heterozygote deficiency values for α-Gpd, and Me-2 loci, shown in Table 2, are much larger than reported values in the literature for other bivalve species. It is possible that some of these values are the result of nongenetic factors such as difficulty in scoring heterozygotes, differential staining activity, or posttranslational modifications. In this sense, as Huelvan (1985) noted in *P. maximus*, the α-GPD behaves as a monomer (heterozygotes present two bands), although this is described as a dimer; in the case of multimeric enzymes, such as the tetrameric malic enzyme, these often exhibit atypical heterozygotes that make its resolution difficult.

In the same sense, still another alternative explanation to the relationship between genotype-dependent spawning and heterozygote deficits could be postulated. Large heterozygote deficiencies occurring at multiple loci could reflect partial selfing in the population. Therefore, the relationship of delayed timing of reproduction and genomic homozygosity would not be of a direct causal nature but rather both would be consequences of this inbreeding. Nevertheless, information on the eventual extent of selfing on that population is lacking.

Finally, we would like to stress that even if genotype-dependent spawning could be one of the sources of heterozygote deficiency in populations of marine bivalves, it certainly would not be the sole one, because many other reasons have been invoked (Mallet et al. 1985, Foltz 1986, Gaffney et al. 1990). Therefore, quantification of the relative importance of the contribution of genotype-dependent spawning to the heterozygote deficiency phenomenon supposes another question left.

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FEEDING PREFERENCES OF THE JUVENILE SOUTH AFRICAN ABALONE *HALIOTIS MIDAE* (LINNAEUS, 1758)

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ABSTRACT An investigation was conducted to determine the feeding preferences of juvenile abalone *Haliotis midae* with respect to food value and chemical and mechanical defenses. Chemical defenses were examined by quantifying the amount of polyphenols, whereas the mechanical defenses were examined by measuring surface toughness. The algae tested were *Ecklonia maxima*, *Laminaria pallida*, and *Porphyra capensis*. The results showed that *E. maxima* was the most defended and of intermediate food value, *L. pallida* was poorly defended and of low food value, and *P. capensis* had almost no defenses and was of the highest food value. Abalone preferred *P. capensis* to either of the other two algae. Food preference was influenced by the diet on which the animals had been fed before the experiment. The influence of plant defenses on food preferences was not consistent between experiments or, in some instances, as plant defense theory may have predicted. Results showed that food value, plant defenses, and prior diet interacted to determine the food selectivity of juvenile abalone but that food value was of the greatest importance.

KEY WORDS: Juvenile *Haliotis midae*, food preference, plant defenses, nutritive value

INTRODUCTION

Feeding preference experiments have been conducted on various marine invertebrate herbivores found in kelp beds (Leighton 1966), including arthropods (Himmelman and Carefoot 1975, Jensen 1983, Nicotri 1980, Steinberg 1984, Steinberg 1988, Tugwell and Branch 1992), molluscs (Himmelman and Carefoot 1975, Nicotri 1980, Stenweck and Watling 1982, Jensen 1983, Steinberg 1984, Steinberg 1985, Steinberg 1988, Watson and Norton 1985a, Shepherd and Steinberg 1992, Tugwell and Branch 1992), and echinoderms (Vadas 1977, Anderson and Velimirov 1982, Bonsdorff and Vahl 1982). These studies have suggested that nutrient content, structural resistance to ingestion, and the presence of secondary plant compounds affect the selective preferences of a number of marine herbivores (Nicotri 1980, Jensen 1983).

Jensen (1983) suggested that food preferences could also affect growth rates. He observed that, in general, herbivores grew better on their preferred foods. For this reason, food preference is important for mariculture; this has been shown in recent studies on commercially valuable animals, such as abalone (Paul et al. 1977, Tutschulte and Connell 1988, Shepherd and Steinberg 1992, Fleming 1995a, Fleming 1995b). It is known that abalone begin to feed after settlement (Wood 1993), initially consuming benthic diatoms (Tutschulte and Connell 1988, Shepherd and Steinberg 1992, Wood 1993, McShane et al. 1994). As they grow, they begin feeding on green, red, or brown macroalgae and may change from one species of macroalgae to another as they mature. Barkai and Griffiths (1986) studied natural populations of adult *Haliotis midae* (Linnaeus, 1758) and found that *Ecklonia maxima* [(Osbeck) Papenfuss] dominated the gut contents, followed by red or green algae, but that this domination of *E. maxima* depended on the locality of the animals. However, juveniles preferred *Laminaria pallida* (Greville Ex Jagardh) (Simpson 1992).

Some previous feeding experiments have not clearly defined the method of investigating preference (Nicotri 1980, Peterson and Renaud 1989). Feeding preferences can be investigated in two ways: first by attractiveness, which entails multichoice feeding experiments, and second through edibility and energy assimilation, which investigates growth in relation to consumption (Nicotri 1980, Peterson and Renaud 1989). In this study, the attractiveness (as defined above) of the three algae was assessed by quantifying the relative importance of food value, chemical defense, and physical defense in relation to quantity of food consumed. Results are discussed in terms of their potential effect on the farming of *H. midae*.

METHODS

Experimental Organisms

Laboratory-reared *H. midae* juveniles of 28.12 ± 2.27 mm shell length (aged 1–2 y) were housed in flow-through seawater aquaria at 17 to 19°C, under low-light intensity with a 12-h day/night cycle. All experiments were run over a winter period, from July to October 1993.

Fresh, drift, adult, nonreproductive fronds of *E. maxima* (Phaeophyta), *L. pallida* (Phaeophyta), and fresh adult *Porphyra capensis* (Kuetzing) (Rhodaphyta) were collected locally from July to October 1993. Frond ends were selected so as to exclude any meristem, reproductive fronds, and epibiota. Note that *E. maxima* and *L. pallida* are subtidal brown algae (kelps) (Branch and Branch 1988), which are part of *H. midae*'s natural diet, and a food source used on abalone farms. However, *P. capensis* is a high-shore intertidal red alga (Branch and Branch 1988), which is not part of *H. midae*'s natural diet but is a potential food source in abalone farms in South Africa.

Feeding Experiments

Multichoice feeding experiments were designed to show differences in the consumption of the three algal species by the juveniles. The tank design was based on a latin square system, and three replicates were run in each tank (see Fig. 1). Before the experiments, animals were divided into three groups of 60 juveniles and fed ad libitum on similar amounts of one of the three algal species for a 2-wk period to assess the effects of prior dietary history on food preference. Animals were then transferred to the

feeding tanks (see Fig. 1) and starved for 3 days, which allowed acclimatization and ensured that the animals were equally hungry at the start of the experiment (Steinberg 1988).

Fresh, surface-dried algae were used for the experiments. Sufficient algae (80–120 g of *E. maxima* and *L. pallida*, 20–40 g of *P. capensis*) was used to ensure equal shading in the compartments. Ten animals per experiment were allowed to feed for 72 h. Algae remaining after the feeding experiments were surface dried and reweighed. A control without abalone was used to monitor autogenic changes of the algae over the duration of the feeding trials which was later subtracted from the mass of algae consumed over the experimental period (Peterson and Renaud 1989).

Nutritive Value of Algae

The nutritive value of the algae was quantified first by bomb calorimetry, which gave an overall indication of calorific value, and second, through a protein content analysis. Algal material was oven dried at 60°C for 24 h, ground, and sieved (300 m), and calorific values of 0.3 to 0.5g samples were determined with a CP500 Bomb Calorimeter, calibrated with purified benzoic acid.

A Kjeldahl digestion was performed on each algal sample, followed by a phenol-hypochlorite colorimetric determination of ammonium-nitrogen (Smith 1980). Total nitrogen was converted to a total protein value according to the method of Allen et al. (1974).

Chemical and Physical Algal Defenses

Algae are known to have secondary plant metabolites (polyphenols) that function as a defense against herbivores. Total plant polyphenols were measured by use of the method described by Tugwell and Branch (1989), in which "type-two" non-diffusible phloroglucinol, high molecular weight polymers were determined in the three algae. A purified extract of *Fucus vesiculosus* was used as a standard (Tugwell and Branch 1989).

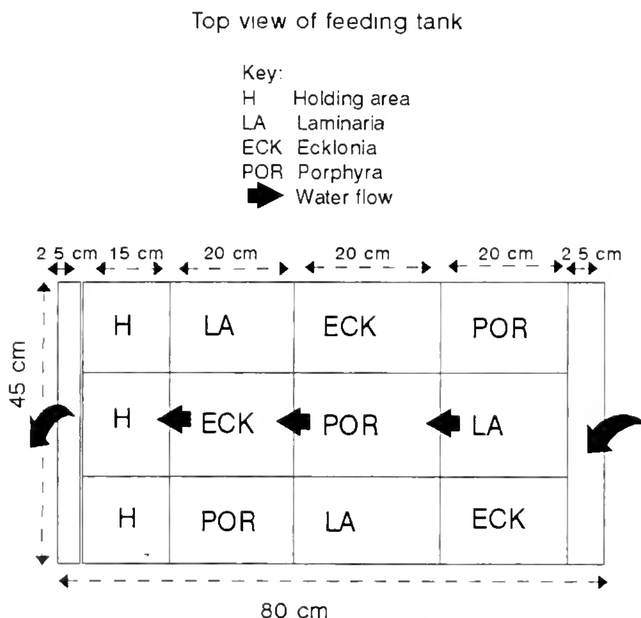

Figure 1. The feed experiment tank design, showing the dimensions from above of the 15-cm high tank with a 9-cm water depth. The positions of the algal species and the starting position of the abalone are also indicated. POR, *P. capensis;* LA, *L. pallida;* ECK, *E. maxima*.

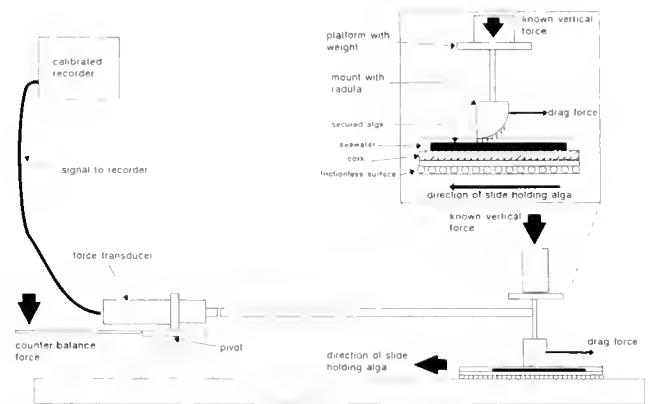

Figure 2. A diagrammatic representation of the apparatus used to assess the surface toughness of the algae tested.

Resistance to mechanical breakdown could be important as a defense mechanism for algae. This susceptibility to tissue removal by the rasping radula of juvenile *H. midae* was estimated by mimicking radulae use, as described by Padilla (1985, 1989). Freshly dissected radulae of *H. midae* juveniles were attached to perspex mounts with glue (Fig. 2). A strip of fresh algae was pinned onto the slide and submerged in seawater to mimic in situ conditions. Radulae were cleaned with a jet of water between every replicate run to prevent clogging by mucus secreted from the algae or by removed tissue. The basis of the method quantified the minimum normal, horizontal, and resultant forces required to remove tissue from the various algal species. Use was made of excised radulae attached to perspex mounts and connected to a force transducer that translated applied forces onto a calibrated Gilson Polygraph recorder (Fig. 2).

The results obtained indicated a linear increase of force that asymptoted (Fig. 3) once the vertical force was great enough to break the algal tissue, thus providing a constant drag force. Padilla (1985, 1989) and Tugwell (pers. comm.) have advised that the intercept between the linear slope and the horizontal tangent of the asymptote was the point of minimum penetration force. From the two coordinates (horizontal and vertical force vectors), the resultant force vector was calculated (Sternheim and Kane 1986) and expressed as milli-Newtons (mN) (Fig. 3).

Statistical Analysis

Calorific value and protein and polyphenol tests were analyzed by the use of a one-way analysis of variance (ANOVA) and a Newman-Keuls multiple-range test (Zar 1984). For the toughness experiment, a Kruskal-Wallis ranking analysis was performed. The feeding experiments were analyzed by use of a model 1 two-way ANOVA followed by a Tukey multiple-range test. A value of $p < 0.05$ was considered significant.

RESULTS

Nutritive Value

P. capensis had a significantly higher calorific value (16.68 ± 0.74 KJ/g) than *L. pallida* (11.74 ± 0.41 KJ/g) or *E. maxima* (11.92 ± 0.51 KJ/g), but there was no significant difference between *L. pallida* and *E. maxima* (Table 1). The Kjeldahl digests revealed a similar trend, where *P. capensis* (271.65 ± 17.15 mg/g) contained significantly more protein than either *L. pallida*

TABLE 1.

The average calorific values of the three algal species; *P. capensis* was significantly different from the other two species (F = 31.4, df = $F_{5,36}$).

Species	Calorific Value (KJ/g)	Standard Deviation (KJ/g)
E. maxima	11.92	0.51
L. pallida	11.74	0.41
P. capensis	16.68[a]	0.74

[a] Significant difference, $p \ll 0.01$

(120.45 ± 9.39 mg/g) or *E. maxima* (123.01 ± 15.43 mg/g). These results suggested that, of the algae tested, *P. capensis* was the best food for abalone in terms of protein and energy value.

Chemical and Mechanical Defenses

The quantities of polyphenolic compounds present in the algae tested are all significantly different from each other. *E. maxima* contained significantly more polyphenolic compounds (18.58 ± 3.49 mg/g) than either *L. pallida* (2.87 ± 0.86 mg/g) or *P. capensis*, the latter species containing no polyphenols (see Table 3).

The minimum required horizontal, vertical, and resultant forces are shown in Table 4 (see below). The required force increased significantly from *P. capensis* (651.46 ± 12.98 mN) through *L. pallida* (814.15 ± 30.31 mN) to *E. maxima* (1,653.58 ± 201 mN).

Feeding Experiments

The multichoice feeding experiments (Fig. 4) indicated that juvenile *H. midae* showed a significant preference for *P. capensis* over *E. maxima* and *L. pallida*: there did not appear to be any significant preference between the latter two (F = 15.9 df = $F_{2,4}$, $p \ll 0.01$). Because prior feeding history affected feeding preference, each group was considered separately.

In group 1, previously fed on *P. capensis*, the juveniles preferred *P. capensis* (1.41 ± 0.97 g/10 animals per day), followed by *E. maxima* (0.95 ± 0.4 g/10 animals per day) and *L. pallida* (0.85 ± 0.17 g/10 animals per day). In group 2, previously fed on *L. pallida*, *P. capensis* was most preferred and *L. pallida* was least preferred (Fig. 4). Group 3 fed previously on *E. maxima*; however, *E. maxima* (−0.05 ± 0.14 g/10 animals per day) seemed to be the least preferred species. Although there was a clear overall selection of *P. capensis*, preference for *E. maxima* and *L. pallida* was less clear. What appears to be a negative consumption rate of *E. maxima* is attributed to autogenic changes of the algae, which the replication of controls was unable to take into account (Peterson and Renaud 1989).

By comparing the food consumption of juvenile abalone with

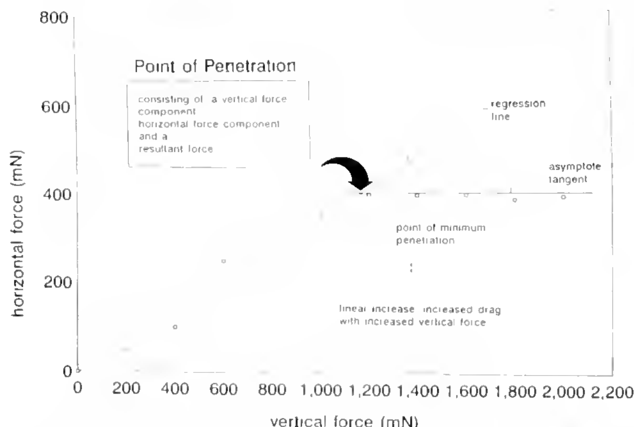

Figure 3. The results of the surface toughness experiment were obtained and analyzed to get the required forces.

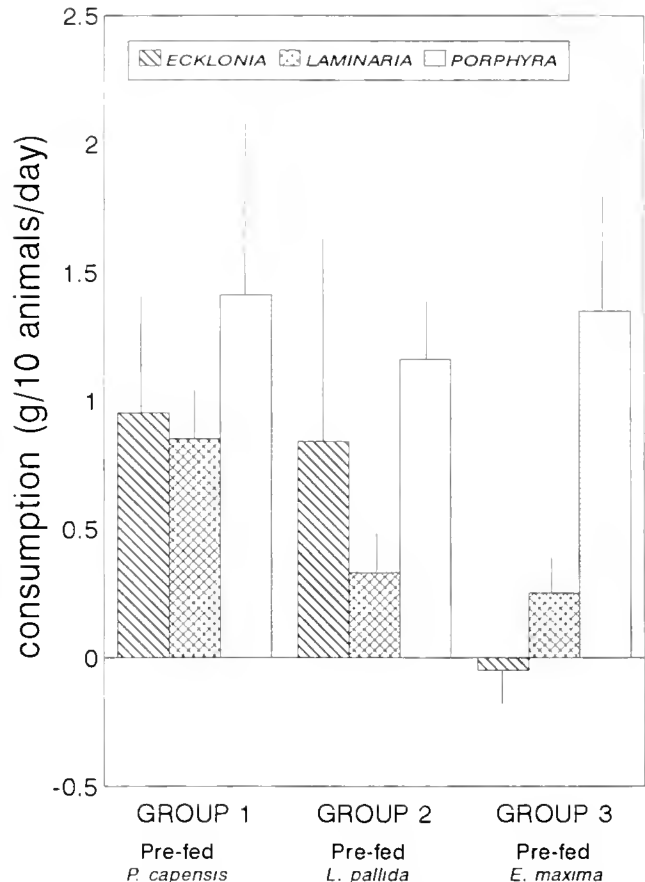

Figure 4. The results of the feeding experiments. Bars are the standard deviations about the mean. The groups denote the dietary histories of the juveniles before the experiment. Group 1, *P. capensis*; group 2, *L. pallida*; group 3, *E. maxima*. There was an overall significant preference for *P. capensis* throughout all of the groups, although no clear preference could be seen between *L. pallida* and *E. maxima*. Groups 1 and 3 contrasted significantly, but group 2 was not significantly different from either group 1 or group 3.

TABLE 2.

The average protein content of the three algal species; *P. caapensis* was significantly different from the other two species (F = 181.6, df = $F_{4,29}$).

Species	Protein Content (mg/g)	Standard Deviation (mg/g)
E. maxima	123.01	15.43
L. pallida	120.45	9.39
P. capensis	271.65[a]	17.15

[a] Significant difference, $p \ll 0.01$.

TABLE 3.

The results of the total plant polyphenols, where *E. maxima* is significantly higher than either of the other two algae ($F = 281.2$, $df = F_{6,60}$).

Species	Total Polyphenols (mg/g)	Standard Deviation (mg/g)
E. maxima	18.58[a]	3.49
L. pallida	2.87[a]	0.86
P. capensis	0[a]	0

[a] Significant difference, $p \ll 0.01$.

TABLE 4.

The results of the surface toughness tests, with *E. maxima* the most difficult to penetrate and *P. capensis* the least.[a]

Species	A	B	C	D
E. maxima	702.24	1,494.69	1,653.58[b]	201.00
L. pallida	373.12	749.00	836.78[b]	30.31
P. capensis	279.16	590.36	653.04[b]	12.98

[a] A, average minimum required horizontal force; B, average minimum required vertical force; C, average minimum required force (resultant) of penetration; and D, the standard deviation of the resultant force. All of the forces displayed are in milli-Newtons (mN) ($T_s = 7.2$, $n = 3$).
[b] Significant difference, $p \ll 0.05$.

calorific values, algal toughness, protein content, and polyphenol content (Fig. 4 and Tables 1–4), an attempt was made to explain positive or negative trends. In general, high calorific values (Table 1) and high protein content (Table 2) were associated with high consumption rates, as seen in the overall preference for *P. capensis*. If polyphenol content (Table 3) and surface toughness (Table 4) represent plant defenses, then the predicted trend of high defenses and low consumption (Steinberg 1984, Steinberg 1985, Steinberg 1988, Tugwell and Branch 1989, Tugwell and Branch 1992, Winter and Estes 1992) was not clearly shown by the whole experiment. After feeding on *P. capensis*, abalone juveniles showed a significant preference for *P. capensis* but no preference for *L. pallida* and *E. maxima*, despite *E. maxima* having high algal toughness and polyphenol content, suggesting that defenses may not be important in this case. A similar trend was obtained for the abalone that fed previously on *L. pallida*.

In the group of juveniles that fed previously on *E. maxima* (group 3 in Fig. 4), however, the predicted defense trend was quite clear (Fig. 4 and Tables 3 and 4), because there was a large consumption of the least defended species (*P. capensis*). In this instance, the plant defenses appeared to be more important in food selection.

DISCUSSION

The nutritive value of food has usually been assumed to be of primary importance in determining the feeding preference of herbivores (Paine and Vadas 1969, Himmelman and Carefoot 1975, Vadas 1977, Nicotri 1980, Jensen 1983, Steinberg 1984, Steinberg 1985, Steinberg 1988). Different studies have, however, produced conflicting results, which may be because of the development of adaptive specializations by herbivores to counteract lower food value or plant defense mechanisms (Watson and Norton 1985a, Uki et al. 1986).

The clear preference for *P. capensis* by juvenile *H. midae* appears to optimize both the high calorific and protein values of the species and its weak plant defenses. Further evidence of the high quality of *P. capensis* was shown by Simpson (1994), where an improved growth rate (50.6 mm/day, at 17°C) in abalone was achieved on a small consumption (3.98% body weight/day, at 17°C) of *P. capensis*.

The results of this study are inconclusive with regard to the effect of phenolic compounds on the feeding behavior of juvenile *H. midae*, although the generally low preference for *E. maxima* could be linked to its high polyphenol content. Other forms of defense available to algae include shape (Steneck and Watling 1982, Watson and Norton 1985b) and surface toughness (Padilla 1985, Padilla 1989, Putman 1990). McShane et al. (1994) considered toughness as the only factor affecting feeding selectivity in the abalone *Haliotis rubra*. In this study, the results of the toughness experiments suggested that the preference of juvenile abalone for *P. capensis* could be partly the result of the relative ease with which the plant tissue may be removed, because *P. capensis* has a thin membranous blade consisting of one cell layer (Branch and Branch 1988).

Prior dietary experience appeared to have an effect on food consumption (Fig. 4). Abalone avoid the well-defended algae, especially if they had already been exposed to this species. The results of this study could have implications for abalone farming and suggest that a good strategy could be to feed animals on a protein-rich, poorly defended alga such as *P. capensis*, which is a rapid-growing, high-shore species. It is not presently cultured in South Africa, but from this study, it appears to be a potential new food source on abalone farms. This may further encourage the development of either polyculture on abalone farms or the formation of *Porphyra* farms, which have been a major industry in the Far East for many years (Branch et al. 1994).

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ANALYZING THE GROWTH AND FORM OF MOLLUSC SHELL LAYERS, IN SITU, BY CATHODOLUMINESCENCE MICROSCOPY AND RAMAN SPECTROSCOPY

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ABSTRACT We describe two novel methods to analyze the microstructure of mollusc shell layers in situ in shell sections. Living abalone, *Haliotis rubra* (Leach), were immersed in seawater baths to which manganese chloride tetrahydrate was added. Distinct cathodoluminescent marks were produced within shell layers formed during the immersion period. Electron and proton microprobe analysis of shell sections confirmed that concentrations of manganese ions within aragonitic and calcitic shell layers are associated with yellow-green and orange-red luminescence, respectively, which allows us to distinguish mineral types. Colored cathodoluminescent marks within shells show that the mineralization of aragonite and calcite may occur simultaneously in the prismatic layer, but also that mineralization under one area of the mantle may switch between polymorphs over short periods. Using cathodoluminescent marks, we show that Raman laser spectroscopy can distinguish mineralogical types in situ and that the dark nacreous layers, variously described as "growth checks" or conchiolin layers, contain aragonite rather than calcite.

KEY WORDS: Abalone, shell, growth layers, cathodoluminescence, manganese, Raman

INTRODUCTION

Advances in the study of molluscan shell microstructure were greatly facilitated by the use of electron microscopy to record crystal growth and by x-ray diffraction to identify mineralogy (Watabe and Wilbur 1961, Mutvei 1969, Wise 1970a, Wise 1970b, Erben 1972). Since the advent of this instrumentation, few studies have introduced novel methods to track and/or analyze mollusc shell layers in situ. Observations on biomineralization in situ should further our knowledge of the biological control of shell microstructure (Wilbur and Saleuddin 1983, Fritz et al. 1994) and facilitate studies of how the mineralization process is affected by physiological and environmental conditions. This may lead to an understanding of how to determine the age of molluscs with shell layers (Lutz and Rhoads 1980) and an ability to control and adapt the process in the biofabrication of materials (Heuer et al. 1992).

The aforementioned pioneering studies have shown that nacre layers in gastropods and cephalopods (*Nautilus*) are formed by the progressive nucleation of crystals at the top of conical stacks of aragonite platelets and the lateral thickening and eventual coalescing of older crystals at the base (Mutvei 1970, Wise 1970a, Wise 1970b, Erben 1972, Mutvei 1978, Nakahara et al. 1982, Wilbur and Saleuddin 1983). In contrast, in bivalves, growing sheet nacre has a steplike structure, and at each step, there is a gradient of maturing crystals overlying fully formed layers (Wise 1970a, Wise 1970b). In both forms of nacre, the crystals are bound within thin protein sheets of conchiolin (Mutvei 1969, Wise 1970a, Wise 1970b, Erben 1972, Uozumi and Togo 1975, Mutvei 1978, Nakahara et al. 1982, Wilbur and Saleuddin 1983). The prismatic layers of molluscs consist of a diverse array of crystal forms and shapes, but mostly of calcite and/or aragonite elongate crystals (Uozumi and Togo 1975, Nakahara et al. 1982, Wilbur and Saleuddin 1983, Mutvei et al. 1985, Dauphin et al. 1989).

More recent studies in the literature have focused on three major areas. (1) Greater emphasis has been placed on understanding biomineralization, to guide the development of new ceramics (Heuer et al. 1992; Sarikaya and Aksay 1992, Fritz et al. 1994, Zaremba et al. 1996). Biomineralization studies have attempted to elucidate shell fabrication in molluscs by relating the order of crystallization to the underlying organic matrix (Watabe and Wilbur 1960, Wheeler et al. 1988, Cariolou and Morse 1988, Donachy et al. 1992, Mann et al. 1993, Falini et al. 1996). (2) Shell layers have been analyzed to yield data on ambient environmental conditions during shell formation (Jones et al. 1983, Tan et al. 1988, Kalberer et al. 1993, Hirao et al. 1994). These studies assume that the mineralization of shell within the extrapallial fluid of the mantle cavity (Crenshaw 1972) is directly controlled by the ambient environment (Pilkey and Goodell 1963). Elemental ordering within shell layers is complex and appears to be a result of ontogeny, physiological and mineralogical controls, and environmental conditions (Crick and Ottensman 1983, Crick et al. 1985, Carriker et al. 1991, Mann 1992). (3) The study of growth layers has been valuable in aging individual animals (Shaul and Goodwin 1982, Deith 1985, Villiers and Sire 1985, Tanabe 1988, Erasmus et al. 1994, Shepherd et al. 1995). The use of growth layers to age animals requires the periodic deposition of contrasting shell layers, which is controlled by either daily or seasonal variations. In all of this recent work, there have been few new methods applied to the analysis of shell layers, with some notable exceptions (Donachy et al. 1992, Manne et al. 1994, Falini et al. 1996).

Abalone, *Haliotis* spp., have been widely used to investigate the microstructure of gastropod shells (Mutvei 1969, Wise 1970a, Wise 1970b, Erben 1972, Fritz et al. 1994, Zaremba et al. 1996). The outer prismatic layer consists of both aragonite and calcite (Mutvei et al. 1985, Dauphin et al. 1989). Within the nacre are dark proteinaceous (conchiolin) layers, which form growth rings under the spire, as well as oblique growth lines behind the growing edge of the shell (Hayashi 1955, Sakai 1960, Mutvei 1969, Muñoz-Lopez 1976, Day and Fleming 1992). The dark layers appear to be deposited periodically and have been used to age individual

animals (Muñoz-Lopez 1976, Prince et al. 1988, Erasmus et al. 1994, Shepherd et al. 1995). The structure of these layers has been variously described as containing deposits of calcium (Erasmus et al. 1994), of prismatic nature (Shepherd et al. 1995), or as an organic/calcite heterolayer (Zaremba et al. 1996). In this article, we describe two novel methods to identify and map the calcification of shell layers over short periods and to investigate the mineralogy of layers within the shells of molluscs. We have used these methods to investigate the formation and composition of shell layers in black-lip abalone, *Haliotis rubra* (Leach).

MATERIALS AND METHODS

Abalone collected from Port Phillip Bay (Australia) were transported to the recirculating seawater system at the Zoology Department of The University of Melbourne. Mature abalone, ranging from 80 to 100 mm, were placed in 20-L buckets supplied with flowing seawater and were left overnight to acclimate to a temperature of 17 ± 2°C. The following morning, the water supply was removed, and aeration and water circulation were maintained by an airlift method whereby water was drawn up through a polyvinyl chloride pipe in the buckets by the air supply. Manganese chloride tetrahydrate ($MnCl_2 \cdot 4H_2O$) was added at a concentration of 189 mg/L for 48 h to the buckets so as to produce a 10:1 ratio of Ca:Mn in seawater. A 48-h immersion period was used to provide ample time for shell growth, so that the Mn^{2+} ion would be incorporated into the shell. Previous shell marking with fluorochromes had shown that 24-h immersion periods produce detectable marked bands within abalone shells (Day et al. 1995). After the immersion period, the buckets were emptied, rinsed, and then supplied with flowing seawater. The abalone were maintained in flowing seawater in the buckets for 18 days, to allow them to deposit further layers of shell on top of the layer marked with manganese. The abalone were then sacrificed, and 30-mm-long by 10-mm-wide sections were cut from the growing margin of the shell and embedded in polyester resin. A cross-section was cut through each resin block, and one surface was ground flat and polished. The block was cut again, parallel to the polished surface, to produce a 2-mm-thick section for analysis.

Cathodoluminescence Microscopy

Manganese bands in shell layers of abalone were detected by the use of cold, low-intensity cathodoluminescence (CL) microscopy (Herzog et al. 1970, Marshall 1988). Under vacuum, the polished shell sections were bombarded by a beam of electrons generated from the cathodoluminoscope (Model ELM2B; Nuclide Corporation). A beam energy of 8 keV and 0.6-mA current under a vacuum of approximately 50 μtorr provided optimal conditions for observing CL. Cathodoluminescent marks were viewed and photographed with a Wild M400 microscope at magnifications of 20–35×. Exposure times of 2–8 min were required to photograph CL marks, with Kodak 1600 ASA Ektachrome film.

Electron Microprobe Analysis

Shell sections were analyzed with a Cameca SX50 electron microprobe operating at 15 kV and 25-nA beam current to determine if the marks observed under CL coincided with increased levels of manganese. Sections were first photographed and then sputter coated with a thin layer of carbon. Spot measurements with an 8-μm-wide microprobe beam and a collection time of 20 min were used to calculate the percent weight of elements. The electron microprobe was programmed to measure levels of calcium (Ca), magnesium (Mg), iron (Fe), strontium (Sr), and manganese (Mn), because these ions constitute the major minerals within carbonates (Tucker and Wright 1990). Means and standard deviations (SD) of the elemental composition of shell layers, presented as parts per million (ppm), were calculated from two to five spot analyses. The detection limit of ions within carbonates with the electron microprobe was 50 ppm. The beam was aligned over the CL marks by the use of photographs of the sections.

Ion Beam–Induced Luminescence

Ion beam–induced luminescence (IBIL) spectroscopy was used to determine the emission spectra of marked layers with protons to excite samples instead of electrons, because the goal is to determine trace element composition from the proton-induced x-ray emission (Bettiol et al. 1994, Yang et al. 1995). IBIL was obtained by focusing a 3-MeV proton beam from a 5U Pelletron accelerator at samples mounted on a eucentric goniometer. The wavelength of light emitted was measured with an Ocean Optics SD1000 spectrometer linked to the microscope eye-piece by a 400-μm-core optic fiber. The intensity of peaks, which were normalized, depends on the concentration of Mn^{2+} in the crystal lattice and the beam current.

Raman Laser Spectroscopy

Because the manganese bands were visible only under electron radiation, photographs of sections double exposed under white light and an electron beam were used to align the Raman laser above CL marks. Measurements were taken with the 514.5-nm (green) line from an argon ion laser with the beam focused to a spot size of 1 μm through a ×100 objective and a DILOR *XY* confocal micro-Raman spectrometer with optical channel collection with a CCD array detector. Light is emitted by resonating bonds in a crystal lattice under the laser beam. Raman spectroscopy identifies crystal structure by mapping peaks in the intensity of this light over a range of wavelengths within the visible spectrum. Because there were distinct peaks for aragonite at 151.5, 179.9, and 205.8 cm^{-1}, which differed from those of calcite at 154.0 and 281.4 cm^{-1}, we limited the analysis to between 100 and 300 cm^{-1}.

RESULTS

CL Microscopy

Shell cross-sections from abalone labeled with manganese showed distinct cathodoluminescent bands behind the growing surface (Fig. 1). The length and thickness of the CL bands varied between abalone. Within the prismatic layer, most CL bands were either entirely orange-red or consisted of short yellow-green bands at the shell edge merging into longer orange-red bands. Only yellow-green bands were observed in the nacre layer. In one shell, a sequence of yellow-green/orange-red/yellow-green/orange-red banding was seen across the previous growth margin of the prismatic layer before continuing as a yellow-green band within the nacre. Color changes along the CL bands demonstrate the simultaneous deposition of aragonite and calcite across the mantle. Furthermore, rapid changes in mineralization from aragonite to calcite and vice versa are evident, as evidenced by the change in CL color at some positions from the top to the bottom of the band (Fig. 1). Nacre CL banding was more continuous than within the prismatic layer and formed a distinct saw-tooth line (Fig. 2). The saw-

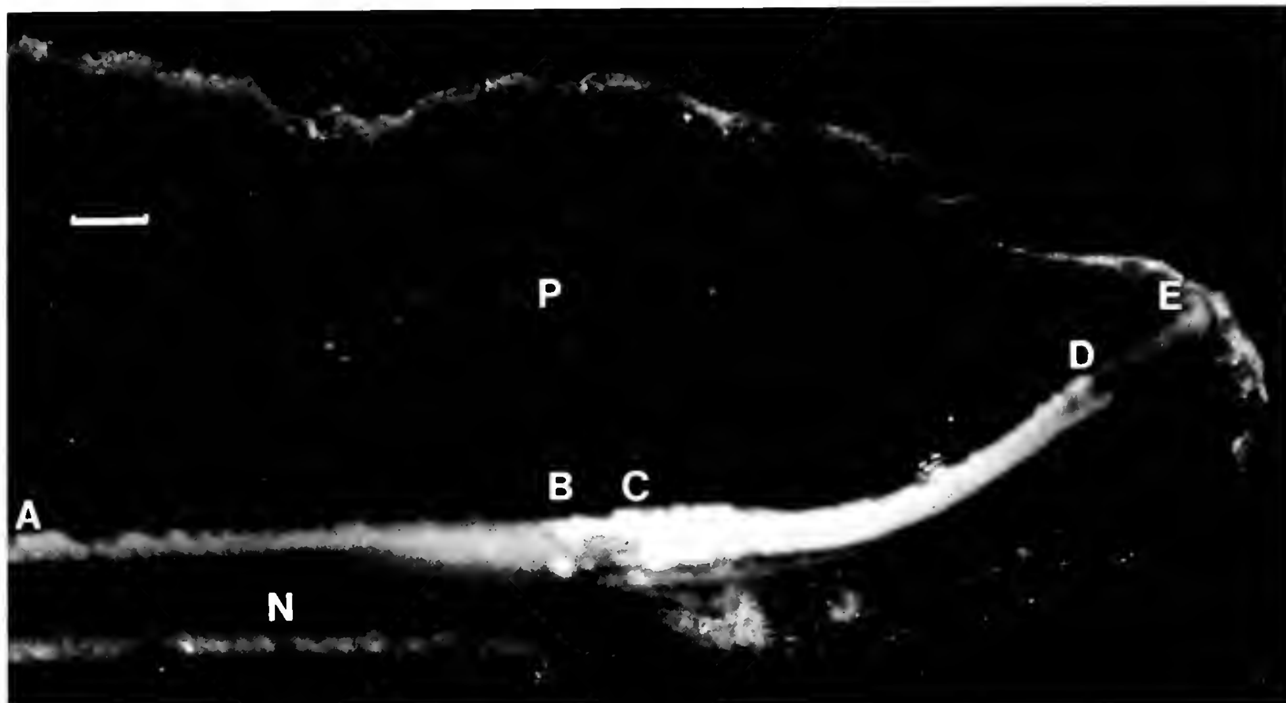

Figure 1. Cross-section of the shell margin of *H. rubra*, showing prismatic (P) and nacre (N) layers and cathodoluminescent bands formed when immersed in a 10:1 ratio of Ca:Mn in seawater for 48 h. Growth layers in nacre (A–B) emitted a yellow-green color, indicating aragonite, whereas labeled prismatic layers (C–D and D–E) emitted orange-red and yellow-green colors, indicating calcite and aragonite, respectively. In region B–C, mineralization switched from calcite to aragonite. Scale bar = 100 μm.

toothed edge of the CL bands has dimensions similar to aragonite growth cones (15–20 μm) and appears to represent the deposition of manganese carbonate at the edges of tablets in the cones. Banding within the prismatic layer was usually smooth, although irregular shell marking was present in some sections.

Electron Microprobe Analysis

Electron microprobe analysis showed that orange-red prismatic bands contained 2,000 ± 800 ppm of manganese, and yellow-green bands in nacre contained 800 ± 50 ppm, whereas nonlabeled areas of both contained no manganese (Table 1). An approximately fourfold increase of magnesium in both labeled prismatic (11,500 ± 900 ppm) and nacre (500 ± 50 ppm) layers was found when compared with nonlabeled layers. The concentration of iron, which is a known quencher of manganese-activated luminescence (Sommer 1972a, Hemming et al. 1989), remained under 300 ± 50 ppm for all measurements. Calcium and strontium levels within labeled and nonlabeled layers showed no systematic relationship.

IBIL

We measured a broad spectral band centered on 540 nm (yellow-green) for aragonite in the nacre layer, in agreement with reported CL emission peaks of manganese in the aragonite of other shellfish (Sommer 1972a, Sommer 1972b, Barbin 1992) (Fig. 3). A broad peak of manganese-activated luminescence, centered around 605 nm (orange-red), was found in the prismatic calcite layer. The shift of this emission spectrum to the right, as compared with previous literature (590 nm), may be attributed to the level of other trace elements within the luminescent calcite layer (see Sommer 1972a [Fig. 2, pp. 264], Machel 1985). Yang et al. (1995) used IBIL to measure the emission peaks of natural CL bands observed in shells from Barbin's (1992) study. They obtained manganese-activated spectral peaks of 560 and 620 nm from aragonite and calcite, respectively, but considered the difference between these emission peaks and those reported in earlier studies to be insignificant. Thus, the emission peaks from aragonite and calcite measured under IBIL in this study and that of Yang et al. (1995) are in broad agreement with previous literature on CL. This confirms that proton radiation and electron radiation activate the same energy states within the crystal lattices (Machel 1985).

Raman Laser Spectroscopy

Raman laser spectroscopy was used to verify mineral types in labeled layers and to identify nonlabeled layers. Spectral analysis of calcite and aragonite controls showed peaks in intensity at 154 and 281 cm^{-1} for calcite and 151, 180, and 206 cm^{-1} for aragonite, in agreement with published Ramam spectra (White 1974, Urmos et al. 1991). Increased fluorescence was detected by the laser when focused on manganese-labeled layers visible under CL. We measured the fluorescence over a number of 50-μm transects and confirmed that the increased fluorescence formed a band coinciding with a CL mark. We then used the position of maximum fluorescence to indicate the marked layer. Layers with yellow-green CL bands had an aragonite spectrum, whereas layers with orange-red CL bands produced a calcite spectrum (Fig. 4). Raman spectra of layers producing yellow-green CL in nacre and prismatic regions had very similar peaks. Unstained shell layers surrounding orange-red CL bands within the prismatic layer produced a calcite spectrum under the laser (Fig. 4A). Nonlabeled nacreous

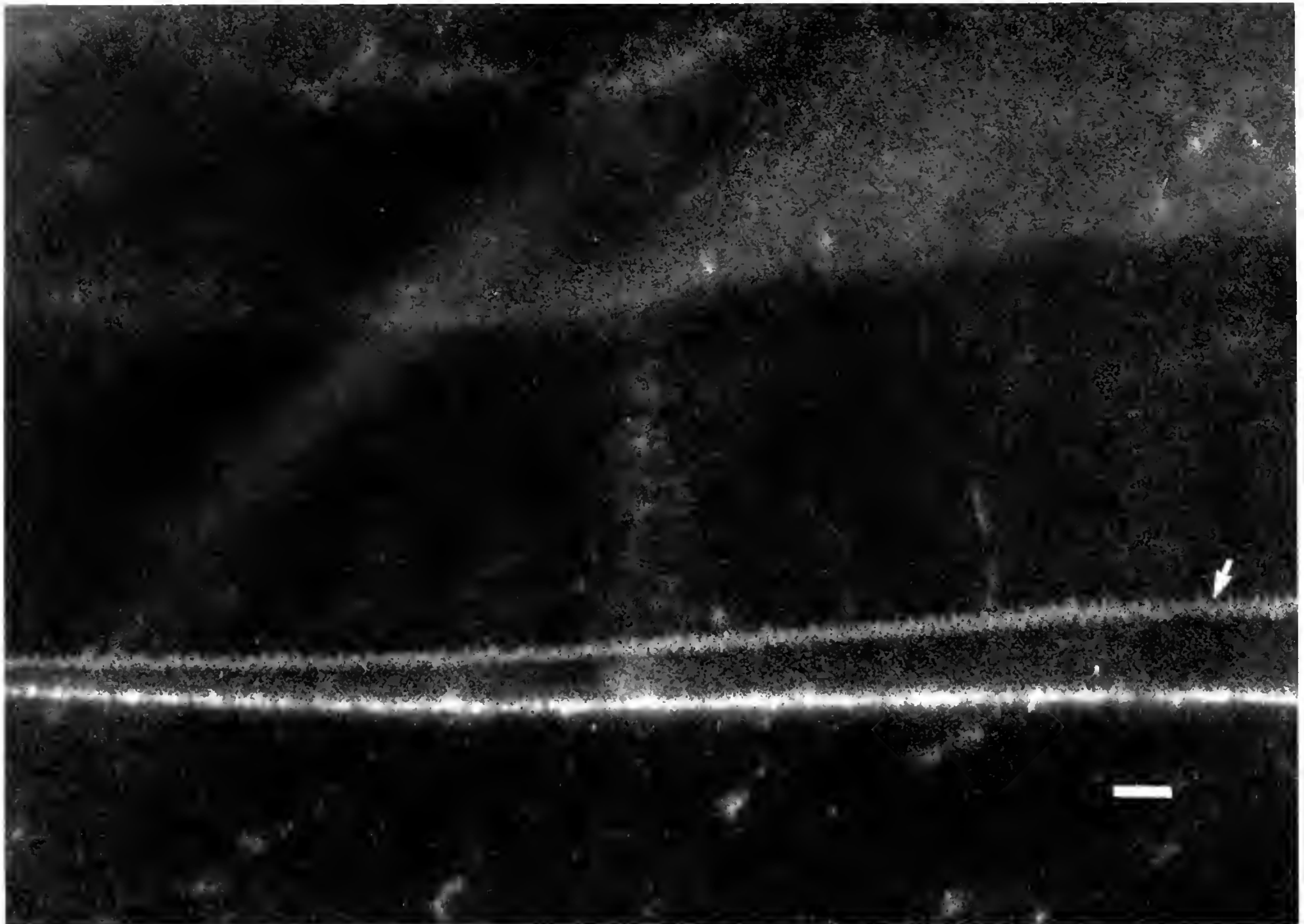

Figure 2. Cross-section of abalone shell, immersed in a 10:1 ratio of Ca:Mn in seawater for 48 h, showing the saw-toothed (arrow) form of manganese-activated luminescence in the nacreous layer, apparently indicating progressive manganese labeling of conical stacks of aragonite tablets. Scale bar = 50 μm.

shell and the dark proteinaceous nacre layers or growth lines of this species produced an aragonite spectrum (Fig. 4B).

DISCUSSION

Previously, manganese luminescence has been detected in aragonitic and calcitic layers of mollusc shells (Sommer 1972a, Sommer 1972b, Barbin et al. 1991a,b, Barbin 1992, Mazzoleni et al. 1995, Yang et al. 1995). Those studies induced luminescence within shell layers containing natural levels of manganese derived from the ambient environment, whereas we have used manganese as a marker to record mineralization. CL bands are formed by the substitution of manganese for calcium in the carbonate lattice (Sommer 1972a). Variations in the color of manganese luminescence from carbonate polymorphs are a result of the activation of different energy states of the 3d electrons and the bond length and position of the Mn^{2+} ion within the host crystals (Sommer 1972a, Yang et al. 1995). Manganese carbonate in aragonite has an orthorhombic crystal structure in which the Mn^{2+} ion is coordinated to nine oxygen atoms. In calcite, the manganese ion forms a rhombohedral unit with bonds to six oxygen atoms (Sommer 1972b,

TABLE 1.

Electron microprobe analysis of manganese-labeled and nonlabeled shell layers.

Ion	Calcite Layers, Nonlabeled (ppm)		Calcite Layers, Labeled (ppm)		Aragonite Layers, Nonlabeled (ppm)		Aragonite Layers, Labeled (ppm)	
	Mean	SD[a]	Mean	SD[a]	Mean	SD[a]	Mean	SD[a]
Ca	396,000	240	382,000	2,200	397,000	1,267	398,000	50
Mg	2,600	150	11,500	900	200	100	500	50
Mn	0	50	2,000	800	0	50	800	50
Fe	200	100	130	50	300	50	50	50
Sr	1,000	50	1,200	250	3,500	1,800	1,600	50

[a] n = 2–5 spot analyses, and minimal standard deviation of 50 ppm = minimal detection limit

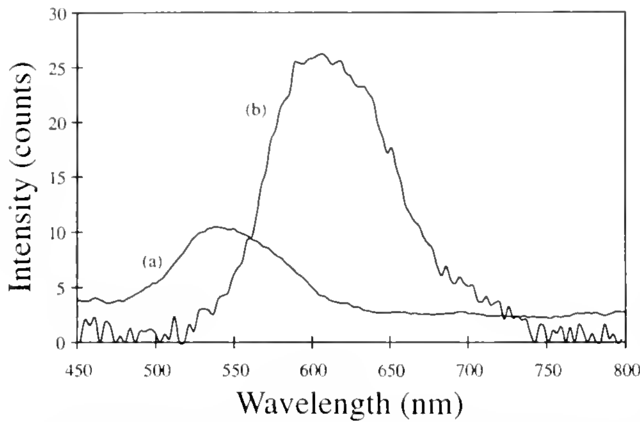

Figure 3. Spectrometer measurements under IBIL of the yellow-green (A–B) and orange-red (C–D) luminescence from nacreous (N) and prismatic (P) layers, respectively, in the abalone section in Figure 1, showing broad bands with peaks at 540 nm for aragonite layers (a) and 605 nm for calcite layers (b).

Tucker and Wright 1990). It is presumed that because Mn^{2+} has an ionic structure similar to that of Ca^{2+}, it follows the same biological pathway of mineralization. The CL banding in *H. rubra* appears to record the natural deposition of aragonite and calcite crystals by the mantle epithelium without disturbing the process and to label growing aragonite tablets of nacre as the conical stacks expand.

An area of great interest concerns the junction of aragonite and calcite deposition (Zaremba et al. 1996). CL, with manganese as a chemical marker, defines where this junction occurs. The continuous CL band across the prismatic and nacre layers (Fig. 1) demonstrates the simultaneous deposition of shell in these two layers. There has been some uncertainty regarding the timing of aragonite and calcite mineralization within the prismatic layer. Earlier work indicated that aragonite and calcite are calcified in the prismatic layer either simultaneously or by rapid changes in mineralization under the control of mantle epithelium cells (Mutvei et al. 1985, Dauphin et al. 1989). We have observed both mechanisms. Continuous CL marks across the prismatic layer confirm that calcite and aragonite were deposited simultaneously, but also show that the mineralization at one position on the growth surface can change from calcite to aragonite or vice versa within short periods.

Raman spectroscopy has been used to compare synthesized and biogenetic carbonates of corals, as well as the carotenoids in protein layers of pearls (Merlin and Dele'-Dubois 1986, Urmos et al. 1991). In this study, we used Raman spectroscopy to unequivocally identify carbonate polymorphs of shell layers in situ and to show that dark nacreous layers contain aragonite rather than calcite in *H. rubra*. This is in contrast to calcite in the same shell layers in *Haliotis rufescens*, identified by Zaremba et al. (1996), and raises the interesting possibility that aspects of shell microstructure may differ between closely related species.

Traditional techniques of determining carbonate polymorphs (i.e., staining or extraction of shell fragments followed by x-ray diffraction) may not provide sufficient resolution. Both the precision of extracting shell and the differentiation of stained layers are restricted to optical microscope magnifications. Raman spectroscopy elucidates crystal form at high resolution without disturbing the fine structure. The analysis is very quick, taking only a few minutes to determine the microstructure, and we have identified shell mineralogy to a beam width of 1 μm, with minimal specimen preparation time.

Applying these new techniques to research on the biofabrication of flat pearls (Fritz et al. 1994, Zaremba et al. 1996) or other crystal-containing materials (Berman et al. 1993, Mann 1993) should provide greater insights into the biologic mechanisms of shell construction and perhaps lead to better ways to synthesize such composite materials for commercial use. Because manganese marking records both the form of microstructure and the timing of its deposition, it should find a use in manipulation trials where optimal conditions for growing crystals are sought. Raman spectroscopy and CL microscopy can be usefully combined, because fluorescence from manganese within the shell layers can be detected under the laser beam. Manganese-activated fluorescence has previously been detected in calcite under ultraviolet excitation (Fonda 1940, Pedone et al. 1990) and presumably is also activated by the 514.5-nm laser wavelength.

Manganese-marking experiments should also be useful in studying the relation between shell mineralogy and ambient conditions. As discussed above, many studies have analyzed mollusc shells and have interpreted the results as a record of environmental conditions. The assumption that mineralization across the mantle epithelium is directly related to environmental conditions is not always valid (Crick and Ottensman 1983, Crick et al. 1985). The use of manganese to mark the abalone shell in conjunction with CL and electron microprobe analysis shows that ambient conditions

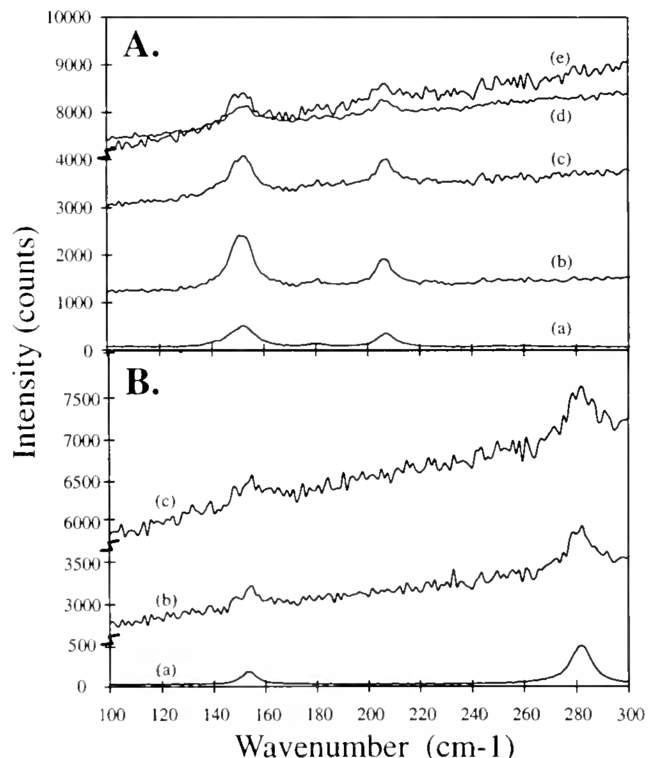

Figure 4. Comparison of Raman spectra of layers in the abalone section in Figure 1 with controls. (A) Raman spectra of aragonite control (a), nonlabeled nacre (b), dark conchiolin layer (c), manganese-labeled aragonite in the nacre layer (d), and manganese-labeled aragonite in the prismatic layer (e), showing vibration peaks at 151, 180, and 206 cm^{-1} in each layer type. (B) Raman spectra of calcite control (a), nonlabeled prismatic calcite (b), and manganese-labeled prismatic calcite (c), showing vibration peaks at 154 and 281 cm^{-1}.

affect the elemental composition of shell layers, but the results also show there is some mineralogical control, because the incorporation of trace elements within the shell matrix varies between aragonite and calcite. In aragonite, the substitution of ions with radii greater than calcium, such as Mn^{2+}, is favored, whereas in calcite, ions with radii less than Ca^{2+}, such as Mg^{2+}, are preferred. Thus, CL or Raman spectroscopy should be used to identify microstructure types when the elemental composition of shell layers is analyzed. Furthermore, our results demonstrate that there were interactions between elements when the composition of shell layers was altered. In layers where we elevated the concentrations of manganese, the concentration of magnesium also increased (Table 1). The effect of the environment on the elemental composition of shell layers may also vary with the physiology and ontogeny of the animal. Controlled experiments in which manganese is applied as a marker in conjunction with other trace elements could reveal how to interpret previous environments from the record held in shell layers.

The pattern of shell deposition determines the shape of the growing shell, but the relation between deposition and shell shape has received very little attention. Shell repair mechanisms are also poorly understood, beyond descriptive studies (see Watabe 1983). Although we have found that fluorochromes did not mark all areas of calcification within the shell (Day et al. 1995), manganese marking can be used to quantify the rate and position of shell deposition over the whole growing surface and thus should be useful for studies of shell growth and repair.

Shell layers have been widely used to estimate the age of molluscs, particularly species such as abalone that are commercially harvested (Ramón and Richardson 1992, Erasmus et al. 1994, Shepherd et al. 1995), because a knowledge of the age composition of the stock is invaluable for management. Validation of the periodicity of the growth layers by comparison to growth curves (e.g., Prince et al. 1988) is unsatisfactory, especially for adults (Day and Fleming 1992). Validation should be ideally based on the use of chemical markers to "date stamp" layers, so as to determine the timing and regularity of subsequent layers (Beamish and MacFarlene 1983). Because manganese marks the shell layers in the spire of abalone that are thought to reflect age (Muñoz-Lopez 1976, Prince et al. 1988, Shepherd et al. 1995), it is suitable for such age validation studies.

We have shown that these new techniques allow the biosynthesis of minerals in mollusc shells to be followed in time and space and cast light on the complex functioning of the mantle epithelium of molluscs in ordering the deposition of calcite and aragonite on a microscale. These techniques should have significant implications for future research on molluscan shell microstructure in relation to the biosynthesis of materials, the elucidation of previous environments recorded in shells, the process of shell construction and repair, and the validation of methods to age molluscs.

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TWO PARASITIC COPEPODS, *PSEUDOMYICOLA SPINOSUS* AND *MODIOLICOLA GRACILIS*, ASSOCIATED WITH EDIBLE MUSSELS, *MYTILUS GALLOPROVINCIALIS* AND *MYTILUS CALIFORNIANUS*, FROM BAJA CALIFORNIA, NW MEXICO

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ABSTRACT Mussel culture and fisheries are two increasing activities in Baja California, NW Mexico. One of the risks for these activities is the presence of harmful parasites like certain copepod species. This study was carried out to determine the parasitic copepods associated with edible mussels, *Mytilus galloprovincialis* Lmk. and *Mytilus californianus* Conrad, from Baja California, NW Mexico, and to establish certain aspects of their distribution, temporal fluctuation, and damage to their host. The study was carried out at sites of contrasting environmental conditions: exposed rocky shores, protected shores, protected and polluted areas, and culture area. Two species of parasitic copepods were found inhabiting the mantle cavity and gills of mussels: *Pseudomyicola spinosus* Raffaele and Monticelli (Mycolidae) and *Modiolicola gracilis* Wilson (Clausidiidae). This is the first record of these copepods in Mexican waters. *M. gracilis* was found in *M. galloprovincialis* and *M. californianus* from all localities studied in numbers from 0 to 5 individuals per host and a maximum prevalence of 26.66% in the first species, and from 0 to 15 specimens per mussel and a maximum prevalence of 70% in the second species. Its presence was relatively constant through the year, with a slight increase in autumn and winter. *P. spinosus*, by contrast, was scarce or absent in *M. californianus* and *M. galloprovincialis* from exposed rocky shore environments. Its number and prevalence were low in the mussel culture area. However, it was very abundant in *M. galloprovincialis* from the protected and polluted environments, where its numbers ranged from 0 to 59 copepods per mussel and a prevalence of 100%. Rates of infestation in mussels increased in autumn. Macroscopical damages associated with the presence of copepods were not detected, and the histopathologic analysis did not reveal any damage to the tissues of the host. However, there were more parasites in larger mussels, and most parasitized mussels showed a low condition index. *P. spinosus* could be considered a potential threat to the mussel culture.

KEY WORDS: *Pseudomyicola spinosus*, *Modiolicola gracilis*, *Mytilus californianus*, *Mytilus galloprovincialis*, copepods, parasites, pathology

INTRODUCTION

The California mussel, *Mytilus californianus*, locally named "Choro," and the blue mussel, *Mytilus galloprovincialis*, are used for human consumption in Baja California, NW Mexico. The first species has been gathered for centuries in the region (Linik 1977, Téllez 1987) and actually supports a local fishery. The second is cultured with submerged longlines and is sold to national and international markets. Mussel culture activities are expected to increase in Baja California. However, there are no studies concerning the parasites affecting both edible mussel species in the area, as well as their potential hazard for commercial production. Our previous analysis revealed that some copepods could be found in the mantle and gills of mussels. The poecilostomatoid family *Mycolidae* includes 15 species of copepods; most of them are parasitic in marine bivalve molluscs. They have been identified as agents of mass mortality of some commercial species in different regions of the world and have been considered to be pests (Korringa 1951, Davey et al. 1978, Ho 1995). In some countries of Europe and Asia, the marine copepod parasite fauna is relatively well known (Avdeev 1977, Davey et al. 1978, Paul 1983, Do and Kajihara 1986, Theisen 1987, Ho and Kim 1991, Poquet et al. 1994, Robledo et al. 1994). In Mexico, however, the identity, distribution, prevalence, and effects of parasite copepods on bivalve molluscs are unknown. The aim of this study was to ascertain the specific identify of the copepods from natural and commercial stocks of edible mussels, *M. galloprovincialis* and *M. californianus*; to document their distribution in Baja California, their temporal fluctuation, and their effects on the condition index of the mussels; and to describe any pathologic effects on the host.

MATERIALS AND METHODS

Seasonal fluctuations of copepods were studied between January and December 1995 in mussels from Baja California, NW Mexico. During this period, 30 adult *M. galloprovincialis* were collected each month from La Mina del Fraile, an exposed rocky shore (mean total shell length, 57.0 mm; standard deviation [SD], 5.76); Tres Hermanas, a mussel culture area (mean total shell length, 64.0 mm; SD, 6.39); and Ensenada Pier, an urban and polluted area (waste water discharge and fuel from ships) (mean total shell length, 58.0 mm; SD, 9.02) (Fig. 1). Additionally, 30 adult *M. californianus* were gathered monthly from a fishery area in La Mina del Fraile (mean total shell length, 63.0 mm; SD, 8.17). In May 1995 and March 1996, other localities were sampled to extend the information on the distribution of parasitic cope-

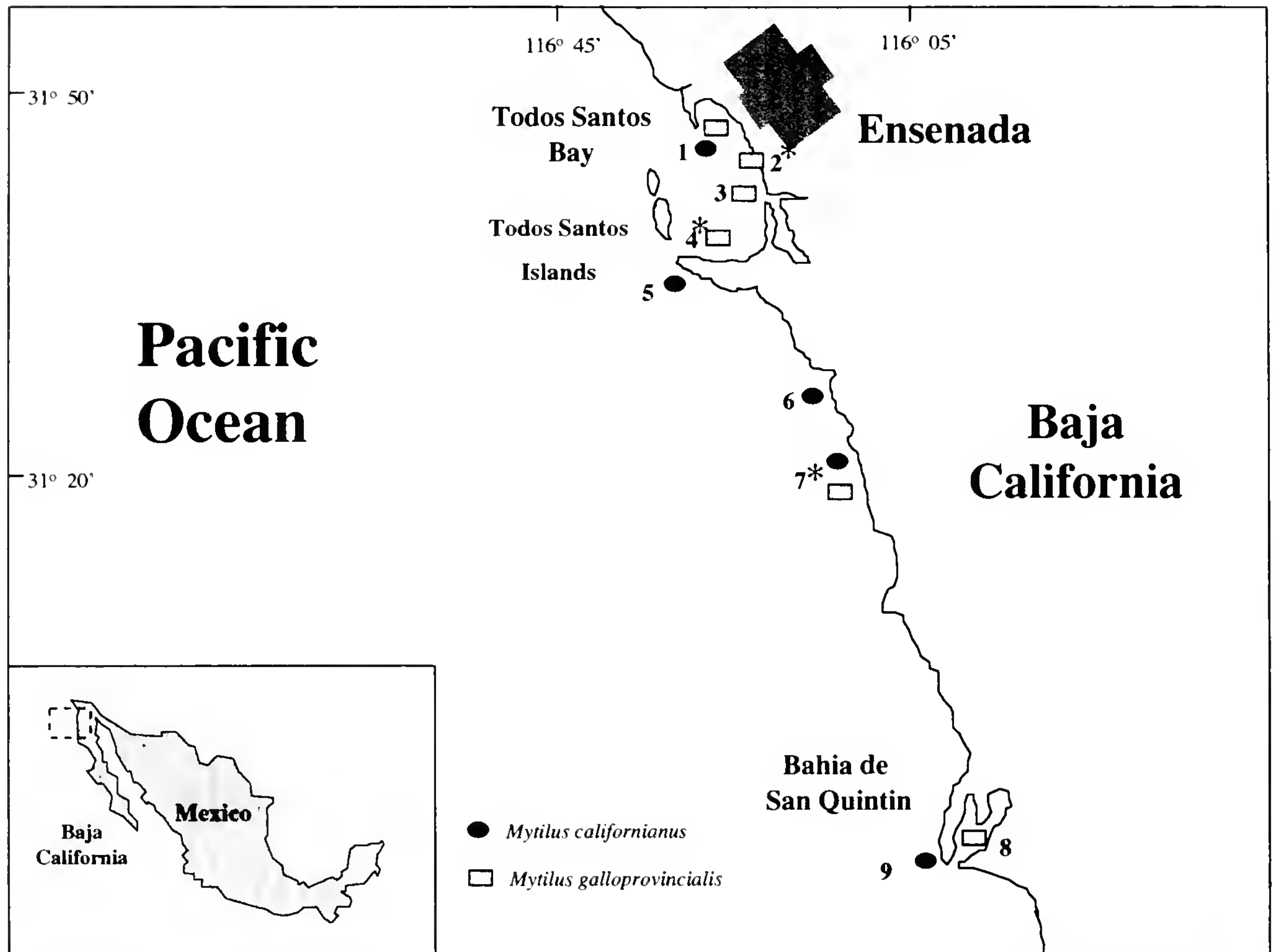

Figure 1. Map of the study area showing collection sites and species. 1, Inner cliff of Ensenada (polluted area); 2, Ensenada Pier (polluted area); 3, Estero Beach (sandy and estuarine area); 4, Tres Hermans (mussel culture area); 5, La Bufadora (exposed tourist area); 6, El Acuario (exposed nonexploited area); 7, La Mina del Fraile (exposed exploited area); 8, San Quintín (sandy and protected area); 9, San Quintín (exposed exploited area), Baja California. (*Localities where annual study was carried out).

pods: *M. galloprovincialis* from Estero Beach, a sandy and estuarine locality; *M. californianus* from La Bufadora, a tourist, exposed rocky shore; *M. californianus* from El Acuario, a nonexploited exposed rocky shore; *M. galloprovincialis* and *M. californianus* from the inner cliff of Ensenada, a protected and polluted area; *M. californianus* from an exposed rocky shore in Bahía de San Quintín, an exploited area; and *M. galloprovincialis* from the inner Bahía de San Quintín, a protected area (Fig. 1).

After any fouling organisms were removed, each mussel was sized (total shell length) and weighed (total weight [TW]), after which they were placed in Petri dishes and opened. Intervalvar water and mussel flesh were examined for the presence of copepods under a dissecting microscope. Copepods were preserved in a solution of 70% ethanol for identification. Body size, length: width ratios, and length:egg sac length ratios were obtained for all of the identified copepods. Measurements were taken with a micrometer eyepiece fitted in a stereoscopic microscope. TW, wet meat weight (MW), and shell weight (SW) of mussels were recorded to obtain a condition index (CI) where CI = [MW/(TW − SW)] × 100 (Aguirre 1979). Prevalence was estimated as the number of infested mussels/number of mussels examined ×100. In November 1995, 226 *M. galloprovincialis*, ranging in size between 20 and 75 mm, were collected from Ensenada Pier for an analysis of the number of parasite copepods and their relation to mussel size (linear regression). The relationship of copepods and the CI of mussels was analyzed in 175 mussels ranging from 60- to 70-mm shell length (linear regression). In both situations, mussels were grouped in length classes and the mean number of copepods per length class, or CI of mussels, was calculated.

For the study of tissue damage, fractions of gills, mantle, and labial palps, in mussels where copepods were found, were removed and fixed in Davison's fixative (Shaw and Battle 1957) for 24 h; tissue samples were then embedded in paraffin wax and sectioned at intervals of 5 μm, and histologic sections were stained with hematoxylin and eosin. Slides were examined under an optical microscope at 40× and 20× magnifications.

RESULTS

Identity of Copepods

Pseudomyicola spinosus

One of the copepod species found in *M. galloprovincialis* and *M. californianus* was identified as *Pseudomyicola spinosus* (Raf-

faele and Monticelli 1885). The material studied included 9 adult females, 10 adult males, and 15 female copepodids from the gills and mantle cavity of *M. galloprovincialis* from Ensenada Pier and from *M. californianus* from the inner cliff of Ensenada. All specimens were ethanol preserved, and vials are deposited in the United States National Museum, Smithsonian Institution (USNM-274221).

For females, the morphological features of *P. spinosus* from *M. galloprovincialis* and *M. californianus* agree with the diagnosis of *P. spinosus* as redescribed by Ho (1980) and Do and Kajihara (1986). Adult female length:width ratios were from 3:4:1 to 4:2:1, length:egg sac length ratios were from 2.5:1 to 2.7:1, and the number of eggs was from 20 to 32. There were two female specimens with atypical size, length:width ratios (4.4:1 and 3.2:1), length:egg sac length ratios (3.3:1 and 1.7:1), and number of eggs (22 and 26). The bodies of these specimens were 18% longer and 15% broader than the "normal" forms, and their egg sacs were almost 10% longer (see Do et al. 1984 for atypical forms).

The male morphology of the studied specimens agrees with previous descriptions of the species: adult length:width ratios were from 3.2:1 to 3.7:1. As recorded for the females, two adult male specimens showed variation in their body proportions:length: width ratio, 4.4:1. Atypical males were more slender than "normal" individuals, being 12% longer and 9% narrower.

Modiolicola gracilis

The other copepod found in *M. californianus* and *M. galloprovincialis* was identified as *Modiolicola gracilis* (Wilson 1935), which was first reported from Monterey Bay, CA. This is another poecilostomatoid parasitic copepod belonging to the family *Clausidiidae*. Although this is a common copepod in California populations of *Mytilus*, this is the first record of the species in Mexico.

The material studied included four adult females and two adult males from the gills and mantle cavity of *M. californianus* and *M. galloprovincialis* from La Mina del Fraile. All specimens were ethanol preserved, and vials are deposited in the United States National Museum, Smithsonian Institution (USNM-274222).

Seasonal Fluctuation and Distribution

The prevalences of the copepod species at the study localities are shown in Figure 2. The prevalence of *M. gracilis* was different among all localities and species, except between *M. galloprovincialis* from Tres Hermanas and *M. californianus* from La Mina del Fraile (Kruskal-Wallis test, $H = 19.7$, $p < 0.01$, followed by all

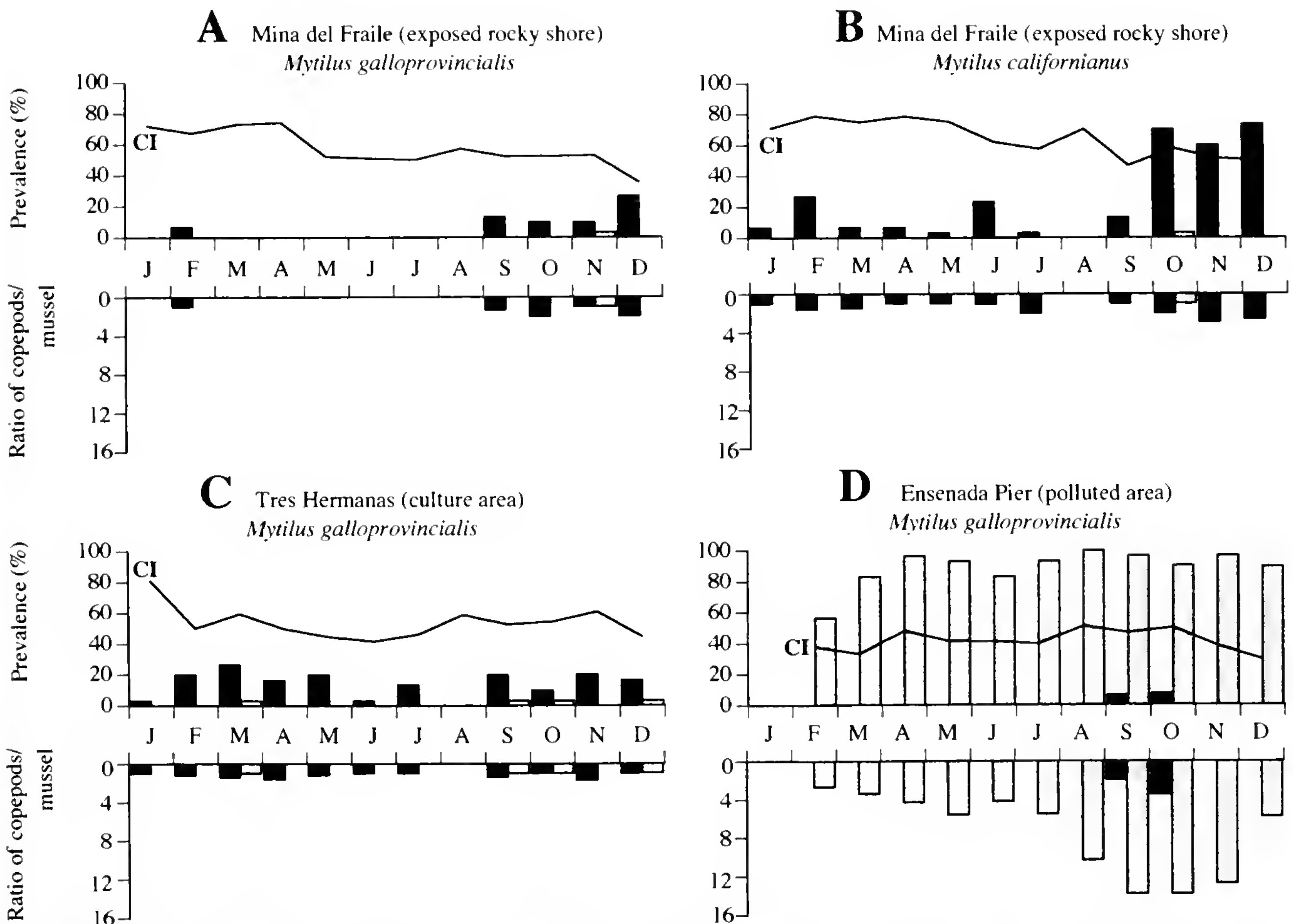

Figure 2. Prevalence of *M. gracilis* (■) and *P. spinosus* (□), mean number of copepods per sampled mussels (ratio of copepods:mussel), and CI in *M. galloprovincialis* from (A) the exposed rocky shore of La Mina del Fraile (exploited area), (C) the mussel culture area of Tres Hermanas, aod the (D) polluted area of Ensenada Pier aod in *M. californianus* from (B) the exposed rocky shore of La Mina del Fraile (exploited area), Baja California, Mexico, from January to December 1995. Differences in prevalence and CI were statistically significant ($p > 0.01$) (see text).

pairwise multiple comparison procedures, Dunn's method). The prevalence of *P. spinosus* was different among all localities and species, except between *M. galloprovincialis* and *M. californianus* from La Mina del Fraile (Kruskal-Wallis test, $H = 36.18$, $p < 0.01$, followed by all pairwise multiple comparison procedures, Dunn's method). *M. gracilis* was currently found in La Mina del Fraile and Tres Hermanas. However, this species was very scarce in Ensenada Pier. In La Mina del Fraile, the prevalence of *M. gracilis* increased in autumn. From spring to middle summer, it was not observed in *M. galloprovincialis*. An increase was not detected in the prevalence of *M. gracilis* during the autumn in *M. galloprovincialis* from the culture area, but the presence of this copepod continued throughout the year, with the exception of August. By contrast, *P. spinosus* was occasionally found in mussel species from La Mina del Fraile and was frequent in the mussel culture area, but at low prevalence. Interestingly, *P. spinosus* was recorded throughout the study period in *M. galloprovincialis* from the Ensenada Pier, and its prevalence was around 80%.

Figure 2 also shows the mean number of copepod species per infested mussel in each locality studied. Variations in the mean number of *M. gracilis* per infested individual were closely related to the variation in its prevalence throughout the study period. The maximum mean number of these copepods occurred in autumn in La Mina del Fraile, when the maximum numbers of *M. gracilis* in *M. galloprovincialis* and *M. californianus* were 5 and 15, respectively. In Tres Hermanas, there were fluctuations in the mean number of copepods per host that were closely related to fluctuations in prevalence. The maximum number of copepods per *M. galloprovincialis* was three. The range and mean number of *P. spinosus* per infested mussel in the Ensenada Pier were higher from summer to autumn than the rest of the year. The maximum number of copepods per mussel was 59 in October 1995. Ovigerous females of *M. gracilis* were recorded in all samples from La Mina del Fraile and from Tres Hermanas. Ovigerous females of *P. spinosus* were found throughout the study period in the Ensenada Pier, where copepodid stages were also recorded in September and October 1995. Both copepod species, *M. gracilis* and *P. spinosus*, were found together in the same host. There were differences in the CI of mussels from localities and species throughout the study period (analysis of variance, $F = 9.51$, $p < 0.001$, followed by Student-Newman-Keuls mean comparisons method). There were no significant differences between the CI of *M. galloprovincialis* and *M. californianus* from La Mina del Fraile and between the CI of *M. galloprovincialis* from La Mina del Fraile and Tres Hermanas, but significant differences were recorded between *M. galloprovincialis* from Tres Hermanas and *M. californianus* from La Mina del Fraile, where the prevalence and mean number of copepods per mussel were similar. The CI of *M. galloprovincialis* from the Ensenada Pier area was less than those of mussels from the other localities, and this was statistically significant (Fig. 2).

Table 1 shows the results of the presence of copepod species in the study area. *M. gracilis* is a common copepod associated with both *M. californianus* and *M. galloprovincialis*. It was found in *M. californianus* from La Mina del Fraile, El Acuario, Bahía de Todos Santos, and Bahía de San Quintín. In *M. galloprovincialis* this copepod was found in all of the localities studied (see also Fig. 2). In both mussel species, the prevalence and range of *M. gracilis* were low. Contrary to this, *P. spinosus* was very scarce on exposed rocky shores but it was very abundant in number and prevalence in protected and polluted environments like the Ensenada Pier.

There was a significant positive correlation between the size of *M. galloprovincialis* and the presence of copepods ($y = -6.98 + 0.28x$, $R^2 = 0.81$, $F = 37$, $p < 0.1$). Mussels from 45- to 75-mm shell length had the highest number of copepods. The lowest CI of *M. galloprovincialis* was recorded in the Ensenada Pier, the most copepod-infested area. The relation between low CI in mussels with the highest number of copepod parasites was corroborated by the correlation between the number of copepods and the CI of their host in this locality ($y = 34.13 - 0.51x$, $R^2 = 0.86$, $F = 18.6$, $p < 0.1$). Neither macroscopic analysis nor histologic analysis of mussel gills, labial palps, or mantle revealed any damage.

DISCUSSION

This is the first record of *P. spinosus* and *M. gracilis* in Mexican waters. *M. gracilis* has been found in *M. galloprovincialis* and *Mytilus edulis* L. from European and Japan waters and in *M. edulis* from Monterey Bay, CA (Wilson 1935, Ho 1980, Poquet et al. 1994). In accordance with Poquet et al. (1994), this species does not show any morphological adaptations to parasitism; however, it presents some characteristics in its cuticule (external microvilli-like projections) that suggest a mechanical function in adhesion rather than nutritional absorptive function. Histologic studies, however, have failed to show any histopathologic lesions. Poquet et al. (1994) pointed out that the determination of specific functions of the integumental glands and the characterization of the chemical composition of the various cuticular layers could lead to the identification of possible long-term alterations in host tissues. The recorded prevalences of *M. gracilis* in this study were higher than those recorded in *M. galloprovincialis* and *M. edulis* from the Ebro Delta River (E. Spain) (Poquet et al. 1994).

M. gracilis was not recorded in August, when the highest temperatures prevailed. The slight increase in the prevalence of this parasite recorded from September to December in La Mina del Fraile suggests that the reduced temperatures that occur during these months may be favorable for this parasite. Permanently submerged mussel culture conditions seem to be favorable for the regular prevalence of *M. gracilis*.

P. spinosus has been associated with 54 species of bivalve molluscs in temperate and tropical waters (Ho 1995, Humes 1968, Ho and Kim 1991). Measurements of the Baja California specimens revealed variations among adult and preadult individuals. The size of normal female specimens falls within the range known for *P. spinosus;* however, their bodies are relatively longer and more slender than specimens from Japan. The egg sac proportions of Japanese and Baja California specimens showed only slight differences (Do et al. 1984). The atypical female is noticeably longer than normal individuals and can be distinguished by its larger size and relatively longer egg sacs than those of the "normal" females. In the Japanese material (Do et al. 1984), the proportion of body length:egg sac length is about 3:1, whereas in the atypical form, the value is only 1.75:1. Atypical males of Baja California were comparable to Japanese atypical males (Do et al. 1984). In both cases, the body is more slender than in the normal individuals, and when comparing the length:width ratio of both atypical forms, Baja California specimens appear to be even more slender (4.4:1) than the Japanese specimens (3.9:1). Do et al. (1984) suggested that these slender body forms appear to be characteristic of active swimmers. However, we did not observe *P. spinosus* swimming; it remained crawling through the mantle and gills.

TABLE 1.

Copepods infecting *M. californianus* and *M. galloprovincialis* from sampling localities in Baja California, Mexico, in May 1995* and March 1996#.[a]

Locality	Species	n	Mussel Mean Length (mm)	SD	Range of Copepod Species	Mean per Infected Mussel
*La Mina del Fraile, exposed rocky shore (exploited)	*M. californianus*	30	60.47	7.71	0–1 *M. gracilis*	1
*La Mina del Fraile, exposed rocky shore (exploited)	*M. galloprovincialis*	30	42.31	4.90	0	0
*El Acuario, exposed rocky shore (not exploited)	*M. californianus*	29	66.91	10.51	0–3 *M. gracilis*	1.5
*La Bufadora, exposed rocky shore (touristic)	*M. californianus*	30	51.59	7.59	0–1 *M. gracilis*	1
*Tres Hermanas (culture)	*M. galloprovincialis*	30	71.27	9.21	0–2 *M. gracilis*	1.2
*Estero Beach (sandy and estuarine)	*M. galloprovincialis*	30	56.69	8.38	0–4 *M. gracilis*	2
*Ensenada Pier (polluted)	*M. galloprovincialis*	30	61.75	6.13	0–15 *P. spinosus*	5.6
#Inner Cliff of Ensenada (polluted)	*M. galloprovincialis*	30	58.23	5.46	0–20 *P. spinosus*	5.8
					0–4 *M. gracilis*	1.7
#Inner Cliff of Ensenada (polluted)	*M. californianus*	30	64.32	7.60	0–11 *P. spinosus*	2.8
					0–2 *M. gracilis*	1.3
#San Quintín, exposed rocky shore (exploited)	*M. californianus*	30	60.12	7.23	0–3 *M. gracilis*	1.7
#San Quintín, sandy and protected	*M. galloprovincialis*	15	57.89	8.12	0–2 *M. gracilis*	1.5

[a] Mussel mean length (n = number of mussels examined). SD, range of copepod species per sample, and mean number of copepod species per infested mussel are shown.

As we found, Do and Kajihara (1986) also detected ovigerous females and adult males of *P. spinosus* throughout the year in *Mytilus edulis galloprovincialis* Lmk. In accordance with those authors, we found a relatively constant prevalence of this parasite through the year. Do and Kajihara (1986) estimated that at least five to six generations of *P. spinosus* can coexist in a year. They reported a higher prevalence and mean number of parasites per mussel (85% and 2.4, respectively) than in this study.

The exact pathologic effects attributable to several parasitic copepods have remained largely uncertain (Do and Kajihara 1986). In the case of a notorious, intestinal copepod, *Mytilicola intestinalis* Steuer, Cole and Savage (1951), Sparks (1962), Dare (1981), and Paul (1983) have recorded the adverse effects of this parasite on the condition of mussels, *M. edulis;* the European oyster, *Ostrea edulis* L; and the Japanese oyster, *Crassostrea gigas* Thunberg. In the case of *P. spinosus*, only Dinamani and Gordon (1974) reported the mechanical and pathologic effects in the gut epithelium of the rock oyster, *Crassostrea glomerata* Gould, but no definite evidence of damage to the gills or labial palps was found in any oysters examined (Do and Kajihara 1986). Although we did not find any macroscopical or microscopical damage in the gills, labial palps, and mantle of the host, the CI of mussels from the Ensenada Pier, where copepods were very abundant, was the lowest of all mussels studied throughout the year. It is possible that damage to the host cannot be detected by macroscopical and microscopical studies of the gills, labial palps, and mantle, but that it may be assessed at the physiologic level or by studying the gut of the host, where this copepod may be found (Korringa and Lamberg 1951). The reduction in the CI in heavily infected mussels for *P. spinosus* represents a potential thread to mussel culture if the *P. spinosus* population increases under culture conditions. The transfer of mussels from infested areas to noninfested culture areas must be avoided.

The occurrence of both *P. spinosus* and *M. gracilis* in the same host (*M. californianus* or *M. galloprovincialis*) indicates that both copepod species may coexist. The presence of the different copepod species in the same host has been observed previously in populations of *M. edulis* in Newport Bay (Ho pers. comm.).

The number of copepods seems to be related to larger organisms. Similar observations on this relation have been recorded by Costanzo and Calafiore (1987) in *Modiolicola insignis* Aurivillius; they pointed out that smaller mussels (under 33-mm shell length) were likely to escape infestation. Further studies are being carried out in order to determine the reasons for the differential prevalence in the host mussels and under environmental conditions and to determine whether *P. spinosus* and *M. gracilis* are found in the gut of *M. californianus* and *M. galloprovincialis* and are producing any lesions.

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MICROGEOGRAPHIC VARIABILITY IN FEEDING, ABSORPTION, AND CONDITION OF MUSSELS (*MYTILUS GALLOPROVINCIALIS* LMK.): A TRANSPLANT EXPERIMENT

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ABSTRACT Mussels, *Mytilus galloprovincialis* Lmk., were reciprocally transplanted between three cultivation rafts in the Ría de Arousa (Galicia, northwest Spain). After an 8-wk period, rafts were visited and individual measurements of clearance rate, absorption efficiency, and condition index were performed in the field under ambient conditions of temperature, salinity, and food availability. Clearance rate standardized to a common shell length was not significantly affected by the location of mussels in the Ría but varied according to their origin. Absorption efficiency was mainly affected by raft position, reflecting the spatial variability in the quality of available food. Origin-related differences in absorption efficiency showed the same trend as recorded for clearance rates. No gradient of environmental factors has been recorded in the Ría that might account for differences in physiologic parameters persistent after transplantation (i.e., origin effects). Associated evidence suggests that observed differences may have resulted from spatial variability in the degree of parasitic infestation. Condition index was dependent on both raft position and mussel origin and closely reflected the described variability in physiological parameters. Remaining variability in condition index can be attributed to different conditions before transplantation took place.

KEY WORDS: Feeding, absorption, condition, transplant, *Mytilus*

INTRODUCTION

The rate of feeding and absorption efficiency (AE) constitute the physiologic parameters controlling energy intake in most animal groups. Marine bivalves exhibit great variability in these parameters in response to different types of factors, both environmental and endogenous. Although rates of feeding are primarily dependent on the availability of food resources, relationships between ingestion rate and particle concentration are mediated by clearance or pumping rate, and this has been found to depend on a variety of environmental factors (see review by Bayne and Newell 1983, Hawkins and Bayne 1992). Absorption efficiency is mainly affected both by the concentration (negatively) and quality (positively) of suspended food matter (Bayne and Newell 1983, Bayne *et al.* 1987, Bricelj and Malouf 1984, Navarro *et al.* 1991, Navarro *et al.* 1994).

Variability in parameters of energy gain has been interpreted in some instances as an adaptation to the specific feeding conditions characteristic of the environment where mussels live. Such adaptations have been evidenced, for example, by seasonal cycles of clearance rate (CR) and AE measured in the laboratory under a standardized feeding regimen (Hawkins *et al.* 1985) or by differences in the CR of individuals from populations inhabiting media characterized by very different seston concentrations and compositions (Theissen 1977, Bayne et al. 1984, Bayne et al. 1987). Another important factor affecting growth potential has been recently reviewed by Newell and Barber (1988) and refers to the negative effect of diseases and parasites on parameters controlling energy acquisition. Although studies dealing with this aspect are scarce, preliminary results have begun to provide the physiological basis for understanding the deleterious effect that diseases and parasitic infection exert on growth rate and reproductive potential. In addition, genetic factors have been shown to influence physiological variability between individuals of the same population (Koehn and Shumway 1982, Hawkins et al. 1986). However, in transplant experiments designed to simultaneously test the effects of genetic and environmental factors on growth rates, local environmental conditions affected the major proportion of recorded differences (Dickie et al. 1984, Mallet and Carver 1989), but see Peterson and Beal (1989) for a somewhat different result.

When trying to quantify the extent to which observed differences in the physiological components of energy balance in mussels from different locations are dependent on environmental factors, reciprocal transplantations appear to be the best-suited procedure. Widdows et al. (1984) compared the seasonal cycle in energetic parameters of native and reciprocally transplanted mussels, *Mytilus edulis*, from two populations. After 2 mo, the clearance rate of transplanted mussels differed by only 20% from those in native mussels, but the remaining differences persisted even after another 3 mo. Authors suggested that either full acclimation needed longer periods of time, or that some genotypic component was responsible for the residual difference. In contrast with these results, Okumus and Stirling (1994) have recently observed that physiological differences between mussels cultivated at two Scottish sea lochs disappeared after a 4.5-mo period of reciprocal transplantation.

In a previous study (Navarro et al. 1991), we reported differences in parameters of feeding and absorption for mussels (*Mytilus galloprovincialis*) growing on different sites in the Ría de Arosa. This study describes transplant experiments that were undertaken to discriminate between alternative causes of these differences, associated either with the present environment or with different origins. Mussels cultivated in rafts at three sites were reciprocally transplanted, and after 8 wk, CR and AE were measured in the field under ambient conditions of temperature, salinity, and food availability. The degree of persistence of physiological differences after transplantation was assumed to provide some insight on the nature of those differences. Condition index was also determined to provide an independent measure of physiological status.

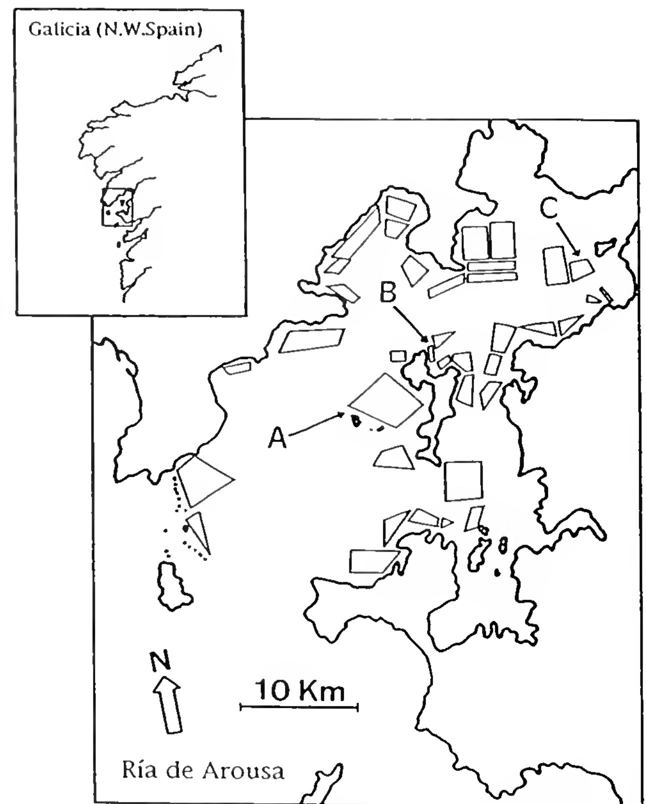

Figure 1. Detailed map of the Ría de Arousa showing the sites where rafts were located. Squares denote raft groupings.

MATERIAL AND METHODS

Animals, Sites and Transplantations

Experiments described in this study were performed in November 1989. According to previous information on the physiological behavior of cultured mussels (*M. galloprovincialis* Lmk.), three rafts located in the Ría de Arosa (Galicia, northwest Spain) were chosen for this study (see Fig. 1). Raft A was moored at the head of a huge grouping (141 rafts) located in an area of maximum oceanic influence within the Ría. Raft B belonged to a small grouping (16 rafts) close to the Isla de Arosa, a zone that has been identified by Otto (1975) as an upwelling area characterized by high values of primary production. Raft C was located in a grouping (40 rafts) positioned within the inner part of the estuary, where oceanic influence is minimal.

Eight weeks before physiological measurements, 150 individual mussels were randomly sampled from a rope located in the central part of the front of each raft and were divided into three groups that were each placed within a separate net bag. One bag remained immersed in the raft of origin, and the other two were transplanted, one to each of the other two rafts. After this procedure, 50 mussels of each origin (Rafts A, B, and C) remained immersed under the same environmental conditions in each of the three rafts (A, B, and C) during the transplant period. Bags were located in the central part of the front of each raft and at a 1-m depth. At the end of the transplant period, rafts were visited on consecutive days and measurements were performed under ambient conditions. Temperature and salinity remained similar between rafts at the time when physiological determinations were made (mean temperature = 15.37 ± 0.52°C; mean salinity = 35.2 ± 0.43‰) (±1 SD).

Procedures

Twelve mussels were taken from each bag and arranged in individual trays within a feeding tank. Water collected from the same depth where mussels had previously been hung was pumped to the feeding tanks (one per origin) through multiple inflow lines. This arrangement was designed to avoid gradients of seston concentrations into the tanks. Flow rate was kept above 2.5 L/min because at this rate, differences in seston concentration between tanks caused by variable filtering rates were calculated to be negligible.

After 30 min of acclimation to flow-through conditions, duplicate 2-L seawater samples were collected at hourly intervals for 4 h. Two hours after the first water sample was taken, the tanks were cleaned and the feces produced by each individual mussel were completely collected on three occasions over the following 4 h.

Measurements

Seawater samples and aliquots of known volumes from each fecal sample were filtered onto preashed (450°C for 4 h) and weighed GFC filters and rinsed with isotonic ammonium formate. Total dry matter was established as the weight increment determined after drying the filters to constant weight at 110°C. Organic matter corresponded to the weight loss after ignition at 450°C in a muffle furnace. After this procedure, total particulate matter (TPM, mg/L), particulate organic matter (POM, mg/L), and particulate inorganic matter (PIM, mg/L) were determined within the water passing through each feeding tank.

The egestion rates of inorganic matter (mg/h) were determined as means of triplicate fecal samples collected from each individual

mussel and were assumed to represent inorganic ingestion rate (i.e., that there was no absorption of ash in the digestive tract). CR (L/h) were then estimated indirectly, with PIM concentration (mg/L of seawater) as the reference for available inorganic matter. The validity of this procedure for estimating CR has been recently tested in both cockles (Urrutia et al. 1996) and mussels (A. J. S. Hawkins unpublished results) and has been used for measuring CR in mussels under ambient conditions of food availability (Hawkins et al. 1996).

AE was measured by the ratio method of Conover (1966), on the basis of organic fractions of food and feces. The same three replicate samples used for measuring egestion rates were used to determine the mean absorption efficiencies for each individual.

After measurements were completed, the soft tissues of each mussel were excised, dried at 85°C, and weighed to the nearest mg (DTW, in grams). After ignition to constant weight at 450°C in a muffle furnace, ash-free dry tissue weight (AFDTW, in grams) was also determined. The length (L) of each mussel shell was measured to the nearest millimeter, and dry shell weight (DSW, in grams) was measured after drying in an oven to constant weight. Condition index (CI) was then calculated for each mussel as:

$$CI = 100 \times AFDTW/DSW.$$

Size Standardization and Statistical Analysis

In general, physiological rates need standardization to eliminate size-dependent variability. The most commonly used reference for size is soft body mass; however, the weight standardization of CR may be somehow arbitrary because this rate is considered to be dependent on filtration (gill) area (Hughes 1969). To obviate problems derived from possible variations of gill area per unit body weight in mussels from different origin, the measurements of CR presented here were standardized to both body mass and shell length.

Mass-specific (1 g) CR was obtained according to the formula:

$$CR_s = CR_e \times (1/W_e)^b,$$

where CR_s is the CR of the standard-sized mussel, CR_e is the uncorrected CR, 1 is the standard mass (= 1 g), W_e is the mass of the experimental mussel, and b is the power that scales CR with the body mass. In this work, we have used a value b = 0.53, estimated by Pérez and González (1984), for *M. galloprovincialis* from the Ría de Arosa. For shell length standardization we used the formula:

$$CR_s = CR_e \times (80/L_e)^b,$$

where CR_s and CR_e are as in the former expression, 80 is the standard length (= 80 mm), L_e is the length of the experimental mussel, and b (= 1.85) is the power that scales the CR with the shell length for mussels from Arosa (Pérez and González 1984).

Standard analysis of variance (ANOVA) procedures (Zar 1984) were applied to test the simultaneous effect of raft location (Raft) and raft of origin (Origin) on the parameters measured in this work: CR, AE, and CI.

RESULTS

Seston Characteristics

Parameters describing the characteristics of seston at the three rafts are presented in Table 1. Recorded TPM values are well below those found for the same rafts in the previous spring: from 1.065 mg/L in raft 6 (raft A in this work) to 2.180 mg/L in raft 4 (raft C) (Navarro et al. 1991). Because no data on seasonal changes in seston concentration are available, we cannot conclude whether these differences are due to a seasonal cycle or whether they are merely the consequence of short-term variability in this parameter. However, the organic contents of the seston were very similar to values previously obtained on different occasions for seven rafts within comparable areas of the same estuary (Cabanas et al. 1979, Navarro et al. 1991), and which reflects a remarkable spatial and temporal constancy of this parameter for the Ría de Arosa.

Although no direct measurements of particulate volumes were performed on seston samples taken in this work, approximate estimations of this parameter were obtained by transforming recorded TPM (in milligrams per liter) to volume (VOL, in cubic millimeters per liter) equivalents using the equation:

$$VOL = 0.4006 + 0.5005 \times TPM \quad (r^2 = 0.832, \quad n = 12, \quad p < 0.001),$$

which was fitted to previous simultaneous determinations of VOL and TPM (Navarro et al. 1991). Food quality was then calculated as Q = milligrams of POM per cubic millimeter, mean values (X ± SD) for which were as follows: Raft A, 0.543 (±0.066); Raft B, 0.484 (±0.044); and Raft C, 0.361 (±0.056).

CR

Mass-specific CR are presented in Figure 2A (X ± 95% confidence interval), and results of ANOVA are in Table 2. Both Raft and Origin exert significant effects on this parameter. However, a comparison of the F values suggests that, among the factors considered here, the main influence was Origin. Maximum and minimum CR corresponded to mussels from origins B and C, respectively.

When standardization is performed for a common length (Fig. 2B), the trend observed for different Origins remains as described for mass-specific CR. Nevetheless, this factor only explains 37% of the recorded variance in CR (Table 3). The effect of Raft,

TABLE 1.

Seston characteristics recorded in the course of physiological measurements.[a]

RAFT	TPM (mg/L)	POM (mg/L)	PIM (mg/L)	*f*
A	0.702 ± 0.379	0.408 ± 0.255	0.294 ± 0.132	0.568 ± 0.070
B	0.871 ± 0.076	0.405 ± 0.054	0.466 ± 0.052	0.465 ± 0.042
C	0.532 ± 0.041	0.241 ± 0.031	0.291 ± 0.022	0.451 ± 0.070

[a] f, organic content (decimal fraction). Data are means ± SD.

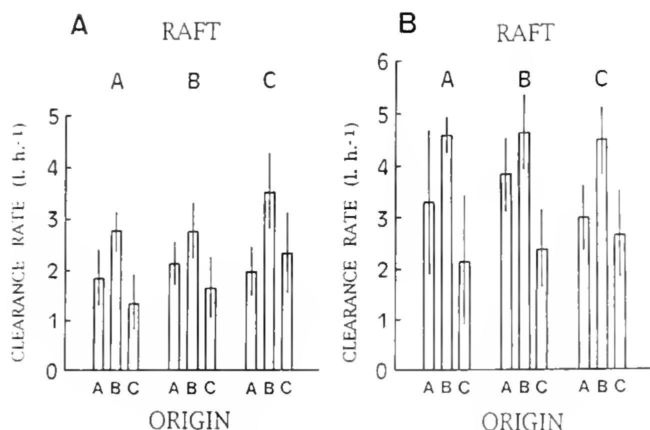

Figure 2. CR of mussels transplanted from origins A, B, and C measured at rafts A, B, and C. (A) Mass-specific (1 g) CR. (B) Length-specific (80 mm) CR. Vertical lines represent 95% confidence interval.

however, becomes nonsignificant, which reflects the higher mass-specific CR of mussels with lower length-specific mass (refer to Discussion). It is worth noting that, in both ANOVA, interaction terms were found not to be statistically significant. Thus, although effect of Raft position in the Ría appears doubtful, the effect of Origin is a significant phenomenon, revealing that either genetic differences or the previous history of mussels elicites fundamental physiological effects.

AE

Mean AE values for each transplant condition and corresponding ANOVA are shown in Figure 3 and Table 4, respectively. Both Raft and Origin exert significant effects on AE. In this case, Raft is responsible for the higher proportion of recorded variance (59%), reflecting the fact that AE is highly affected by the environmental conditions prevailing in the area where rafts are located. Nevertheless, the effects of Origin were also highly significant, although this factor only explains 9% of observed variance. Even more remarkably, origin-dependent differences in AE exhibit the same trend as described for CR data: the maximum AE corresponding to mussels from origin B and the minimum AE corresponding to those from origin C. As for CR, the interaction term was found to be not significant.

In Figure 4, we have plotted mean AE values against the seston qualities estimated for each raft from TPM recorded at the time of physiological determinations. Similar measurements obtained in the previous spring for the same three rafts are included for comparison, together with an exponential curve that had previously been fitted to data from mussels of Arousa (Navarro et al. 1991).

TABLE 2.

Summary of ANOVA for testing significance of differences among mass-specific CR.

Factor	d.f.	SS	MS	F	p
Raft	2	6.996	3.498	4.003	0.021
Origin	2	32.090	16.045	18.359	0.001
Interaction	4	3.814	0.954	1.091	0.365
Error	99	86.520	0.874		

SS: sum of squares, MS: mean squares, F: F value.

TABLE 3.

Summary of ANOVA for testing significance of differences among length-specific CR.

Factor	d.f.	SS	MS	F	p
Raft	2	1.370	0.685	0.482	0.617
Origin	2	87.057	43.529	30.656	0.001
Interaction	4	4.479	1.120	0.789	0.535
Error	99	140.573	1.420		

The AE values presented here and the included curve follow a similar trend, suggesting that raft-dependent variability in this parameter results from existing differences in the quality of suspended particulate matter available to the mussels from each raft. On the other hand, once the effect of variability in food quality is removed, differences in AE associated with Origin alone remain of similar magnitude to those recorded in the previous spring.

Condition Index

Large differences in CI appear associated with both Raft and Origin (Fig. 5 and Table 5), the interaction term being nonsignificant as in previous physiological determinations. The effect of Raft accounts for most variability in CI (27%), revealing a clear gradient between the inner (raft C, CI = 12.75) and outer (raft A, CI = 18.53) zones of the Ría. Although not as great as the effect of Raft, a highly significant influence is also exerted by Origin (Table 5), which explains 20% of recorded variability in CI.

DISCUSSION

Effect of Raft

The effects of Raft on CR were not apparent when standardized for a common shell length, but were significant when standardized

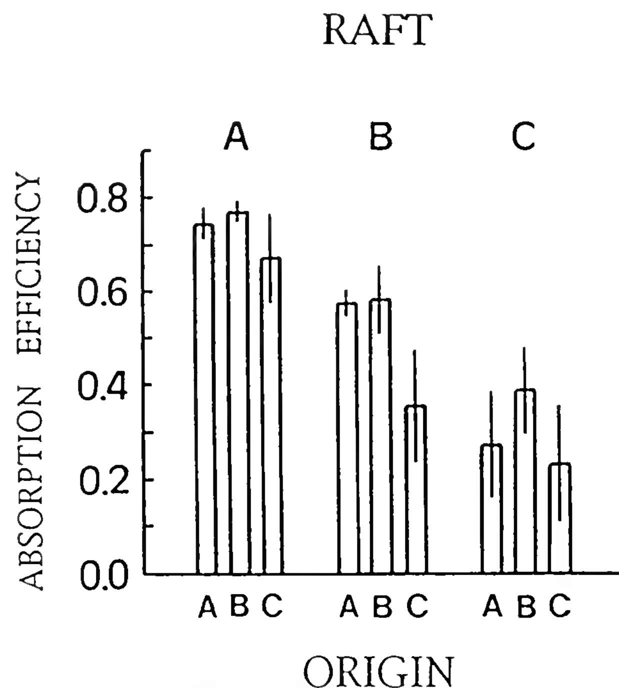

Figure 3. AE of mussels transplanted from origins A, B, and C measured at rafts A, B, and C. Vertical lines represent 95% confidence intervals.

TABLE 4.

Summary of ANOVA for testing significance of differences among AE.

Factor	d.f.	SS	MS	F	p
Raft	2	3.170	1.585	98.211	0.001
Origin	2	0.499	0.249	15.452	0.001
Interaction	4	0.102	0.025	1.578	0.186
Error	99	1.598	0.016		

to soft-body mass. We suggest that the apparent effects of Raft on mass-specific CR do not represent true differences in the feeding behavior of mussels in different areas of the Ría de Arousa.

As bivalves grow bigger, an increasing proportion of produced biomass is allocated into energy stores and reproductive products (this is evidenced by increasing reproductive effort with size or age; Bayne et al. 1983, Peterson 1983, Peterson 1986, Thompson 1984, Iglesias and Navarro 1991). Thus, large variations in total body mass may occur between individuals with structural organs (shell, gills, etc.) of similar size. This appears to be true in this study: the individuals presented here ranged from 68 to 89 mm in shell length (mean, 78 mm), which is close to the maximum size attainable in the Galician Rías (90–110 mm). Further, during the autumn, a season characterized by comparatively low rates of shell growth (Aguirre 1979, Pérez and Román 1979), the mussels used in this study showed dry masses that varied from 698 to as much as 4,031 mg (mean, 2,093) and that correlated poorly with shell length ($r^2 = 0.318$ for the log-log relationship). Under these circumstances, of such variable growth of soft tissues, standardization to a common dry-flesh weight may result in misleading estimations of CR, so that the differences observed among rafts would be spurious. In particular, significant higher mass-specific CR in mussels transplanted to raft C probably resulted from their lower dry weights for similar shell sizes.

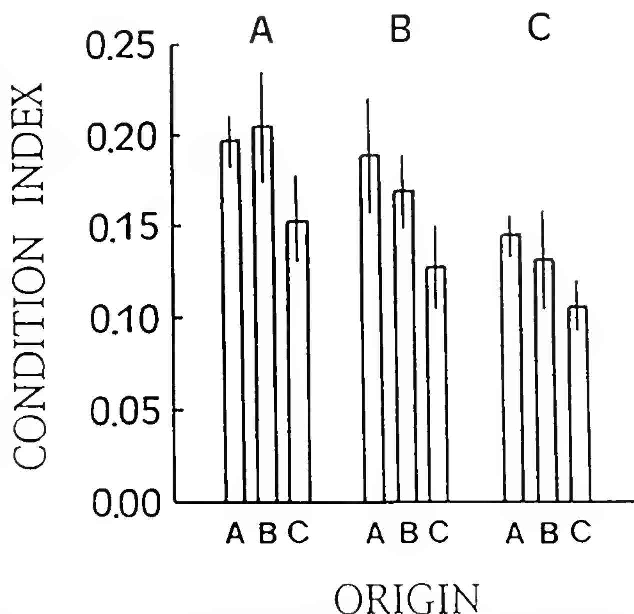

Figure 5. CI of mussels transplanted from origins A, B, and C and measured at rafts A, B, and C. Vertical lines represent 95% confidence interval.

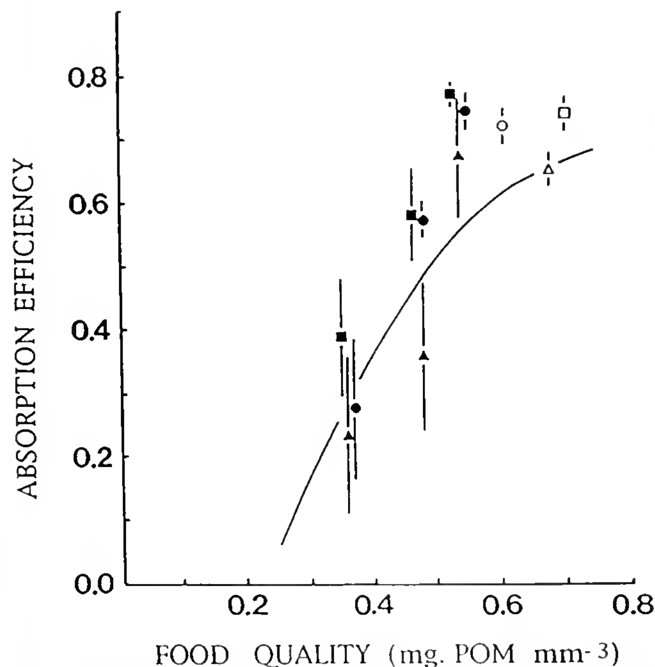

Figure 4. AE as a function of food quality (q) recorded at sites of rafts A (q = 0.543), B (q = 0.484), and C (q = 0.361). Points are mean values ±95% confidence interval. Circles, Origin A; squares, Origin B; triangles, Origin C. Solid symbols, data obtained in these experiments; hollow symbols, AE recorded in the previous spring (April 1989) for mussels from sites A, B, and C (Navarro et al. 1991). The equation of the curve: $AE = 0.807\ (1 - e^{-3.604\ (q\ -\ 0.252)})$ (q, food quality) has been taken from the same previous work (Navarro et al. 1991).

AE was influenced by raft position. As documented in previous studies with species of *Mytilus*, AE changes in response to short-term variations in the quality of suspended matter, expressed as POM availability per unit particulate volume, both in the laboratory (Bayne et al. 1987) and in the field (Navarro et al. 1991). The curve shown in Figure 4 illustrates this dependence for mussels from the Ría de Arousa. Points included in the figure that represent mean AE for each transplant condition agree reasonably well with the curve. Therefore, we can conclude that differences associated with Raft are the consequence of different food qualities occurring at each raft when experiments were performed.

Raft-dependent variability in CI was highly significant. As dis-

TABLE 5.

Summary of ANOVA for testing significance of differences among CI.

Factor	d.f.	SS	MS	F	p
Raft	2	600.862	304.431	26.319	0.001
Origin	2	462.458	231.229	19.991	0.001
Interaction	4	42.529	10.632	0.919	0.456
Error	99	1145.118	11.567		

cussed above, a major proportion of energy retained by the specimens used in this study is very likely devoted to storage reserves or reproductive products. Therefore, recorded differences in CI may have resulted from different growth rates achieved by mussels in each zone of the Ría. Certainly, a close corespondence between growth rate and CI has been previously reported for mussels grown in cultured plots (Smaal and Van Stralen 1990). Because CR per unit shell length remained independent from Raft, maximum growth rates (assumed to be represented by CI) corresponding to Raft A and minimum to Raft C must have resulted from increasing food availability and/or quality with geographic position from inner to outer areas of the Ría. This interpretation is supported by associated changes in AE, suggesting that differences in food quality, through its effect on AE, can be considered as the main factor that was responsible for recorded variability in CI.

Effect of Origin

CR is highly affected by Origin, irrespective of the standardization procedure used, and differences associated with this factor present the same trend of variation imposed by Raft: higher CR values for origin B and lower for origin C. Although AE is mainly dependent on raft position, Origin also exerted a significant influence, mean values of AE showing the same trend as that described for CR.

Spatial variability of temperature or salinity is small in Arousa (Landin 1987). Nutritional conditions known to modify physiological traits of mussels (Bayne et al. 1984, Bayne et al. 1987), especially food quality, may be more variable among rafts. However, mussels adapt to new nutritional conditions within much less than 2 mo (Hawkins and Bayne 1992). Therefore, the persistent effect of Origin indicates the importance of endogenous factors, which may include the following influences: (a) genotype; (b) long-lasting environmental effects; (c) parasitism.

Although the parentage of mussels used in this work cannot be ascertained, genetic influences seem highly unlikely. The probability that different transplant groups came from different genetic stocks can be assumed to be negligible, because most farmers in the Ría de Arousa position their seed ropes on the same area at the rocky shore near the mouth of the Ría.

Long-lasting environmental effects mediated through physiologic changes in early life history have been invoked by Peterson and Beal (1989) to explain origin-dependent variability in the growth rates of *Mercenaria mercenaria*. This possibility must not be discarded and is currently being tested in mussels from the Ría de Arousa.

Alternatively, parasitization is well known to alter the physiological status of bivalves (Newell and Barber 1988). In the Ría de Arousa, variable degrees of infestation by parasites have been reported among cultivated mussels (Figueras et al. 1991). Further, Villalba et al. (1993) examined the degree of infestation of mussels by *Marteilia refringens*, a protistan infecting the stomach and digestive diverticula, during 1988 and 1989 in the raft groupings where our rafts A and C were located. According to their results, there is a gradient, from high prevalences in the inner area (site of our raft C) to lower prevalences in the vicinity of Arousa Island (groupings of rafts A and B).

According to Figueras et al. (1991), when invading the stomach and digestive diverticula, *Marteilia* causes tissue disruption in the stomach wall and hemocytic infiltration into the digestive gland. These alterations may, obviously, disturb the normal process of food adsorption, which could be the reason for lower absorption efficiencies in mussels from origin C compared with A and B. Given that *Marteilia* does not damage the filtering system, it is possible that lower CR may constitute a response to reduced functional digestive capacity. Other indirect evidence comes from data obtained by Bayne et al. (1979), who recorded reduced CR at high temperatures in mussels whose digestive systems were infected by the parasite copepod *Mytilicola intestinalis*. In addition, reduced CR in oysters occur with infection by the endoparasite *Haplosporidium nelsoni* (Newell 1985). In conclusion, parasitism may help to explain the persistence of observed differences in AE and CR, but this needs future confirmation.

The effect of Origin on CI can be attributed to two different sources of variation: First, the condition of mussels before transplant took place can account for a certain proportion of observed differences, and second, as a result of the effect of Origin on parameters of energy gain, when exposed to the same environmental conditions, mussels from different origins would have experienced different growth rates. We shall deal with this aspect in the next section.

Relationship Between CI, Feeding, and Absorption

Figure 6 shows CI values for each transplant condition plotted against their corresponding AE. Although these two parameters appear correlated ($r^2 = 0.80$; $p = 0.001$) in the pooled set of data, more information can be gained by considering a particular relationship for each different origin. In each of the three cases, CI seems to be an almost linear function of AE, implying that differences in AE induced by food quality account for a great proportion of raft-dependent variability in growth rates. Alternatively, differences in food availability between rafts were of minor relevance.

Another interesting aspect of the relationships presented in Figure 6 is evident when comparing differences between the slope and the elevation of the fitted lines. The slopes of these lines seem to be related to the mean CR obtained for each Origin: steeper slope appears to be associated with the lowest CR for Origin C. This is as expected, because the degree of dependence of CI on AE must

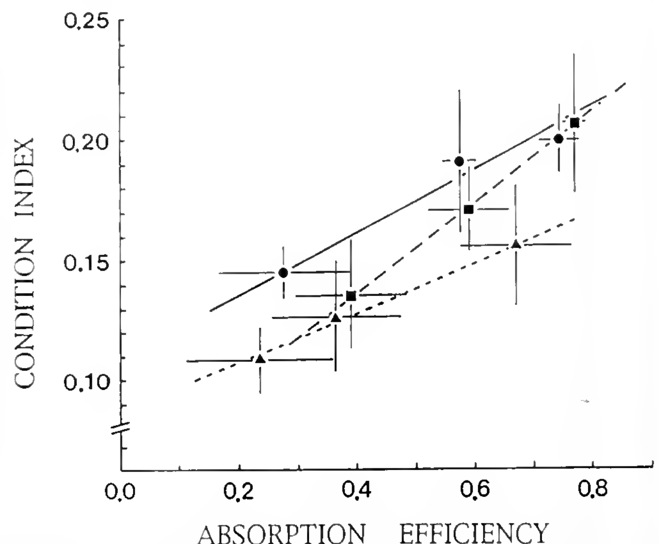

Figure 6. CI plotted as a function of AE (mean values ±95% confidence interval). Lines were fitted by eye to points corresponding to the same origin. Circles, Origin A; squares, Origin B; triangles, Origin C.

itself be a function of the rate of feeding, which under the same feeding regimen, can only be a function of the pumping activity. On this basis then, we suggest that the remaining fraction of origin-dependent variability in CI not explained by parameters of energy gain (i.e., differences in elevation of lines) may be attributed to different CI of mussels from each origin before the transplantation took place.

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FACTORS AFFECTING THE DISTRIBUTION AND ABUNDANCE OF *MYTILICOLA ORIENTALIS* (COPEPODA) IN THE MUSSEL, *MYTILUS TROSSULUS*, IN BARKLEY SOUND, B.C.

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ABSTRACT The copepod, *Mytilicola orientalis*, was found in 0–80% of summer-collected samples of mussels (*Mytilus trossulus*) from two sheltered locations in Barcley Sound, on the western coast of Vancouver Island. It was absent in mussels from two adjacent, wave-exposed locations. Copepod abundance at infected sites was 2 ± 5, which is comparable to that at other sites along the Pacific Coast where this species has been reported. At the most heavily infected location, copepod abundance was highest in the largest mussels (25–35 mm), particularly those collected near the low-tide mark. Mean abundance increased as average host size increased but was not associated with host density. The restricted regional distribution of this copepod in Barcley Sound (and throughout the Pacific Northwest) may be limited by factors that confine transmission to sheltered, muddy estuaries. Within such sites, copepod abundance is highest in large mussels collected near the low-tide mark.

KEY WORDS: copepod, blue mussel, *Mytilicola orientalis*, *Mytilus trossulus*, marine parasite, population biology

INTRODUCTION

Mytilicola orientalis (Mori, 1935) is a copepod that infects the intestine of bivalves, especially the mussel, *Mytilus trossulus* (Gould, 1850), on the Pacific Coast of North America. The sporadic distribution of this copepod along the coast between southern California and Vancouver Island is thought to reflect the spread of infective stages from imported Japanese oysters, *Crassostrea gigas* (Wilson 1938, Bernard 1969, Bradley and Siebert 1978). Although the congener, *Mytilicola intestinalis*, has been intensively studied in European mussels (review by Davey 1989), *M. orientalis* has received little attention. Presumably, its life cycle is similar to that of *M. intestinalis* and involves free-living stages (two naupliar and one infective copepodite) and parasitic adult stages (Gee and Davey 1986). In this study, we document the distribution of *M. orientalis* in mussels from several localities in Barkley Sound, on the western coast of Vancouver Island, British Columbia. On the most heavily infected mussel bed, we also examine how position on the shore (with respect to the tidal cycle), host size, and host density affect copepod abundance.

MATERIALS AND METHODS

The taxonomy of smooth-shelled *Mytilus* is problematic. Previously, most authors considered common mussels in temperate waters to be *Mytilus edulis*. However, McDonald et al. (1991) used genetic and morphological evidence to show that smooth-shelled *Mytilus* of the North Pacific are *M. trossulus* and that *M. edulis* is primarily restricted to western Europe and eastern North America.

Four localities were selected within Barkley Sound and sampled between August 4 and 8, 1994. Two localities (Grappler Inlet and Bamfield Inlet) were sheltered from wave action and had muddy substrata. The other two were at the more wave-exposed mouth of each inlet and had rocky/sandy substrata. At least 10 size-matched mussels (20–30 mm) were collected haphazardly from each of three sites within each locality. The three sites were separated horizontally along the shoreline by at least 40 m. Mussels were returned to the laboratory and were kept alive in flow-through aquaria for a maximum of 24 h before dissection.

We sampled intensively at the most heavily infected locality (Grappler Inlet) to examine the effects of tidal emersion, host density, and host size on copepod prevalence and abundance. To examine the effects of tidal emersion, two 60-m transect lines were placed from the high-tide mark to the low-tide mark. The shore was gently sloping, and the tidal height difference between the top and bottom of the transect line was approximately 1 m. Both transect lines were placed perpendicular to the shoreline and were approximately 40 m apart. The first 10 mussels (25–35 mm) encountered at 0 (high-tide mark), 30, and 60 m (low-tide mark) along the transect line were collected.

Mussel density was estimated at Grappler Inlet with 0.25 m^2 quadrats. First, two 10-m^2 sites on the mussel bed were demarcated with a transect line. The two sites were separated horizontally by approximately 5 m, and there were no obvious differences in emersion period between them. Five quadrats were then placed randomly within these two sites following the procedures of McGrorty et al. (1990). All mussels larger than 5 mm within each quadrat were placed in a bag, returned to the laboratory, counted, and measured for maximum linear shell length (in millimeters). Ten mussels (25–35 mm) from each quadrat were dissected for copepod abundance.

For each host selected for examination, the maximum shell length was recorded before severing the adductor muscle. The effect of host size on copepod abundance was determined by regressing shell length on copepod abundance for all mussels collected from the 10 quadrats on Grappler Inlet. A subsample of 10 small hosts (smaller than 25 mm) was haphazardly selected from

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the total sample (collected from the 10 quadrats) to provide abundance data within the size range of mussels on Grappler Inlet.

Initially, we examined the entire gastrointestinal tract for immature and adult stages of copepods. However, in contrast to Gee and Davey (1986), we did not find immature copepods in the stomach. We therefore limited further examinations to the intestine and rectum. These regions of the alimentary tract were compressed between two glass Petri dishes and examined for copepods under a dissecting microscope.

The effect of tidal exposure on copepod abundance was analyzed by analysis of covariance (ANCOVA), with size of the host as the covariate. For this analysis, copepod abundance data were log transformed. The effects of host size and host density on copepod abundance were analyzed by correlation.

RESULTS

Mytilicola orientalis was only present at the two sheltered, muddy locations within Barkley Sound (Table 1). No copepods were found in either of the wave-exposed locations. Prevalence ranged from 0 to 70% for samples of mussels collected from Bamfield Inlet to 10 to 80% for Grappler Inlet. Overall, copepod intensity (not including uninfected hosts) was 2.3 ± 1.2 in the 80 infected mussels collected from these two localities.

There was a significant increase in mean copepod abundance with increased distance from the high-tide mark (Table 2; Fig. 1). On average, mussels collected from the low-tide mark had 75% higher mean abundance than those collected from the high-tide mark. In data pooled between the two transects, Scheffe's post-hoc comparison showed that mean abundance differed between samples collected at 0 and 60 m but not between pairs of samples collected at other distances.

The density of mussels larger than 5 mm ranged between 16 and 76 mussels/0.25 m^2 in Grappler Inlet. However, variation in host density was not associated with variation in copepod abundance ($n = 10$, $r = 0.288$, $p > 0.05$), but there was a significant positive correlation between copepod abundance and mean mussel size (Fig. 2; $n = 10$, $r = 0.943$, $p < 0.05$). The importance of host size is also shown by the moderately significant correlation between copepod abundance and the numbers of mussels within a quadrat that were >30 mm in length ($n = 10$, $r = 0.618$, $0.01 < p < 0.05$). Mean copepod abundance differed between the two 10-m^2 areas within Grappler Inlet (Fig. 2; $F_{1,9} = 44.9$, $p < 0.001$). This difference in copepod abundance may be due to differences in position with respect to tidal exposure, to differences in average mussel size ($F_{1,9} = 44.9$, $p < 0.001$), or to a combination of both factors.

No copepods were found in mussels smaller than 19 mm (Fig. 3). However, for mussels larger than 19 mm, copepod abundance increased exponentially ($\log y = -0.83 + 0.71 \log x$; $n = 92$, $r = 0.41$, $p < 0.01$). Mussels larger than 30 mm made up only 9.5% of the 358 mussels collected at random from the mudflat; however, these hosts contained 70.4% of the total number of copepods.

TABLE 2.

Summary statistics of ANOVA for the effect of distance from high-tide line on the abundance of *M. orientalis* in mussels from Grappler Inlet.[a]

Source	DF	MS	F	p
Covariate	1	0.213	4.678	0.035
Transect	1	0.018	0.397	0.531
Distance	2	0.240	5.295	0.035
Transect × Distance	2	0.132	2.916	0.063
Residual	53	0.045		

[a] Main effects were adjusted for the effect of the covariate (log host size). DF: degrees of freedom, MS: mean square.

DISCUSSION

The distribution of *M. orientalis* in mussels from Barcley Sound appears to be restricted to hosts from sheltered, muddy estuaries. Such a restricted regional distribution is similar to results from other reported studies of *M. orientalis* along the northern Pacific Coast (Bernard 1969, Bradley and Siebert 1978). The

TABLE 1.

Prevalence and intensity of *M. orientalis* in 25- to 30-mm *M. trossulus* from four locations in Barkley Sound, Vancouver Island, British Columbia.

Locality	Site[a]	n	Prevalence (%)	Mean (±SD) Intensity	Range
Bamfield Inlet[b]	A	10	0	0	0
	B	22	27	1.2 ± 0.4	0–2
	C	10	70	1.1 ± 0.4	0–2
Grappler Inlet[b]	A	10	60	1.5 ± 0.8	0–3
	B	10	20	3.5 ± 0.7	0–4
	C	10	80	3.4 ± 2.3	0–8
Santa Maria[c]	A	10	0	0	0
	B	10	0	0	0
	C	10	0	0	0
Dixon Island[c]	A	10	0	0	0
	B	10	0	0	0
	C	10	0	0	0

[a] Letters refer to three sites sampled within each locality.
[b] Sheltered locality.
[c] Exposed locality.

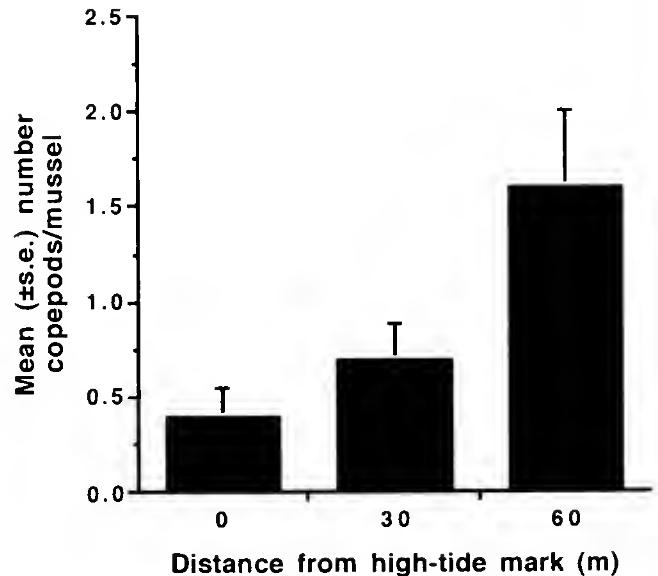

Figure 1. The relationship between distance along a tidal gradient and mean copepod abundance in mussels collected from Grappler Inlet.

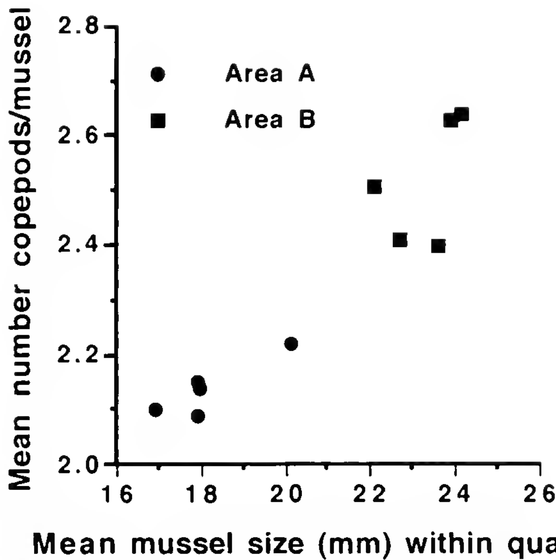

Figure 2. The relationship between the average size of mussels within 10 0.25-m^2 quadrats in Grappler Inlet and mean copepod abundance. Five quadrats were placed in one 10-m^2 area (A) of the mussel bed, and five were placed in an adjacent 10-m^2 area (B). Symbols indicate means calculated from 10-, 20-, and 30-mm mussels collected from within each quadrat.

congener, *M. intestinalis*, is also restricted to mussels from sheltered, muddy estuaries in northern Europe (review by Davey 1989).

Several factors may determine this restricted distribution. The colonization potential of infective larvae will play a role, as it does in other parasite/host systems (review by Kennedy 1993). Details of the infection process have been established for *M. intestinalis* by Gee and Davey (1986). Copepodite larvae can remain infective for up to 35 days, and there is no decline in infectivity for at least the first 6 days after hatching. Gee and Davey (1986) suggest that a photonegative response guides larvae to the substrate, after which the rate of infection is passively determined by a host's field of filtration. Even under experimental conditions of low temperature, relatively low host density, and low copepod density, the transmission of larvae to mussels is remarkably efficient (Gee and Davey 1986). Davey (1989) used such evidence to conclude that the transmission of *M. intestinalis* to mussels occurs within localized sites on a mussel bed, with few (if any) larvae colonizing from external sources. Thus, for both species, factors associated with the estuarine habitat seem to lead to the restriction of *Mytilicola* to sheltered, muddy sites.

An alternative, but not necessarily independent, explanation for the restricted distribution of *M. orientalis* is the sporadic distribution of oyster farms within Barcley Sound. The colonization of copepod larvae from imported Japanese seed oysters to local populations of mussels is frequently cited as the mechanism by which *M. orientalis* originated, and dispersed, along the Pacific Coast (Wilson 1938, Bernard 1969, Bradley and Siebert 1978). There is a small commercial oyster farm near the mouth of Grappler Inlet that could potentially act as the source of larvae. However, we examined small numbers of oysters and other bivalves at this and other commercial oyster farms in Barcley Sound and none was infected (Weber unpub. data).

Our data cannot address the importance of the colonization of mussel beds via imported oysters. However, evidence supporting the traditional view that *M. orientalis* originated from Japanese seed oysters is anecdotal (Wilson 1938, Bernard 1969, Bradley and Siebert 1978). It is equally plausible that the transmission biology of *M. orientalis* restricts it to those few estuaries in the Pacific Northwest where bivalves (especially *M. trossulus*) encounter slow-moving water. In this case, *M. trossulus* would act as the primary source of larvae, which are then available to infect other bivalves. Ecological studies aimed at investigating transmission between mussels and oysters, together with genetic studies on Japanese and North American stocks of copepods, could address this issue.

Within sheltered sites, the abundance of *M. orientalis* is influenced by local conditions. Host size plays a major role. Similar results have been shown for some field studies of *M. intestinalis* in mussels from Europe, but not all (review by Davey 1989). A positive association between abundance and size is often explained by an accumulation of copepods with the age of the host, an increased tolerance of larger hosts to higher copepod intensities, or to the increased filtration rate of larger hosts. Gee and Davey's (1986) experimental and field evidence show that the first two possibilities are unlikely for the *M. intestinalis*/*M. edulis* system, but that higher filtration rates were correlated with higher rates of infection. We cannot distinguish among these three possibilities with our data. Whatever the mechanism, it appears that mussels larger than 30 mm, despite their scarcity in Grappler Inlet (10% of 358 mussels), are disproportionally important to the overall transmission of *M. orientalis* within the mussel bed. The significant correlation between the mean size of mussels within 0.25-m^2 quadrats and mean copepod abundance provides further support for the importance of host size in the local transmission of copepods within suitable sites. The difference in mean copepod abundance between the two adjacent sites on Grappler Inlet may also be explained by the significant difference in mean host size between the two sites.

The location of hosts with respect to the tidal cycle also influenced copepod abundance. Similar results have been shown for *M. intestinalis* in Europe (Hepper 1955). The most simple explanation for this result is the increased duration that mussels are covered by the tide and thus available to larvae. Alternatively, mussels at lower tidal levels may differ in host quality or there may be fewer

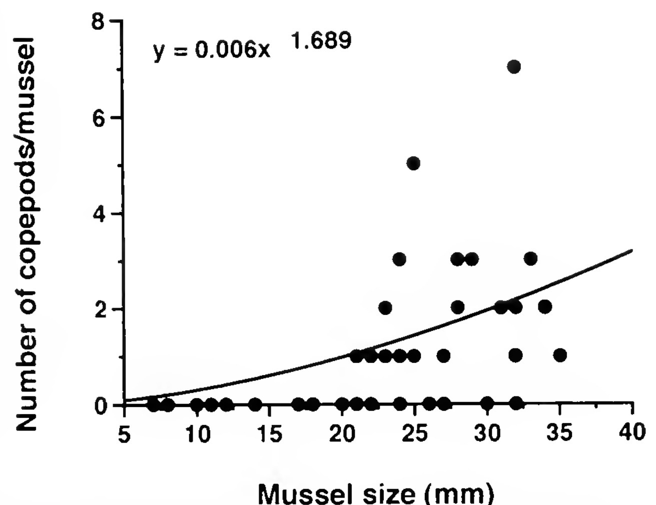

Figure 3. The relationship between host size and copepod abundance for 92 mussels collected from Grappler Inlet.

large mussels at higher tidal levels to act as significant sources of infection.

The infection characteristics of *M. orientalis* in *M. trossulus* seem similar to those of the more intensively studied system in Europe involving *M. intestinalis* in *M. edulis*. In both systems, the regional distribution of copepods in mussels seems to be determined by factors that restrict colonization by free-swimming larvae. Such factors are unknown for this system, but wave action, tidal currents, salinity, and/or substratum conditions may each play a role. In both systems, the local distribution of copepods within a mussel bed is influenced by a mussel's position on the shore (in relation to the tidal cycle) and by the size of individual hosts. Because events associated with local transmission between immediate neighbors play an important role in an individual host's risk of infection, larval site selection may be an important feature in determining an individual's ultimate exposure to copepods.

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APPLICATION OF ULTRASOUND TECHNOLOGY TO MOLLUSCAN PHYSIOLOGY: NONINVASIVE MONITORING OF CARDIAC RATE IN THE BLUE MUSSEL, *MYTILUS EDULIS* LINNAEUS, 1758

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ABSTRACT The noninvasive application of diagnostic medical sonography (ultrasound) to monitor cardiac rate in bivalve molluscs is demonstrated. The cardiac activity of three commercial-size edible blue mussels, *Mytilus edulis* Linnaeus, was visually monitored within a temperature range from 10 to 20°C in 32‰ salinity. Heart rates (17–50 beats/min) were consistent with published studies using various invasive techniques, such as the implantation of electrodes in the pericardial cavity. The acceleration of cardiac rate relative to changes in temperature varied during the course of the observations; acceleration was slower over the temperature range from 10 to 14°C than from 15 to 20°C.

KEY WORDS: Cardiovascular physiology, cardiac rate, heart rate, ultrasound, ultrasonography, Mollusca (mollusc, mollusk), Bivalvia (bivalve), blue mussel, *Mytilus edulis*

INTRODUCTION

Galtsoff (1964), in his review of the American oyster, referred to the challenges involved with *in situ* studies of bivalve heart beat. Investigators drilled windows in the valves of oysters and removed the pericardium to make direct visual observations of the heart. This technique was later modified by covering the windows with glass or cellophane. Invasive methods are currently in use, although the current popular technique involves some form of electrode (platinum, silver, stainless steel) inserted through the shell and into the pericardial cavity. The electrodes are connected to an impedance pneumograph with output to a multichannel recorder (Helm and Trueman 1967, Bayne 1971, Stickle and Sabourin 1979, Grace and Gainey 1987, Deaton 1991, Stentin-Dozey and Brown 1994).

Depledge and Andersen (1990) developed a noninvasive method for continuously monitoring heart and scaphognathite activity in reptant decapod crustaceans. Transducers are cemented to the carapace rather than implanted. Their computer-aided method has also been used successfully with thin-shelled bivalve molluscs and polychaete worms.

Instruments used in diagnostic radiology and cardiology have been shown to have potential application to invertebrate organ systems. Gribble and Reynolds (1993) and Gribble (1994) used angiography to describe the cardiovascular function of a portunid crab, *Portunus pelagicus* Linnaeus. Haefner (1996) described the use of diagnostic medical sonography (ultrasound) to monitor and record heart and scaphognathite activity in *Portunus gibbesii* Stimpson. This article demonstrates the use of noninvasive ultrasound techniques to monitor heart rate in the edible blue mussel, *Mytilus edulis* Linnaeus, in response to temperature changes.

MATERIALS AND METHODS

Edible blue mussels, *M. edulis*, were obtained from a local vendor (source, Great Eastern Mussel Farms, Inc., Tenants Harbor, ME). They were maintained in recirculated artificial seawater (32‰, 9°C, 8.1–8.9 ppm dissolved oxygen (DO) in a 50-gallon temperature-controlled system before experimental handling.

A depression cast of the ventral half of a mussel was made from Instamold (Activa Products, Inc., Marshall, TX) and secured to a weighted platform, which in turn was placed in a plastic aquarium (26 × 16 × 16 cm) filled with 4.8 L of seawater. The placement of a living mussel in the depression guaranteed that the hinge remained in a position to allow unimpeded ultrasound monitoring of the heart. At least 2 cm of water above the mussel provided a depth range through which the probe could be adjusted to achieve an optimal resolution of ultrasound images.

The waterproof transducer probe of the ultrasound unit (Ultrasonix 750 SDX) contained a 7.5-MHz linear array coupled to a 3-MHz single-crystal Doppler array. Dual video monitors provided a rectilinear image scan of the target organ as well as its frequency of movement. Polaroid prints of the video images were produced on a Sony printer.

After the specimen was secured to the platform, the transducer probe was lowered into the water and its surface was cleared of adhering air bubbles. The probe was then positioned parallel to the long axis of the mussel, directly over the dorsal hinge line. This provided the most effective display of the heart, which was detected by its known anatomical position (Fig. 1) and by the sonographic image of its contractions in the proximity of the posterior adductor and byssus retractor muscles (Fig. 2). The Doppler display of pulse frequency, shown to be effective in monitoring cardiac rate in brachyuran crabs (Haefner 1996), was not consistently reliable in our observations on mussels and was not used. In this study, cardiac rate was determined by counting 10 consecutive contractions, as seen on the video display, and timing their duration with a stopwatch. The rates were converted to beats per minute (bpm).

Three mussels (18, 37, and 27 g wet weights, respectively) were monitored at three different temperature regimens in 32‰ salinity (Table 1). In Trial 1, the water temperature in the experimental aquarium was allowed to gradually equilibrate to room temperature. In Trials 2 and 3, the experimental aquarium was placed in a larger basin that served as a water bath. The temperature of the water in the experimental aquarium was manipulated by changing the temperature of the water bath with an aquarium

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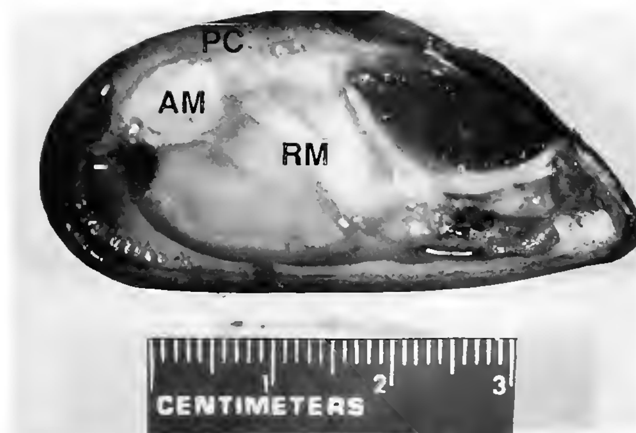

Figure 1. Midsagittal presentation of fresh *M. edulis;* left shell, mantle and gills removed. Internal organs identifiable on the ultrasound image (see Fig. 2) are posterior adductor muscle (AM), posterior byssus retractor muscle (RM), and pericardial cavity (PC).

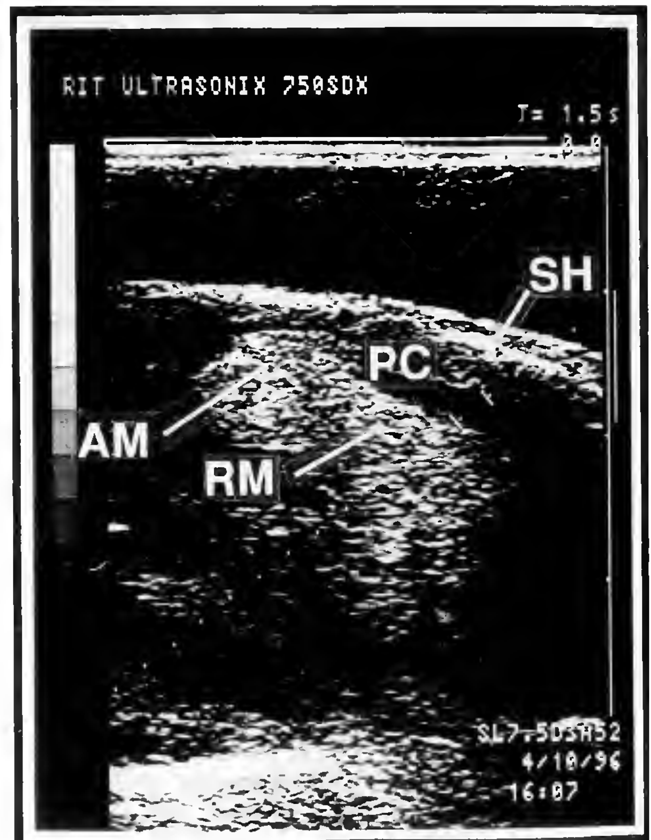

Figure 2. Midsagital rectilinear ultrasound scan of living blue mussel, *M. edulis*. Compare with Figure 1. Face of transducer probe represented by bright bar across top of photograph. The pericardial space (PC) is located at the confluence of the curved shell (SH), posterior adductor muscle (AM), and the origin of the byssus retractor muscle (RM). The heart, indiscernible in this print, can be detected by its pulsating image in a living specimen.

heater or by adding warm water or ice. The acclimation of the mussels to experimental conditions (15–20 min) was considered complete when the shells gaped, an indication of active ventilation (Bayne 1971) and heart function (Coleman 1974). The extent of gape was visually monitored during the trials, and heart rates were recorded only when the valves were agape. With few exceptions, observations were made at 1-min intervals during the course of the trials.

RESULTS AND DISCUSSION

We demonstrated that ultrasound technology can be used to monitor cardiac activity in *M. edulis*. Furthermore, the 17- to 50-bpm range of heart rate for the 10–20°C temperature range (Table 1) is consistent with rates reported by studies that involved invasive techniques (Helm and Trueman 1967, Bayne 1971, Scott and Major 1972, Coleman 1974, Stickle and Sabourin 1979, Grace and Gainey 1987).

The metabolic response of *M. edulis* to increasing temperature, reflected by increased heart rate (Fig. 3), was expected (Coleman 1974). However, there were noticeable differences in the change of heart rate relative to the change in temperature within each trial. Linear regression analyses were performed on subsets of the data (Table 1), corresponding to the observed trends shown in Figure 3: $Y = aX + b$, where Y is the heart rate (bpm), X is the temperature, a is the slope, and b is the intercept.

In Trials 1 and 2 (Table 1), the increases in heart rate during the first 18- to 20-min period (10–14°C) were relatively gradual, as indicated by a values of 3.35 and 1.42, respectively. Cardiac activity accelerated comparatively faster during periods in which the temperature increased beyond 15°C (Fig. 3, Trials 1 and 2).

TABLE 1.

Ultrasonographic monitoring of *M. edulis* in 33‰ salinity.[a]

Trial	Time (min)	Temperature Change: Range (°C)	Temperature Change: Rate (°C/min)	Cardiac Rate, Range (bpm)	Linear Regression: a	Linear Regression: b	Linear Regression: r^2
1	0–20	13–14	+0.05	21–26	3.35	−22.38	0.73
	21–35	15–18	+0.18	27–50	6.80	−72.47	0.89
2	0–18	10–13	+0.14	17–21	1.42	2.28	0.91
	19–35	14–20	+0.35	21–35	2.34	−11.96	0.98
	36–69	19–12	−0.21	35–20	−2.26	−8.32	0.98
3	0–25	16–18	+0.07	34–40	1.96	1.44	0.57
	26–61	18–10	−0.23	36–24	−2.02	3.76	0.96

[a] Temperature regimens of trials with corresponding cardiac rates, and linear regression analyses for $Y = aX + b$, where Y = heart rate (bpm), X = temperature (°C), a = slope of line, b = intercept, and r^2 = correlation coefficient.

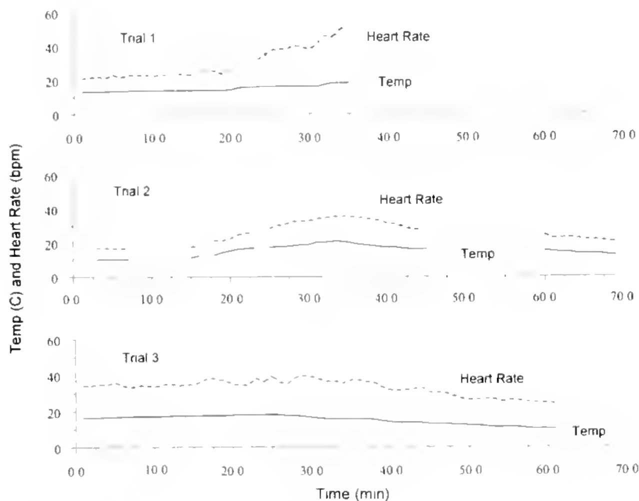

Figure 3. *M. edulis*. Heart rates relative to time and temperature change (see Table 1). Trial 1, 18-g specimen exposed to gradual temperature increase. Trial 2, 37-g specimen exposed to controlled temperature increase and decrease. Trial 3, 27-g specimen exposed to controlled temperature increase and decrease. All three plots are drawn to same scale for comparison.

These greater rates of responses are reflected in the higher *b* values (6.80 and 2.34, respectively) (Table 1).

Lowering temperatures in the latter phases of Trials 2 and 3 resulted in a decrease in cardiac rate. These decelerations were similar in magnitude of slope ($b = -2.26$ and -2.02) to the accelerations in heart rate ($b = 2.34$ and 1.96) in response to the increasing temperatures immediately preceding the decrease.

During the course of the trials, it was noticed that the width of the gape of the valves increased slightly as water temperature increased. This could be related to an increase in ventilation rate, perhaps in response to decreases in oxygen tension as well as an increase in water temperature. Although ventilation rate and dissolved oxygen concentrations were not monitored during the course of this study, it is known that changes in oxygen consumption are correlated with changes in heart rate (Baynes 1971).

As Coleman (1974) stated, "Laboratory measurements of heart rate, valve activity, filtration and respiratory rates show that under normal conditions all are closely related and all would seem to be equally valuable as measurements of activity." The noninvasive ultrasound scanning of bivalves provides conditions that are closer to what Coleman (1974) referred to as "normal" than specimens exposed to the implantation of electrodes, thermistors, and transducers in or near the heart. In this article, we demonstrated that the noninvasive monitoring of cardiac activity in *M. edulis* is possible through the use of ultrasonographic technology. We were also able to relate changes in cardiac activity to observed changes in the behavior of the specimen and/or to changes in the environmental conditions within the test chamber.

Currently, there are limitations to the extended application of medical ultrasound technology to invertebrate organisms. Medical ultrasound instruments are designed to distinguish between organs of differing tissue densities and to monitor stroke volume and blood flow in major vessels. In crabs (Haefner 1996) and in the mussel, it was possible to detect and monitor the movement of a soft-tissue organ (heart) lying beneath a relatively dense shell. However, the resolution of the image generated by the instrument in use was insufficient to make any quantitative assessments of stroke volume. The achievement of an optimal transducer frequency that will provide enhanced resolution of the heart might soon be possible as the technology of ultrasound imaging systems advances.

Unquestionably, limited availability and cost of currently available ultrasound instruments preclude their use in the majority of teaching and research laboratories. However, the ability to combine displays of heart activity with direct observation of specimen behavior under noninvasive experimental conditions warrants further explorations of such application of this technology.

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COMPARISON OF GROWTH, SURVIVAL, AND REPRODUCTIVE SUCCESS OF DIPLOID AND TRIPLOID *MERCENARIA MERCENARIA**

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ABSTRACT Diploid and triploid northern quahogs, *Mercenaria mercenaria*, were cultured intertidally in a South Carolina estuary for about 4 y. No difference in shell length was detected between quahogs identified as triploids and diploids at 6 and 27 mo of age; however, at 47 mo of age, triploid quahogs were significantly larger than diploids. The survival rates of diploids and triploids were similar over the field growout period None of the triploids thermally induced to spawn released gametes, whereas 82% of the diploid quahogs released viable gametes. Gonads of diploid individuals were ripe, with numerous mature oocytes and radiating bands of spermatozoa in the lumens. Gametogenesis in triploids, however, was severely retarded and abnormal. Although the lumen areas in triploid and diploid quahogs were similar, the areas occupied by sex cells were significantly larger in the diploid quahogs.

KEY WORDS: *Mercenaria*, quahogs, ploidy, growth, survival, reproductive success.

INTRODUCTION

Since the early 1980s, triploidy has been induced in a number of bivalves including the eastern oyster, *Crassostrea virginica;* the Pacific oyster, *C. gigas;* the pearl oyster, *Pinctada fucata martensii;* the bay scallop, *Argopecten irradians;* the noble scallop, *Chlamys nobilis;* the great scallop, *Pecten maximus;* the blue mussel, *Mytilus edulis;* the soft-shelled clam, *Mya arenaria;* the northern quahog, *Mercenaria mercenaria;* and the dwarf surfclam, *Mulina lateralis*, (Allen et al. 1982, Stanley et al. 1984, Tabarini 1984, Allen and Downing 1986, Beaumont 1986, Buzzi and Manzi 1988, Beaumont and Kelly 1989, Komaru and Wada 1989, Wada et al. 1989, Guo and Allen 1994a). The methodologies and consequences of triploidy induction in molluscan shellfish are reviewed by Beaumont and Fairbrother (1991). Although enhanced growth of triploids is a well-established characteristic in many of these species, it has not been documented in the soft-shelled clam (Mason et al. 1988) and the northern quahog (Hidu et al. 1988). In fact, triploid quahogs were significantly smaller than diploid controls in Maine waters after three growing seasons (Hidu et al. 1988).

Buzzi and Manzi (1988) produced triploid quahogs as part of a genetics program in South Carolina. Analysis at 6 mo of age revealed no statistical difference in shell length (SL) between juvenile quahogs confirmed as diploid and triploid individuals. Quahogs reach sexual maturity at 35- to 40-mm SL at about 1–1.5 y of age in South Carolina (Eversole et al. 1980). The reallocation of metabolic energy from gametogenesis to growth has been proposed to explain the increased size in triploids (Allen and Downing 1986). This study was designed to evalute the performance of confirmed diploid and triploid quahogs after several breeding seasons. We also histologically examined the gonads and evaluated reproductive potential. The aim of this study was to test the hypothesis that triploidy results in increased size (SL), altered gametogenesis, and sterility in reproductively mature northern quahogs.

MATERIALS AND METHODS

Production and Growout

The quahogs used in this study were produced as a part of a quahog genetics program in South Carolina. In December 1987, Buzzi (1990) used the gametes from three males and one female each to create six unrelated families. Aliquots of eggs from each family were treated with 1 mg/L cytochalasin B in 0.1% dimethylsulfoxide (DMSO) 5 and 10 min after fertilization to disrupt meiosis I (M I) and meiosis II (M II), respectively (Buzzi and Manzi 1988, Buzzi 1990). The two experimental groups (MI and M II) and control groups (a DMSO and an unexposed control) were treated for 20 min. After washing, the experimental and control groups were cultured separately in static conditions for 21 days before being transferred to recirculating downwelling culture systems for an additional 156 days. In May, at 125 days of age, quahogs were measured and the ploidy was determined by flow cytometry (Allen 1983, Buzzi 1990).

The family of quahogs with the highest percentage of triploids (M I and M II) was planted on a private aquaculture lease in Folly River in October 1988. Quahogs from the M I and M II treatments were planted in separate trays at about 900/m^2. In March 1989, quahogs from the M I and M II treatments were combined and planted in one tray because of poor survival. This tray was subsequently relocated to Charleston Harbor in September 1989 because the aquaculture operation failed. Quahogs were replanted on

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*Technical Constribution No. 4248 of the South Carolina Agricultural Experiment Station.

a different aquaculture lease in Folly River in July 1990, where they remained until the termination of this phase of the experiment in October 1991.

Tissue Sampling and Ploidy Determination

In February 1990, quahogs (n = 483) were measured and individually numbered by etching each valve. A subsample of quahogs (n = 25) representative of the size distribution was selected for a test run of the ploidy determinations. The anterior end of the valves was notched with a grinding tool, and a small portion of mantle and muscle was teased free with fine forceps. The tissue was added to a capped vial with 1 mL of Sorensens's buffer (pH 7.8) until a small pellet was visible on the bottom; 2.3 mL of ethyl alcohol was then added and stirred with a vortex mixer. The samples were held on ice until frozen the following day. Results from a test run of these procedures in February 1990 indicated that ploidy analysis was possible and that the short-term survival of the sampled quahogs in a raceway was good.

Tissue samples for ploidy determinations were collected on two other occasions, June and December 1990. A total of 150 ploidy determinations were attempted, and the ploidy was confirmed for all of the quahogs used in histologic examinations, spawing trials, and growth assessments, except in October 1988 and March 1989. Experimental quahogs lacking ploidy confirmation are referred to as either cytochalasin B–treated or control individuals in this publication.

The methods for the preparation of tissues and ploidy determination were adapted from Allen (1983) and Buzzi (1990). Thawed tissue samples were digested for 30 min at room temperature in 0.5 mL of 0.05% collagenase in 1.5-mL polypropylene centrifuge tubes. Digestion was stopped with the addition of 0.5 mL of phosphate buffer. The tissues then were aspirated into a 3-ml syringe and repeatedly forced through an 18-gauge needle to dislodge cells. Cell suspensions were centrifuged for 3 min, and the supernatant was decanted. Cell pellets were digested further in 0.6 mL of a 1:1 solution of 0.003% Nonidet P-40 and 60 μg/mL ribonuclease A. After digestion for 30 min at room temperature and stirring with a vortex mixer, the cell suspensions were transferred to a 1-mL tuberculin syringe and forced through a 48-μm-pore-size Nytex screen into a clean 1.5-mL centrifuge tube. Samples were again centrifuged, decanted, and washed with 0.5 mL of phosphate buffer.

One-half hour before flow cytometry, samples were centrifuged and decanted, 0.6 mL of 50 μg/mL propidium iodide was added, and the samples were stirred with a vortex mixer. Cell preparations were filtered once more immediately before loading in an EPIC 751 flow cytometer equipped with an argon laser. Ploidy determination was accomplished by a comparison of the relative fluorescence of unknown samples with those from known diploids.

Growth

The SL (anterior-posterior axis) of those quahogs identified as diploids and triploids was measured to the nearest 0.1 mm in February 1990 and October 1991. These diploids and triploids were treated as a population, and the mean SL of quahogs were compared by the use of paired t-tests with the appropriate statistic for unequal and equal variances. The numbers of identified diploid and triploid individuals available for SL comparisons were 84 and 37 in February and 42 and 15 in October, respectively.

Survival

Survival was estimated by comparing the relative proportion of triploids to diploids determined by Buzzi (1990) when the quahogs were 4–5 mm in SL and approximately 6 mo of age in May 1988 to the relative proportion of triploids alive in February 1990 (approx. 27 mo of age) and October 1991 (47 mo of age). The null hypothesis that the relative proportion of triploid to diploid quahogs was the same in February 1990 and in October 1991 as that determined in May 1988 was tested with X^2.

Spawning Trial and Gonad Conditions

In April 1991, 22 diploid and 22 triploid quahogs were collected from the growout location and conditioned for a month before the spawning trial. Quahogs in individual vessels were induced to spawn by thermal induction and the introduction of pasteurized sperm (Castagna and Kraeuter 1981). The number and sex of spawners were recorded. Eggs collected from individual spawns were quantified with a counting cell. The number of sperm in a spawn was estimated with a spectrophometer and the linear relationship (sperm/mL = [45.4284 × 10^6] absorbance at 610 nm) developed by Bricelj (1979). Subsamples of gametes were mixed to determine viability and competence through 48 h. The diameters of 100 formalin-fixed eggs were estimated with an AIC-2 image analysis system.

After the spawning inductions, a subsample of 10 triploid and 10 diploid quahogs was sacrificed for histologic examination. Quahogs were shucked and the foot, mantle, and gill tissue around the gonad were cut away before the gonad was placed in Davidson's fixative. The entire gonad was embedded in paraplast, and sections were cut at 7 μm progressively through half of the gonad. Sections were stained with hematoxylin and eosin Y (Howard and Smith 1983). Eleven evenly spaced sections from each gonad of five triploid and five diploid quahogs (three females and two males) were used to calculate the percentage of lumen area in the microscope field and the percentage of lumen area occupied by sex cells. This calculation involved estimating the area of the gonad lumen and sex cells in the lumen with a computerized scanning image analyzer (Heffernan and Walker 1989). An attempt was made to select microscope fields representative of the entire gonad section. The number of oocytes per square millimeter was measured, and the diameters of oocytes and their nuclei were also measured at this time. Only oocytes with a visible nucleolus were measured. Female and male gonad area and the number oocytes per square millimeter were compared separately between triploid and diploid quahogs with *t*-tests. Because only one triploid had mature oocytes, diameter measurements of oocytes and nuclei were restricted to one individual of each ploidy and statistical comparisons were not attempted.

RESULTS

Growth

At 6 mo of age (May 1988), there were no statistical differences in SL between identified diploid and triploid quahogs resulting from the disruption of either meiosis I (M I) or meiosis II (M II) (Buzzi 1990). Mean SL (±SD) for diploids and M I triploids were 5.13 ± 0.40 and 4.83 ± 0.80 mm; values were 5.14 ± 0.44 and 5.11 ± 0.42 mm for diploids and M II triploids, respectively (Fig. 1). Shell measurements of the cytochalasin B–treated quahogs revealed that from May to October 1988, the mean SL

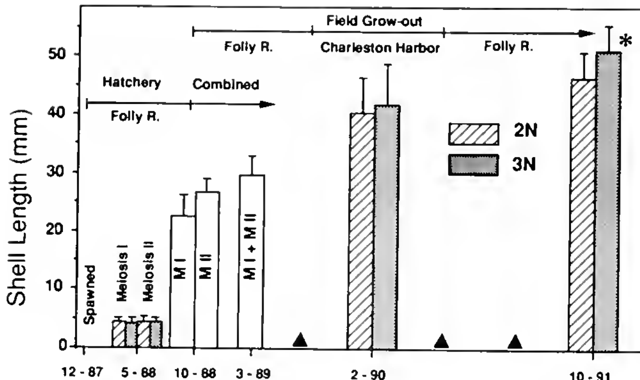

Figure 1. Mean SL and standard deviation of diploid and triploid northern quahogs cultured in Folly River and Charleston Harbor, SC. The growth of quahogs treated to disrupt meiosis I (M I) and meiosis II (M II) was monitored over a 47-mo period. Shaded and cross-hatched histograms represent SL of ploidy-confirmed triploid and diploid quahogs, whereas open histograms represent SL of the cytochalasin B–treated groups. The asterisk indicates a significant difference, and the closed triangles represent major spawning periods (Eversole et al. 1980). See text for further details.

increased to 22.44 ± 3.75 mm (n = 25) in the M I tray and to 26.40 ± 1.92 mm (n = 25) in the M II tray (N. Hadley, unpub. data). When the M I- and M II-treated groups were combined in March 1989, the quahogs averaged 29.38 ± 3.49 mm SL (n = 50). After an additional year in the field (February 1990) and possibly one spawn by the larger quahogs, the mean SL of identified triploids was larger (41.41 ± 7.03 mm, n = 37) than that of identified diploids SL (40.48 ± 6.17 mm, n = 84), but this difference was not significant (t = 0.7278, p = 0.468). The change in mean SL between the first (May 1988) and second (February 1990) ploidy determination was 35.35 mm SL for the diploids and 36.42 mm SL for the triploids. This increase in mean SL was not tested for statistical significance because in May 1988, the ploidy determination involved destructive sampling (Buzzi 1990). A significant difference (t = 2.5212, p = 0.008) in mean SL was detected between those identified triploids (50.13 ± 5.53 mm, n = 15) and diploids (46.21 ± 5.04 mm, n = 42) in October 1991 after 20 more months of growth and at least two major spawning periods (Fig. 1). The increase in mean SL of individuals over these 20 mo was 27% (8.71 mm SL) for the triploids compared with only 14% (5.73 mm SL) for the diploids; this growth difference was significant (t = 3.402, p = 0.007).

Survival

The relative proportion of triploid (M I and M II) to diploid juvenile quahogs was 25.8% in May 1988 (Buzzi 1990). In February 1990, 30.6% (37 of 121) of the ploidy identified quahogs were triploid, and in October 1991, 26.3% (15 of 57) of the quahogs were triploid. We failed to reject the null hypotheses (2 × 3 contingency table; χ^2 = 1.234, p = 0.557) that the proportion of triploids after 21 and 41 mo of growtout was the same as the 25.8% observed by Buzzi (1990). The percent survival of the triploids (55.6%) and diploids (56.0%) between February 1990 and October 1991 was nearly identical.

Spawning Trial

One triploid quahog was misidentified; consequently, one less triploid (n = 21) was exposed to spawning stimuli. Mean SL were similar (t = 0.9558, p = 0.345) for the diploids (47.73 ± 4.55 mm) and triploids (49.41 ± 6.82 mm) in the spawning trial. None of the quahogs identified as triploids spawned, whereas 82% (6 males and 12 females) of the diploids spawned. On average, males released 4.55×10^9 sperm and females released 1.17×10^6 eggs. Formalin-fixed eggs averaged 78.0 μm and were relatively uniform in size (73–89 μm in diameter). Samples of pooled sperm successfully fertilized eggs and produced shelled larvae.

Gonad Examination

The gonads of identified diploid quahogs were ripe; male gonads contained several rows of spermatogonia, spermatocytes, and spermatids with radiating bands of mature spermatozoa arranged with heads facing the follicle wall and tails toward the lumen center (Fig. 2a). Two of the five triploids were tentatively identified as males because these quahogs had darkly stained cells in the lumen (Fig. 2b) that appeared to be products of abnormal spermatogenesis (Loosanoff 1937). These triploid quahogs also lacked definite oogonia and oocytes.

The gonads of diploid females contained free mature oocytes (50–80 μm in diameter) within the lumen, whereas other oocytes were still attached by a thin peduncle to the follicle wall (Fig. 2c). The vitelline coat was fully developed, and a well-defined nucleolus within the nucleus was prominent in mature oocytes of diploids. In contrast, an occasional large oocyte (60–90 μm in diameter) was observed in the gonad of a triploid quahog, but in many cases, the lumen was empty (Fig. 2d). Small clusters of what appeared to be proliferating cells were also observed along the follicle wall. These cells were not as darkly stained as those cells observed in the triploid males. Although the gonads of female and male triploids contained some oogenic and spermatogenic stages in every specimen checked, gametogenesis was greatly retarded and obviously aberrant in the triploid quahogs.

Data for the percent lumen area per microscope field indicate that female and male lumen areas were similar in triploid and diploid quahogs (Table 1). However, significant differences in the lumen area occupied by oogenic and spermatogonic stages were observed between diploids and triploids; in diploids, sex cells occupied two to four times as much area as in triploid quahogs. The mean number of oocytes per square millimeter in diploid gonads was also significantly larger than that for triploid gonads. The difference in the oocytes per square millimeter between the diploid and triploid quahogs was about two orders of magnitude. The mean diameter (±SD) for nucleated oocytes in a triploid was 74.1 ± 12.4 μm (n = 49), compared with 62.6 ± 9.4 μm (n = 99) in a diploid quahog. The diameter of triploid oocyte nuclei averaged 40.4 ± 9.5 μm, compared with 30.3 ± 6.0 μm for diploid quahogs.

DISCUSSION

The survival of quahogs treated with cytochalasin B through the larval period was significantly lower than the controls (Buzzi 1990). After the larval period, the survival of the cytochalasin B–treated quahogs was comparable to that of the controls from spat to 6 mo of age (Buzzi 1990). Two lines of evidence indicate that survival was also similar in the older triploid and diploid quahogs; the relative proportion of triploids to diploids did not change from May 1988, and the percent survival of each ploidy was the same from 27 to 47 mo of age. Comparable survival has been observed for 1-y and older triploid and diploid eastern oys-

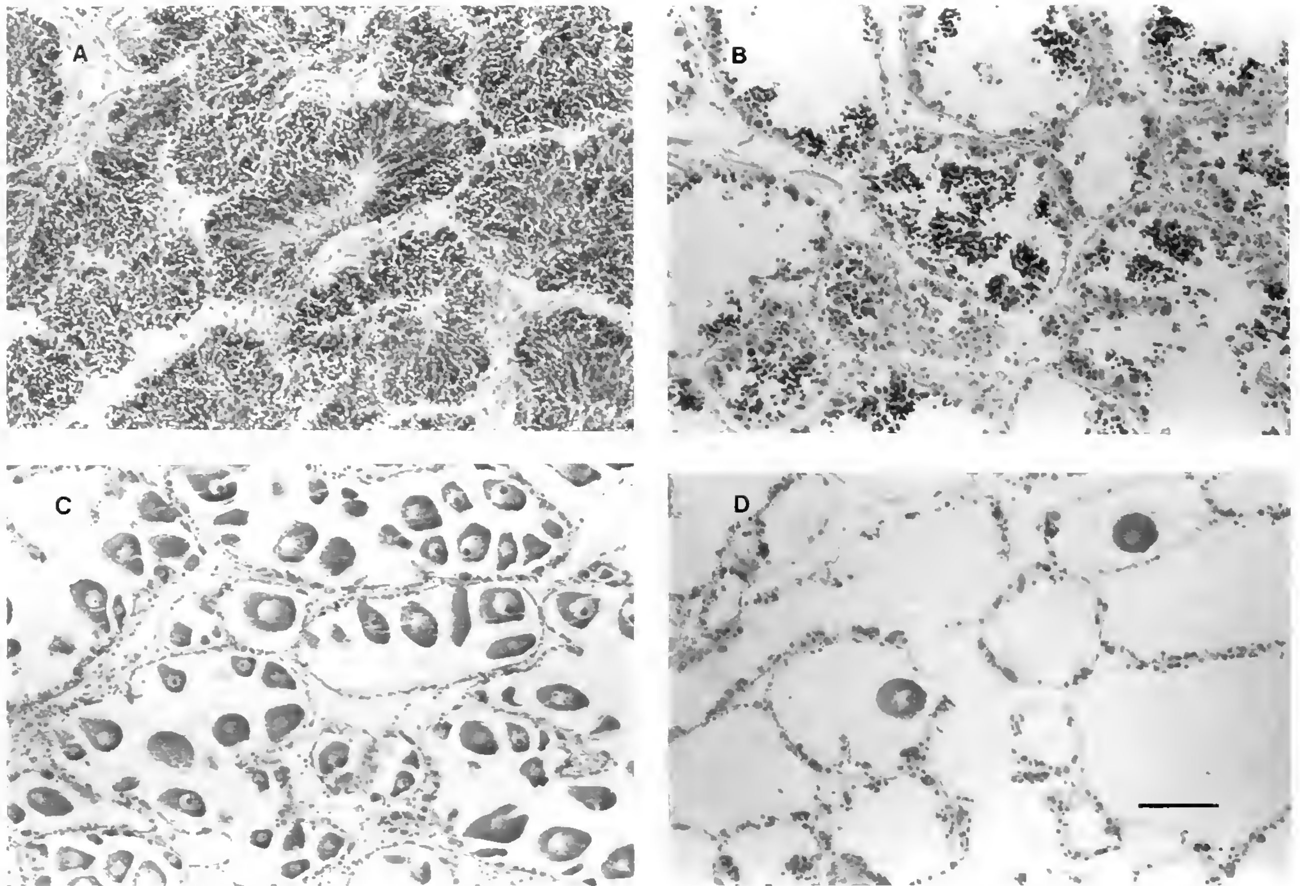

Figure 2. Tissue sections of identified diploid and triploid quahog gonads sacrificed in May 1991 from: a) ripe diploid male, 53.6 mm SL; b) triploid male, 54.7 mm SL; c) ripe diploid female, 55.2 mm SL; and d) triploid female, 47.9 mm SL. Bar = 100 μm.

ters, bay scallops, and soft-shelled clams (Stanley et al. 1984, Tabarini 1984, Allen et al. 1986). In contrast to these observations, the survival of triploid Pacific oysters from spat to 1 y was superior to that of diploids (Allen and Downing 1986). Reduced survival in triploid larvae is commonly observed in molluscs, and it is believed to be caused by exposing the fertilized eggs to harsh induction methods during critical developmental stages (Beaumont and Fairbrother 1991). On the other hand, the reasons for the superior survival of post-set triploids are less obvious. Adult triploid Pacific oysters produce fewer gametes and, as a consequence, have less energetic demands for reproduction than do diploid oysters (Shpigel et al. 1992). These investigators suggested that trip-

TABLE 1.

Mean ($\bar{x}$) ± standard deviation (SD) of the percentages of lumen area per microscope field and sex cell area per lumen and the number of oocytes per square millimeter of gonad.[a]

	Female				Male			
	Diploid		Triploid		Diploid		Triploid	
Parameter	n	$\bar{x}$ ±SD	n	$\bar{x}$ ±SD	n	$\bar{x}$ ±SD	n	$\bar{x}$ ±SD
Lumen per field (%)	3	78.0 ± 9.6[b]	3	69.1 ± 6.5	2	75.0 ± 3.8[c]	2	59.1 ± 14.8
Sex cells per lumen (%)	3	34.4 ± 2.0[d]	3	15.0 ± 5.3	2	85.2 ± 3.7[e]	2	20.0 ± 0.5
Oocyte/mm^2	3	133.9 ± 28.8[f]	3	1.5 ± 2.6				

[a] n represents the number of quahogs used to calculate means. Comparisons were made within category and sex.
[b] t = 1.3349, p = 0.2528.
[c] t = 1.4647, p = 0.2806.
[d] t = 5.9774, p = 0.0039.
[e] t = 24.8668, p = 0.0016.
[f] t = 8.2201, p = 0.0012.

loid oysters gain an energetic advantage and survive better than do diploids under stressful conditions (e.g., elevated temperature). The production of faster growing and larger individuals via triploidy has the potential for increased survival through reduced quahog predation (Whetstone and Eversole 1978).

In contrast to the observations of Hidu et al. (1988), triploid quahogs were significantly larger than the identified diploids in this study. The length of the study and the number of spawning periods experienced by the two study populations differed. For example, quahogs were cultured for four growing seasons in South Carolina and only three growing seasons in Maine (Hidu et al. 1988). It is well known that the quahog grows faster and reaches sexual maturity earlier in the more southerly portion of its geographical range (Ansell 1968, Eversole et al. 1980). By the use of Ansell's (1968) growth data and a 35- to 40-mm SL minimum for maturity (Eversole et al. 1980), it is unlikely that quahogs grown in Maine were capable of spawning during the three growing seasons of the study of Hidu et al. (1988). In contrast, the South Carolina diploid and triploid quahogs reached 35–40 mm SL after two growing seasons and experienced at least two spawning periods before the final diploid-triploid growth comparisons. It is possible that if the Maine study had been extended to five growing seasons, the growth of triploids would have surpassed that of the diploid controls.

The induction of triploidy retarded gametogenesis in the northern quahog. The sex cells in the lumen occupied less area in both sexes, and fewer oocytes developed in the females identified as triploids (Table 1). Gametogenesis was reported to be severely retarded in triploid soft-shelled clams, bay scallops, and eastern oyster (Tabarini 1984, Allen et al. 1986, Barber and Mann 1991), and to a much lesser degree in triploid Pacific oysters and dwarf-surf clams (Allen and Downing 1986, Allen and Downing 1990, Guo and Allen 1994a). Triploid Pacific oysters spawn and in some cases produce viable gametes (Guo and Allen 1994b). Unlike the triploid Pacific oyster, triploid quahogs failed to respond to repeated spawning stimuli, despite the fact that a few large oocytes were in the gonad of one triploid. It appears, on the basis of the overall abnormal nature of the gonads, the scarcity of sex cells, and the failure to respond to spawning stimuli, that triploid northern quahogs should be considered sterile.

Triploid Pacific oysters produced larger eggs and sperm than did their diploid counterparts (Komaru et al. 1994, Guo and Allen 1994b). Because triploid nuclei contain theoretically 1.5 times more DNA than diploid nuclei, the observation that the oocyte and nuclei diameters of triploid quahogs were larger than those in diploid quahogs was not unexpected. In fact, Child and Watkins (1994) used the optically measured diameters of gill tissue and hemolymph cell nuclei to successfully distinguish between known diploid and triploid Manila clams, *Tapes philippinarum*.

Recently, Guo and Allen (1994a) added polyploid gigantism to heterozygosity (Stanley et al. 1984) and energy reallocation (Allen and Downing 1986) as hypotheses to explain increased size in triploid molluscs. Although our study was not designed to test these hypotheses, our data do lend support for the energy reallocation hypothesis, which argues that energy normally destined for gametogenesis is allocated to growth because of partial or complete sterility. In this study, the evidence is good that identified triploid quahogs were sterile; triploids failed to respond to spawning stimuli and exhibited signs of abnormal and severely retarded gametogenesis. Also, the difference in diploid and triploid SL was not observed until after quahogs reached sexual maturity (i.e., 35–40 mm SL) and had opportunity to spawn. Ansell and Lander (1967) calculated the losses attributed to spawning in a 40-mm-SL quahog to be about 20–25% of the total energy used for growth. This amount of energy, if diverted into growth, may be sufficient to account for the difference in SL between known diploids and triploids; however, it may not be the only factor contributing to the observed SL difference. The relative importance of heterozygosity, energy reallocation, and gigantism in increased triploid size may differ among molluscan taxa; understanding these mechanisms in different taxa warrants further study (Guo and Allen 1994a).

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A CARDIAC CELL LINE FROM *MYA ARENARIA* (LINNAEUS, 1759)

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ABSTRACT Using standard tissue culture techniques, we report here the development of a cardiac cell line from *Mya arenaria* that exhibits anchorage dependency and an observable, high mitotic coefficient. The cultures can be subcultured while retaining reduced mitotic activity.

KEY WORDS: *M. arenaria*, cell line, marine mollusc, tissue culture, proliferation

INTRODUCTION

Although it has long been possible to establish *in vitro* primary, anchorage-dependent cultures of various cells of molluscan origin, demonstration of the sustained, observable proliferation and/or passage of these cells, whether from embryonal or adult tissue, has been limited (Perkins and Menzel 1964, Cecil 1969, Hetrick et al. 1981, Li et al. 1966). Generally, marine molluscan cells, either from dissociated tissue or cells that have migrated from explants, will eventually deteriorate (Bayne 1993). This eventuality can be delayed by lowering incubation temperature and/or reducing nutrients. The present exception to this generalization is that represented by the *established* freshwater molluscan cell line developed by Hansen (1979) using embryonal tissue.

Recently, Walker et al. (1996) successfully cultured and cryopreserved malignant hemopoietic cells of *Mya arenaria* using chemically defined medium in spinner culture. By the use of these techniques, cells doubled every 40–50 h for several generations, but eventually deteriorated. Although presenting many advantages, this system does not afford the desirable characteristics of an anchorage-dependent system, in which the direct observation of induced or normal intracellular or intercellular activity can be pursued.

MATERIALS AND METHODS

Preparation of Heart Explants

Mature specimens of *M. arenaria* were obtained from Sandy Hook National Park, NJ, and maintained at the Haskin Research Laboratory, Port Norris, NJ. Clam hearts (ventricles) were dissected from clams with normal blood cells and with neoplastic blood cells (determined cytologically). With a stereo-dissecting microscope, the hearts were removed so that contamination from adventitious microorganisms was minimized. Hearts (ventricles) so obtained were rinsed several times in sterile seawater and subjected to two 20-min rinses in seawater containing antibiotics (penicillin, 200 U/mL; streptomycin, 200 μg/mL). Specimens were placed in a deep Maximov slide, flooded with sterile seawater containing antibiotics, and minced with a scalpel blade into 1-mm^3 pieces. After mincing, the pieces were rinsed twice with seawater containing antibiotics to remove extraneous tissue and debris, and culture medium was added just before culture.

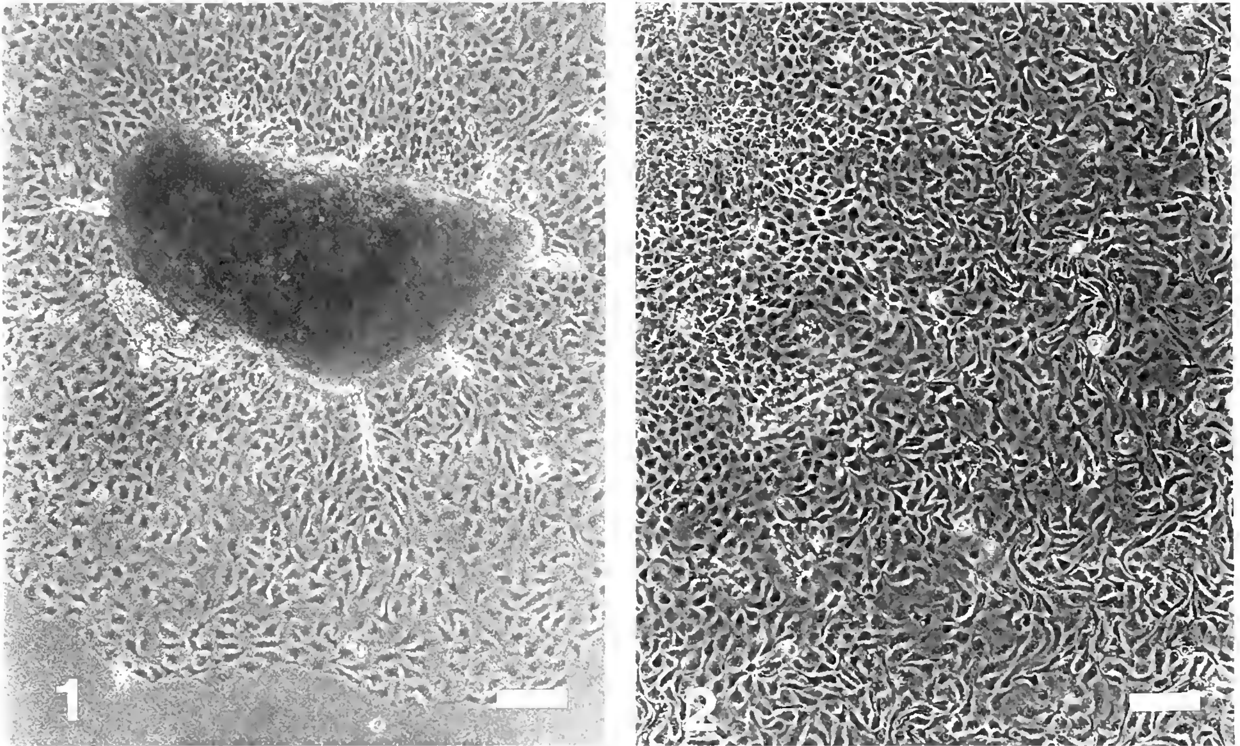
Culture of Heart Explants

Primaria T25 tissue culture flasks (Falcon Labs) were used in all experiments. After dissection and preparation, explants were transferred to the culture flasks by pipette and 3.0 mL of culture medium was added. Incubation was at 15°C under ambient air conditions. Initially, culture medium was changed (50%) weekly. After firm attachment by the explant to the substrate and the initiation of cell migration out of the explant, the medium was changed every 4 days (50%).

Medium Preparation

During the development of this cell line, many media formulations were used and evaluated in an attempt to foster cell maintenance and proliferation. The most successful medium used (hereafter designated as K-P 58) was composed as follows: sterile glass-distilled deionized H_2O, 1,000 mL; MEM Eagle (Earle's salts) with L-glutamine and nonessential amino acids, 4.85 g; $CaCl_2 \cdot 2H_2O$, 1.82 g; KCl, 0.68 g; $MgCl_2 \cdot 6H_2O$, 4.36 g; NaCl, 24.26 g; $MgSO_4 \cdot 7H_2O$, 3.16 g; HEPES buffer, 5.0 g; and glucose, 0.5 g.

To prepare the final medium, the following components were added at the time of use: fetal bovine serum (FBS) and *M. arenaria* sterile hemolymph, each 10%; insulin/transferrin/sodium selenite supplement, 2%. Antibiotics routinely added to the culture were penicillin, 100 U/mL, and streptomycin, 100 μg/mL. The pH of the final medium was adjusted to 7.2 with 1 M NaOH; the final osmolarity was 1100–1150 mOsm/kg. Sigma Chemical Co. supplied the MEM (M0643) and the insulin/transferrin/sodium sel-



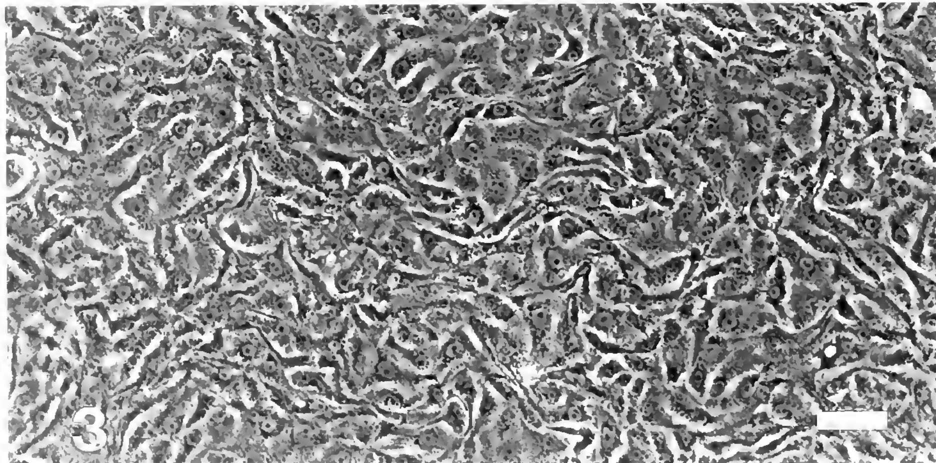
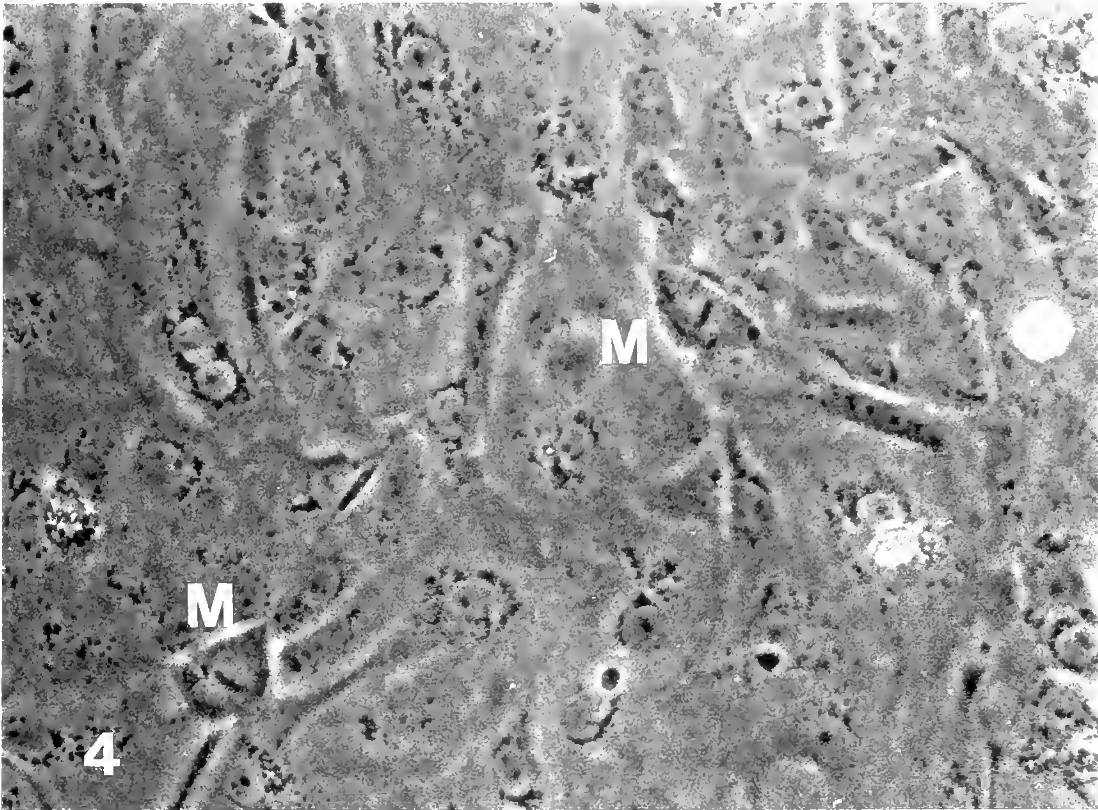


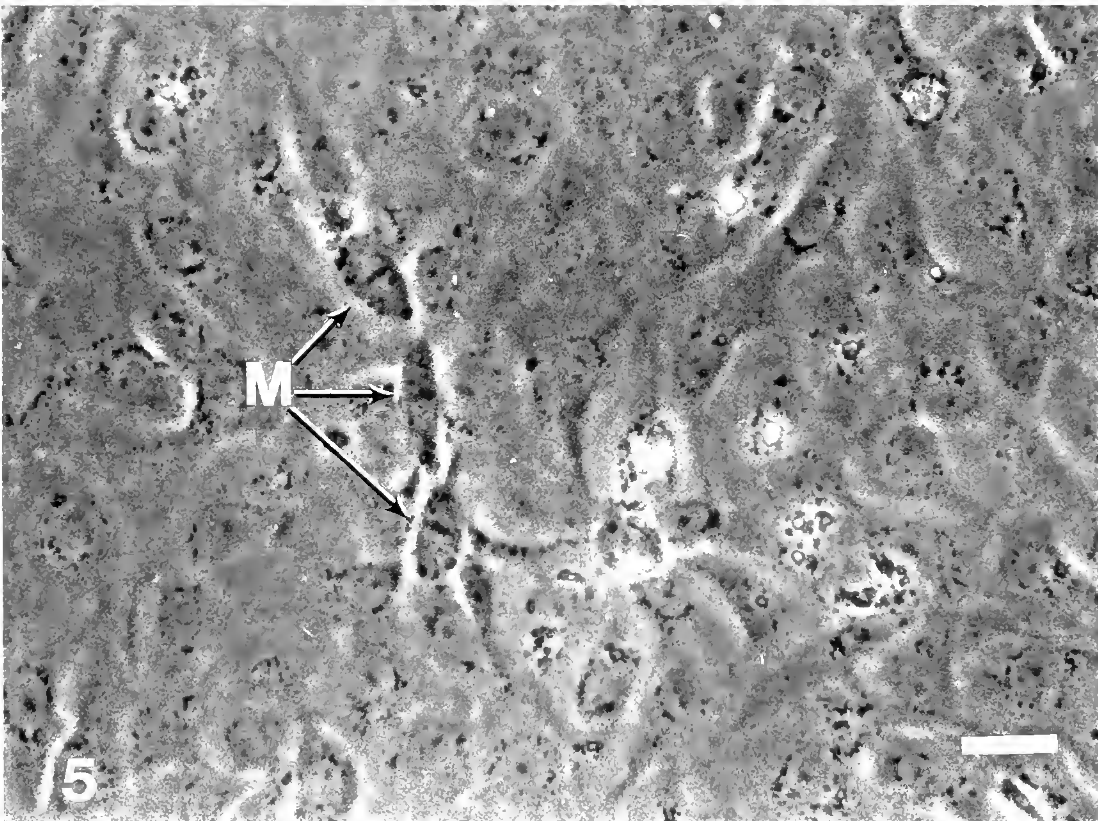
Figure 1. Explant of *M. arenaria* cardiac tissue, 3 wk in culture. Phase contrast microscopy. Scale bar, 0.12 mm.
Figure 2. Cardiac cells of *M. arenaria*, 4 wk in culture. Phase contrast microscopy. Scale bar, 0.12 mm.
Figure 3. Cardiac cells of *M. arenaria*, 4 wk in culture. Phase contrast microscopy. Scale bar, 0.06 mm.

enite supplement (I1884). Sterile Systems (Hyclone) supplied the FBS.

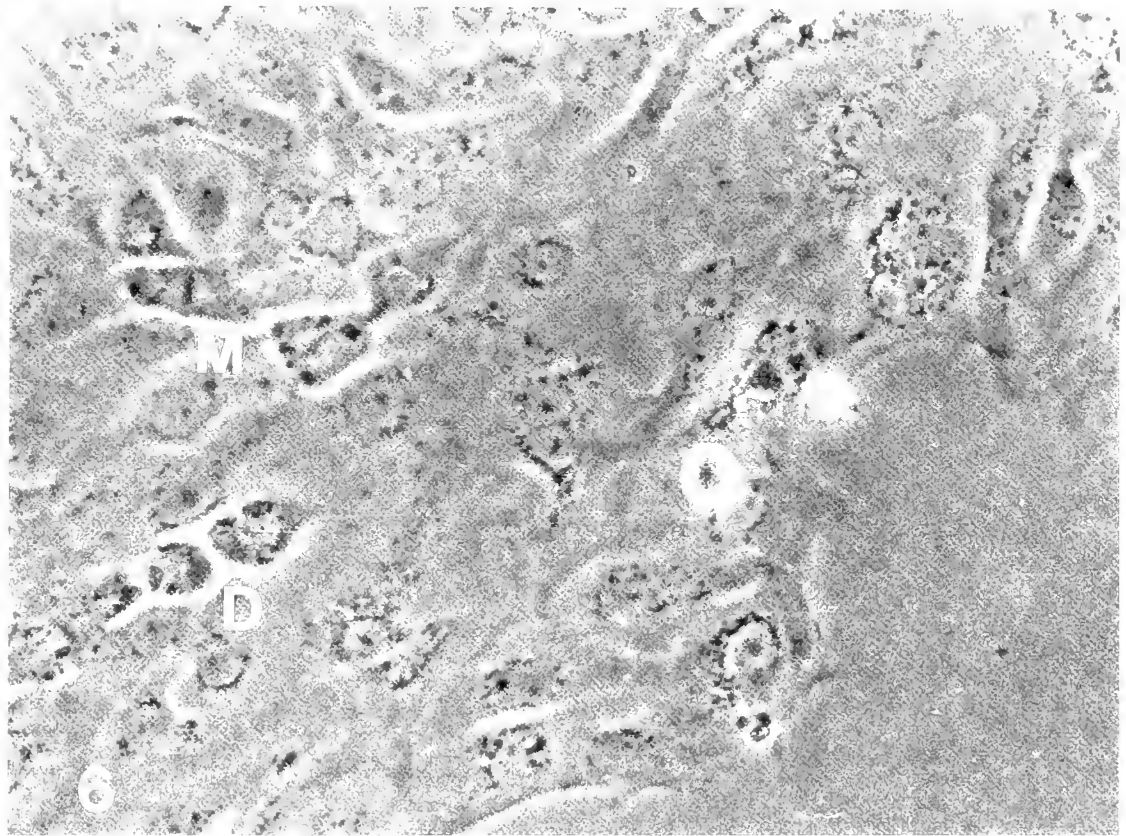
Subculturing

Six weeks after culture establishment, proliferation and cell numbers were sufficient to permit passage. After a brief rinse with calcium-magnesium free seawater (CMF), the cells were exposed to a sterile trypsin-CMF seawater solution. During this treatment, the cultures were monitored microscopically to determine the correct exposure time. It was found that an exposure time of 1–3 min at room temperature was ideal. When exposure time was determined microscopically to be sufficient, but before *sheets* of cells were totally detached, the enzyme solution was carefully decanted and medium with 15% FBS was substituted. The flasks were then agitated to completely free the cell sheets from the substrate, and





Figures 4–7. Cardiac cells of *M. arenaria*, 4 wk in culture. Notice many mitotic figures. M, metaphase; D, daughter cells. Phase contrast microscopy. Scale bar, 0.025 mm. (Figures are continued on next page.)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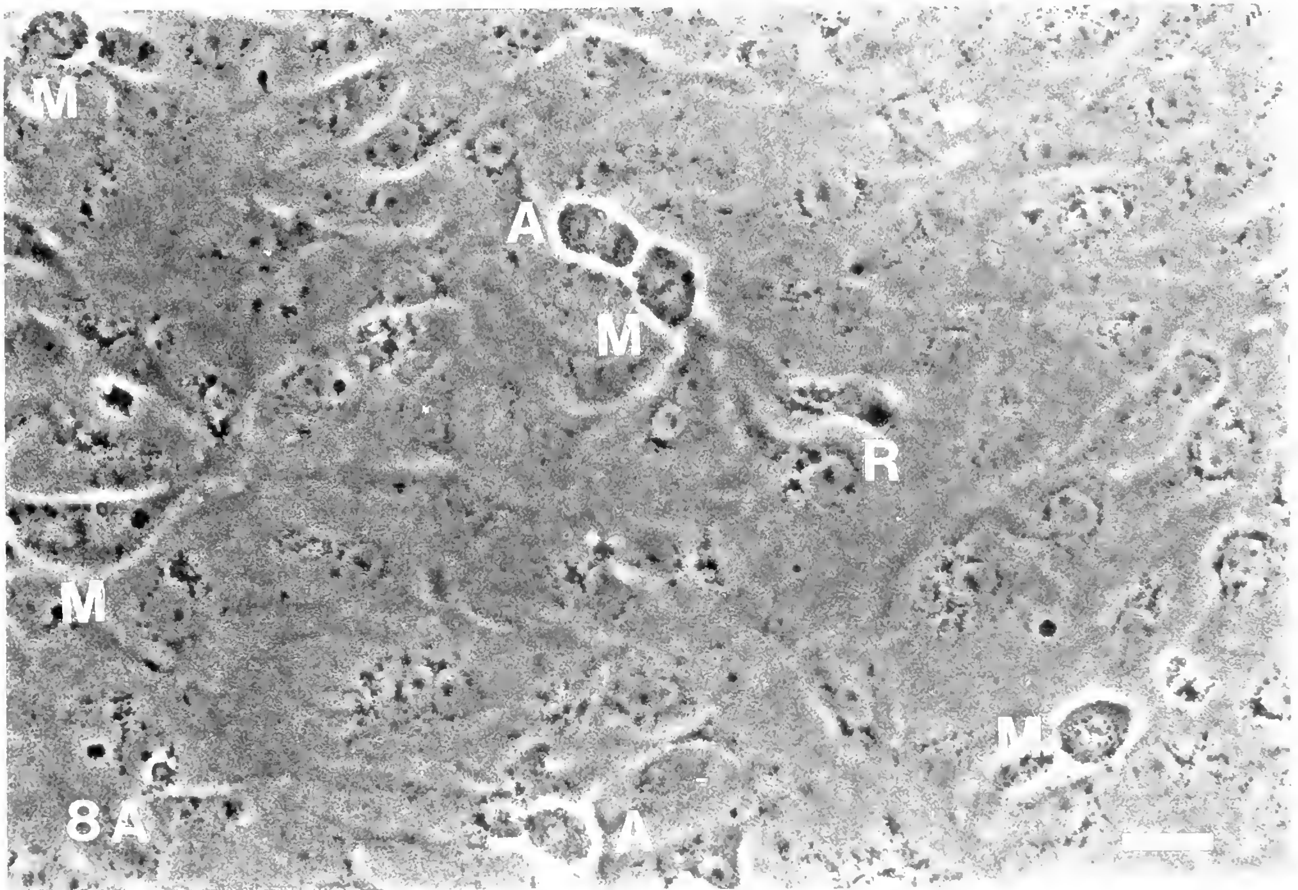


Figure 8. Cardiac cells of *M. arenaria*, 4 wk in culture. Mitoses of different cells are shown in sequence in each photomicrograph. R, reference point; P, prophase; M, metaphase; A, anaphase; D, daughter cells. Phase contrast microscopy. Scale bar, 0.025 mm. (Figure is continued on following pages.)

the contents were decanted into fresh culture flasks to begin the first passage.

RESULTS

Within 48 h of culture initiation, most explants were firmly adhered to the substrate. The migration of cells from the cardiac tissue began 1–3 days later and continued until the explants were in "monolayer form" (Figs. 1–3). Proliferation and visible mitotic activity became evident within 4 wk (Figs. 4–7). Mitotic activity at 15°C was at a lower level than that observed when the culture medium was changed and the cultures were exposed to room temperature for 3–4 h. The mitotic coefficient at room temperature was determined to be 0.75%. Figures 8 and 9 document much of this activity with various sequential stages in the mitotic cycle, as well as chromosome movement, shown in cultures that were 4 wk old. It is important to note that after cell division, the daughter cells immediately reattached to the substrate and remained anchorage dependent. Mitotic activity was present throughout all areas of the monolayered plaques, with the older parts of the plaque (first migrators at the edge of the plaque) showing as much activity as the younger parts of the plaque (later migrators at the center of the plaque). Very little senility was evident in any of the cultures throughout these experiments, and primary cultures continued to proliferate until passage was indicated.

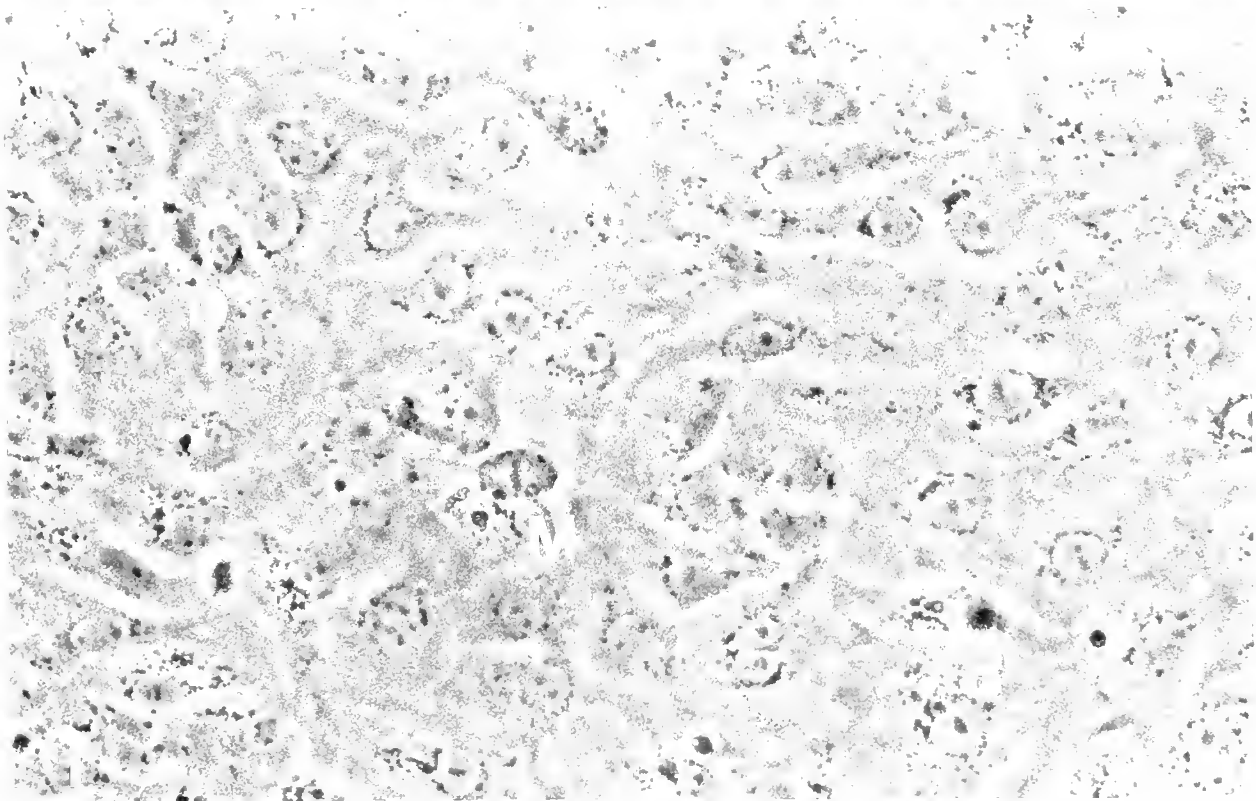
After the passage of primary cardiac cultures, sheets of cells were allowed to remain undisturbed for 3 days, after which fresh medium with 10% FBS was substituted. Shortly thereafter, sheets of cells reattached to the substrate and cellular migration, anchorage dependency, and proliferation became evident in 1 wk (Fig. 10). Current cultures were in primary culture for 6 wk and have been in first passage for 4 wk as of this writing.

The cytological evaluation of cells from both normal hearts and hearts from clams with neoplastic disease revealed no detectable differences with regard to division rates, migration rates, anchorage dependency, contact inhibition, or response to subculturing. The nuclear/cytoplasmic ratios, chromosome structure, and nuclear profiles appear to be the same in cultured cardiac cells from both types of clams.

DISCUSSION

Long-term *in vitro* cultures of prolific marine molluscan cells in anchorage-dependent form afford many opportunities for investigative exploitation by shellfish researchers. Although, by definition (Merchant et al. 1967), successful subculturing of a primary culture is termed a cell line, longevity of that line is indeterminate. Only when successfully passed many times, with a finite and terminal number of generations, or infinitely and immortally, may a cell line be termed an *established* cell line (Merchant et al. 1967). Generally, however, only cell lines with appropriate genetic aberrations or retrograde embryonic genetic expression exhibit immortality.

It is yet to be determined how many successful passages the



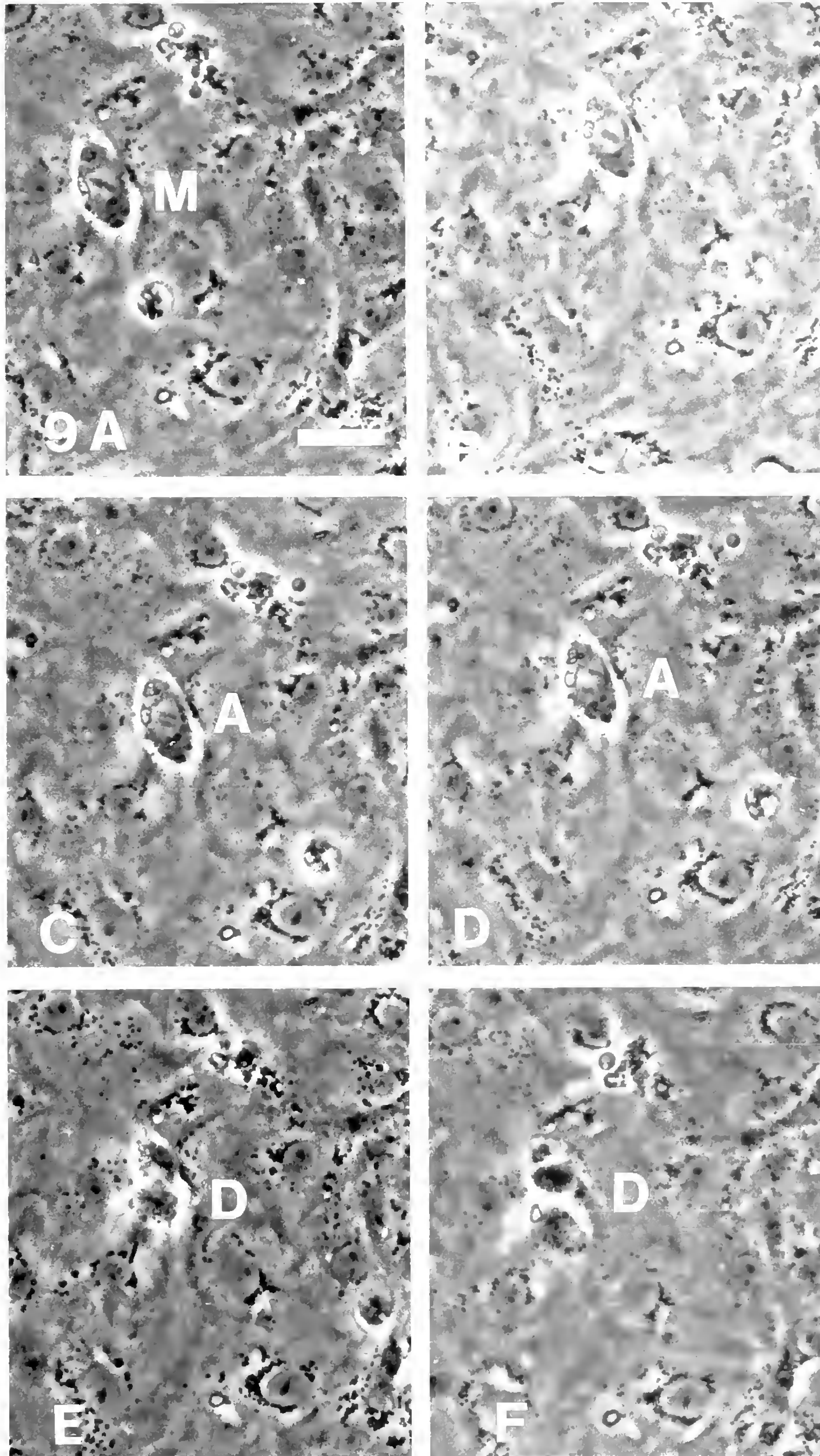


Figure 9. Cardiac cells of *M. arenaria*, 4 weeks in culture. Mitosis of a single cell is shown in sequence. M, metaphase; A, anaphase; D, daughter cells. Phase contrast microscopy. Scale bar, 0.025 mm.

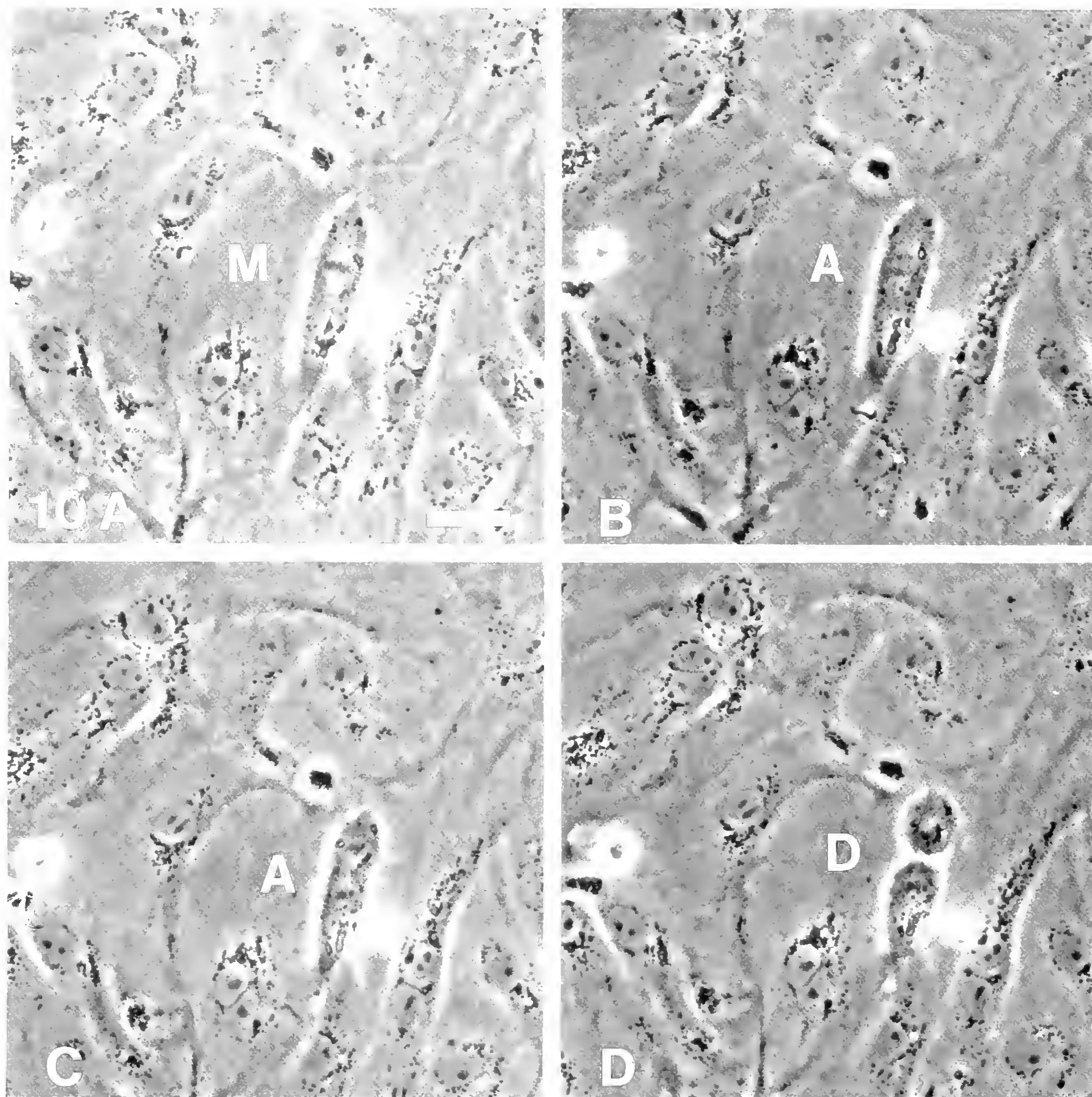


Figure 10. Cardiac cells of *M. arenaria*, 7 wk in culture, after the first passage of primary culture. Mitosis of a single cell is shown in sequence. M, metaphase; A, anaphase; D, daughter cells. Phase contrast microscopy. Scale bar, 0.025 mm.

cultures described herein will undergo and if they can eventually qualify to be termed an established cell line. On the basis of our observation of reduced mitotic activity after the first passage of the cultures, we expect that the generation number of the cardiac cells therein will be infinite, as are all normal cells. However, the demonstration of a long-term, anchorage-dependent cell line with an observable high mitotic index fulfills many desirable criteria for exciting studies that heretofore could not be performed.

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GROWTH AND MORTALITY OF TRANSPLANTED JUVENILE HARD CLAMS, *MERCENARIA MERCENARIA*, IN THE NORTHERN INDIAN RIVER LAGOON, FLORIDA

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ABSTRACT Growth and mortality were examined in hatchery-produced, early-juvenile *Mercenaria mercenaria* transplanted to protected and unprotected plots at a site in the northern Indian River lagoon, FL. Clam density and size were examined in both treatments five times in the year after transplantation. The growth of clams in both treatments was rapid and comparable to that of clams from other areas within the lagoon. Growth in the protected treatment was initially depressed, but after 363 days, clams from both treatments did not differ significantly in shell height (SH). The mortality of clams in both treatments was high, although significantly greater in the open treatment. Clams in the protected treatment died at a high rate until 80 days into the experiment (SH about 8 mm), beyond which no significant mortality occurred. This experiment suggests that (1) growth rates in the northern Indian River lagoon may favor future aquaculture ventures; (2) clams can be grown out in the lagoon (if protected from epibenthic predators) when they are 8 mm SH, much smaller than current aquaculture practice suggests; and (3) placing unprotected juvenile clams in situ at high densities is not an efficient stock-enhancement technique.

KEY WORDS: Growth, mortality, *Mercenaria*, hard clam, aquaculture, Indian River lagoon

INTRODUCTION

Growth and mortality in early life stages are important aspects of bivalve population dynamics. The abundance of early-juvenile bivalves is a critical determinant of the abundance of the adult bivalve population (Muus 1973, Marelli 1990) and is negatively affected by mortality. There is also generally an inverse relationship between size and mortality that is expressed as a prey size refuge (Carriker 1959, Menzel and Sims 1962, MacKenzie 1977, Whetstone and Eversole 1977, Kraeuter and Castagna 1980, Arnold 1984, Peterson et al. 1995). Finally, the livelihood of harvesters and culturists of commercially important clams depends on the availability or production of adequate numbers of legal-sized clams.

The success of bivalve populations is heavily dependent on the survival of postsettlement juveniles, which are the most vulnerable benthic stage (Carriker 1959, Menzel and Sims 1962, Muus 1973, Eldridge et al. 1976, Kraeuter and Castagna 1985). Predation on juvenile clams is often relieved by growth into sizes that offer refuge from predation or by their occupation of a spatial refuge. Spatial refugia occur where physical or biologic structures or physiological regimes interfere with predator efficiency (Gainey and Greenberg 1977, Menge 1978, Pohle et al. 1991, Peterson 1982, Summerson and Peterson 1984, Riese 1985, Bertness 1989). Clam culturists construct artificial refugia with predator-exclusion devices (Eldridge et al. 1976, Menzel et al. 1976, Flagg and Malouf 1983, Kraeuter and Castagna 1985, Vaughan 1989).

We examined three premises regarding the growth and mortality of transplanted juvenile *Mercenaria mercenaria* (Linnaeus 1758) in Florida's Indian River lagoon: (1) Clam growth rate in an area historically depauperate of clams is similar to those in areas that support large clam populations; (2) It is economically feasible for clam culturists to begin the field growout phase of their operation with smaller, less expensive clams than those traditionally used; (3) Broadcasting unprotected juvenile clams (1- to 3-mm shell height [SH]) as a stock-enhancement technique is not effective.

MATERIALS AND METHODS

Approximately 56,000 hatchery-spawned and hatchery-reared early juveniles, or "seed," of *M. mercenaria* were held for 1 wk in a 1,600-L conical tank at the Harbor Branch Oceanographic Institution. The tank contained a 62.5 mg/L solution of tetracycline hydrochloride, and clams were fed from cultured algae. Water was not changed for the first 2 days, and subsequently, the water and food supply were changed daily, but no additional tetracycline was added.

Clams were then concentrated on a 750-μm-pore-size screen, and the entire sample population was measured volumetrically. Thirty-two 6-mL subsamples were removed from the sample population, and each was placed dry in a glass jar. Jars were transported to the field site in a chilled cooler. A portion of the sample population (approximately 8 mL) was preserved and used to estimate the mean size (maximum SH: the maximum measurement from the umbo to the ventral margin) and density of the juvenile clams.

The experimental site was located in Shellfish Harvesting Area B ("body B") in the northern Indian River lagoon, just north of State Road 405 on the east side of the Intracoastal Waterway (Fig. 1). This area had a depauperate *Mercenaria* population during 1986 and 1987 (Arnold and Marelli pers. obs.). Hard clam growth rates in body B have been estimated to equal or exceed growth rates from other areas of the lagoon (Arnold et al. 1991). The study site had a sandy bottom, was approximately 2 m deep, and was vegetated with attached and drift algae (*Gracilaria* sp.). Water temperatures reach a maximum near 30°C in midsummer and decline to 10–15°C in early winter (Arnold and Marelli pers. obs.). Mean salinity is stable, high (30–36‰; McCall et al. 1970), and similar to that of body C, an area with a large clam population (Arnold et al. 1996).

We defined a 2.5- by 2.5-m area on the bottom by laying down a polyvinyl chloride (PVC) template (3/4" schedule 40 pipe) and marking the corners with stainless steel stakes. The template was subdivided with net-mending twine into 16 equal squares (0.39 m^2

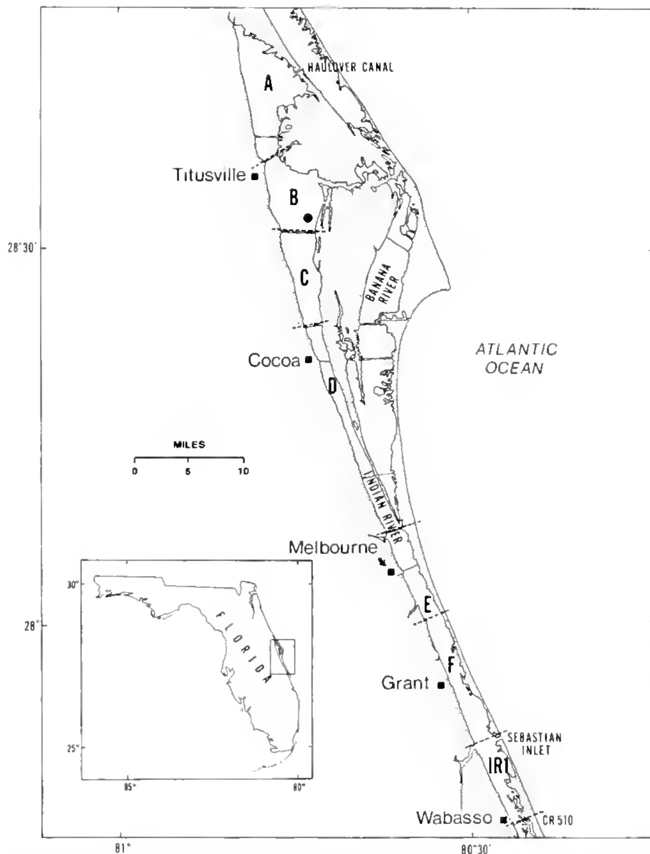

Figure 1. Indian River lagoon, FL, indicating shellfish-harvesting bodies and approximate position of experimental site (●).

each) and was anchored to the substrate during transplanting by four steel rebar pins (9.5-mm [3/8"]). On September 14, 1989, a diver haphazardly poured the clams from one 120-mL jar onto the surface of each of the 16 subplots. The template was then removed. A second plot was prepared with a second PVC template and covered on the lower side with polypropylene mesh (open areas, 10 by 10 mm), approximately 4 m away from the first plot. The cage template was also subdivided into 16 0.39-m^2 squares and was anchored to the substrate by eight rebar pins. The diver planted the clams by pouring the contents of one jar per subplot directly through the mesh onto the substrate. On both treatments, the diver observed that juvenile clams rapidly burrowed into the substrate.

Fifteen days after transplantation, three of the subplots from each treatment were sampled. Samplings were also conducted 80, 183, 273, and 363 days after transplanting. The selection of sampled subplots was random, but no subplot was sampled more than once during the experiment. Subplots were located by laying a subdivided template over the plot. Three cores with surface areas of 0.032 m^2 and depths of 5 cm were removed with a suction dredge from each randomly selected subplot. Material removed was collected in a 303-μm-pore-size mesh bag and preserved in buffered 10% seawater formalin. After the 80-day sampling, six cores were taken from each subplot because declining densities in the open plots might make statistical analysis difficult. All live *M. mercenaria* that displayed a tetracycline band under ultraviolet illumination were counted, and the SH of each was measured. Most authors report clam size as shell length (SL). The relationship between SL and SH was calculated from clams recovered during the early postplanting stages. During each sampling of the caged subplot, the mesh template was cleared of all fouling growth, which was always minimal. Before transplantation and also after the 363-day sampling, three cylindrical cores (37 mm in diameter, 5 cm in length) were taken haphazardly from within both the open and the caged plots. These were analyzed separately for major sedimentary characteristics (% gravel, % sand, % silt-clay, and % organic matter by ignition [Folk 1974]) as a measure of the influence of the treatments on the sediment profile.

We analyzed survivorship using a two-way analysis of variance (ANOVA) with days after transplantation and plot condition (open or caged) as factors. Lack of treatment replication may make interpreting the meaning of between-treatment effects difficult, but highly significant differences would suggest real main effects. Because the experimental design was unbalanced, data were analyzed with the SAS GLM procedure (SAS Version 5; SAS Institute, Inc., Cary, NC). Where F ratios were significant ($p < 0.05$), Hochberg's GT2 method for comparing means was applied (Hochberg 1974) because it is useful when variances are equal but sample sizes are unequal (Day and Quinn 1989). We used ANOVA to analyze shell data and developed a growth model by fitting (via Table Curve 2D software, Version 3.0; Jandel Scientific, Corte Madera, CA) a nonlinear growth function to the SH data. Separate functions were fit to the data from each treatment because prior ANOVA results demonstrated significant growth differences between treatments. Five functions that have been demonstrated to be useful in modeling bivalve growth (von Bertalanffy, Gompertz, power curve, logistic curve, and exponential curve) (Kennish and Loveland 1980, Kaufmann 1981, Walker and Humphrey 1984, Jones et al. 1990, Arnold et al. 1991, Allison 1994, Lefort 1994) were fit to the SH data. The most appropriate function for each of the data sets was selected on the basis of best fit (highest r^2 value) among the five functions.

RESULTS

The mean clam density at planting was 5,221.2/m^2, and the mean SH of these clams was 1.63 ± 0.01 mm (range, 0.7–3.7 mm SH). The relationship between SH and SL was determined to be $SH = -0.209 + (0.967)(SL) + (-9.127 \times 10^{-5})(SL)$. Clams in both treatments experienced high mortality almost immediately (Fig. 2): mortality approached 90% within 15 days in the open plot and exceeded 40% in the caged plot. Mortality in both treatments exceeded 95% within 80 days after planting, but clam densities did not decline appreciably for the remainder of the experiment. Because of the drastic early mortality in both treatments, the clam-density data for the 15-day sampling was eliminated from the analysis and the two-way ANOVA was conducted on the remaining data. Within-treatment densities on sample dates from 80 to 363 days did not differ significantly from each other; however, clam densities in the caged plot were significantly higher than those in the open plot on all sample dates ($p < 0.0001$).

Sample date had a significant effect on clam height ($p \approx 0$), and mean sizes on all dates were significantly different from each other. Treatment also had a significant main effect on clam height ($p < 0.0001$), although there was a significant interaction between date and treatment ($p < 0.0001$). Clam growth initially appeared to be more rapid in the open plot (Fig. 3). For both treatments, a power curve provided the best fit for clam height over time. The open-treatment data yielded a power curve of the form $SH_t = 0.021(t + 78.99)^{1.603}$, where t = days after planting and SH_t =

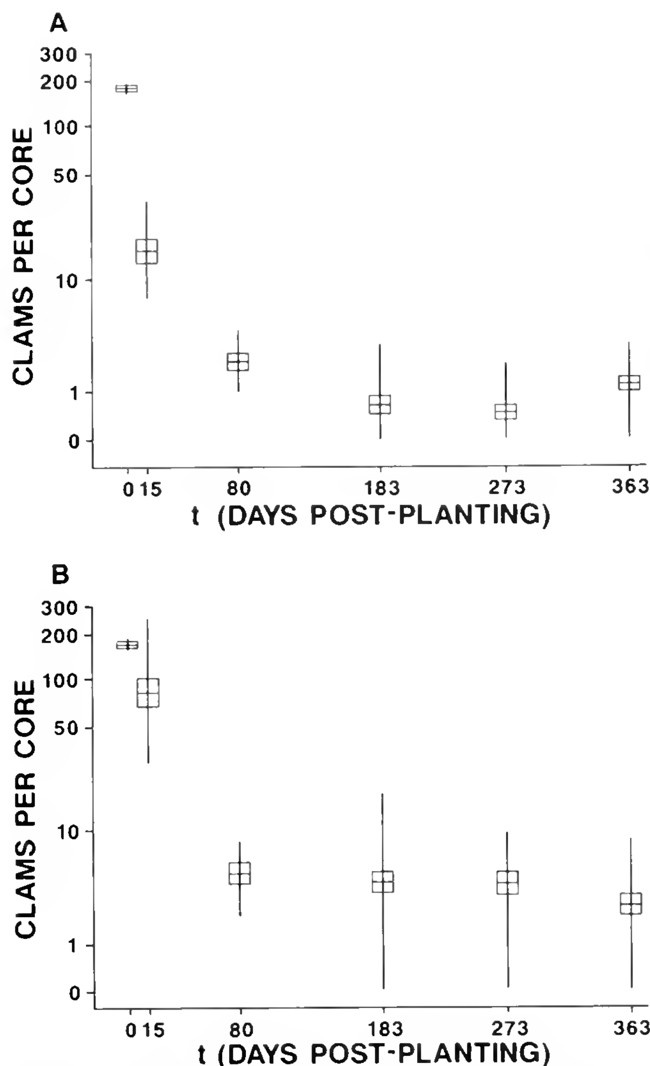

Figure 2. Changes in density (number per 0.032-m² core) over time of *M. mercenaria* transplanted to (A) uncaged and (B) caged experimental plots in Indian River shellfish-harvesting body B, 1989–1990. Symbols indicate range, mean, and ±1 standard error. Densities were significantly greater in the caged treatment on all dates ($p < 0.0001$).

shell height at t ($r^2 = 0.959$). The caged treatment shell growth was best expressed by a power curve where $SH_t = 0.002\ (t + 634.24)^{5.78}$ ($r^2 = 0.945$).

The substrate sediment profile was altered by the cage treatment. Large increases in both silt-clay (>222%) and organic fractions (>42%) and a slight (10.4%) reduction in the sand fraction were identified in the caged treatment, whereas silt-clay increased only slightly (28.8%) in the open treatment.

DISCUSSION

Clams transplanted into Shellfish Harvesting Area B grew at rates consistent with those estimated (from models generated by Arnold et al. 1991) for clams in other Indian River areas, rates that could be amenable to economical aquaculture. Differences in initial clam growth rates observed between caged and uncaged treatments were not detectable at the termination of the experiment. Caging artifacts can alter biologic processes, including growth (Virnstein 1977, Dayton and Oliver 1980, Riese 1985). Although we identified an altered sediment profile in the caged treatment, clam growth was ultimately not affected.

Mortality rates for unprotected clams were high and consistent with the 96–100% mortality over 3–12 mo reported for juvenile clams transplanted by other researchers (Menzel et al. 1976, Kraeuter and Castagna 1977b, Flagg and Malouf 1983, Kraeuter and Castagna 1985). The rapid decline in clam density followed by a slow but steady reduction in the open treatment is indicative of the density-dependent predation reported in other crab-clam assemblages (Mansour and Lipcius 1991, Boulding and Hay 1984). Despite the compromise created by a lack of replication, our data suggest that planting unprotected clams of less than 8 mm SH is neither an efficient stock-enhancement technique nor an economical method of aquaculture. In fact, mortality, although reduced in our caged treatment, was unacceptably high (as per Menzel et al. 1976) in either treatment for an aquaculture operation. Several

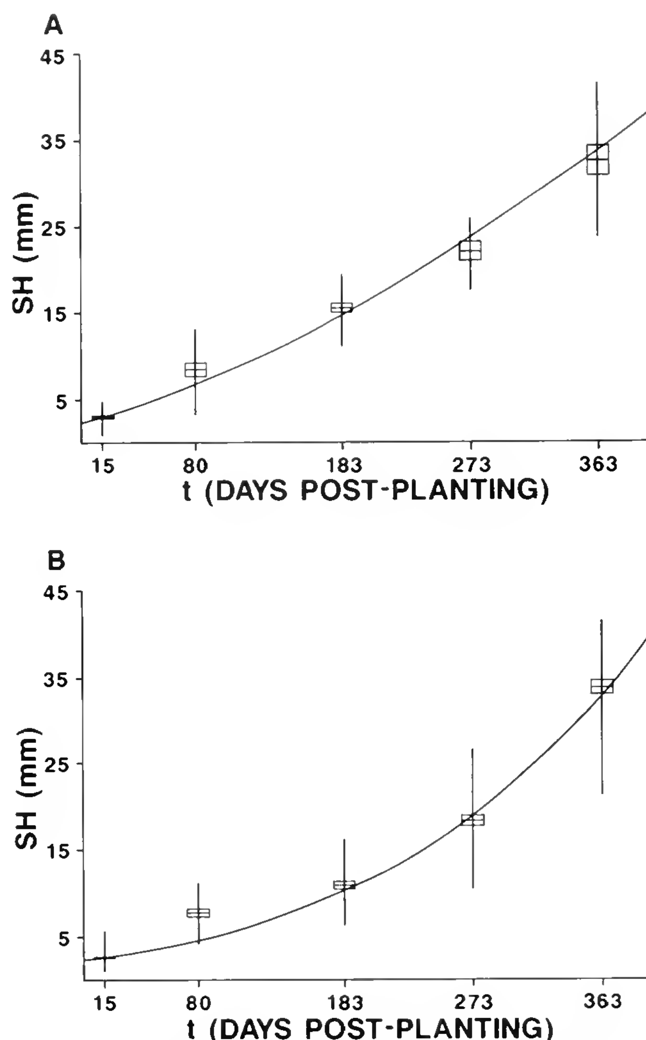

Figure 3. Size (SH) over time of *M. mercenaria* transplanted to (A) uncaged and (B) caged experimental plots in Indian River shellfish-harvesting body B, 1989–1990. Symbols indicate range, mean, and ±1 standard error. Curves represent best fit of models examined and are explained by the equations (a) $SH_t = 0.021\ (t + 78.99)^{1.603}$ and (b) $SH_t = 0.002\ (t + 634.24)^{5.78}$. Initial growth was higher in open plots ($p < 0.0001$), but SH did not vary by treatment at $t = 363$ days.

factors may have contributed to high mortality: initial clam sizes were much smaller than those of clams usually transplanted to Indian River field growout facilities (typically, 14–16 mm SL; Barry Moore pers. comm.), and mesh sizes used by aquaculturists in Indian River growout operations are much smaller (commonly 6.35-mm or 1/4″ mesh; Barry Moore pers. comm.) than the size we used. Although our cage was not specifically designed to exclude crabs tunneling into the treatment, no such behavior was apparent until the end of the experiment, when one stone crab (*Menippe mercenaria*) had taken up residence under the eastern edge of the mesh.

Increasing the initial size of the transplanted clams would reduce mortality. Caged clams experienced predation below the 10-mm mesh from infaunal or small epibenthic predators. Xanthid crabs are known to enter such cages and prey on juvenile *Mercenaria* (MacKenzie 1977, Eldridge et al. 1979, Walker 1984, Kraeuter and Castagna 1985, Bisker and Castagna 1989), as are juvenile blue crabs (*Callinectes sapidus*) (Walker 1984, Bisker and Castagna 1989). From an economic perspective, the smallest clams that can be protected and raised should be planted (Kraeuter and Castagna 1977a). Those authors insist that the greater losses of smaller clams can be offset by the lower cost of raising or purchasing smaller stock. Survival can be enhanced if protected clams are planted at larger sizes or planted in combination with predator-exclusion devices and/or predator-removal techniques (Eldridge et al. 1976, Menzel et al. 1976, Whetstone and Eversole 1977, Eldridge et al. 1979, Walker 1984, Kraeuter and Castagna 1985). Some of those authors also reported reductions in predation when clams achieved a SH of 15–20 mm (Menzel et al. 1976, Whetstone and Eversole 1977, Eldridge et al. 1979, Walker 1984). Our protected clams became effectively immune to predation at a SH of 8 mm (SL = 8.55 mm) under 10-mm mesh, although similarly sized clams in the open treatment were still vulnerable, suggesting a size refuge from predation by infaunal and small epibenthic predators at about 8 mm SH. Unprotected clams also achieved this refuge, but they continued to be exposed to larger epibenthic predators (brachyuran crabs, busyconid whelks, and fish).

Stock-enhancement or aquaculture operations for hard clams must mitigate predatory losses by protecting clams or planting at low densities (see Peterson et al. 1995). The growth and mortality rates we observed may not be directly applicable to clams in other areas, but they do suggest that clams can be economically cultured in Indian River Shellfish Harvesting Area B and, further, that protected clams can be successfully planted at a SH ≥8 mm (SL ≥8.55 mm).

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THE EFFECTS OF LARVAL STOCKING DENSITY ON GROWTH, SURVIVAL, AND DEVELOPMENT OF LABORATORY-REARED *SPISULA SOLIDISSIMA SIMILIS* (SAY, 1822)

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ABSTRACT The optimal larval stocking density was determined for laboratory-reared *Spisula solidissima similis* (Say). Stocking density treatments of 10, 20, 30, and 50 larvae/mL were analyzed for effects on survival, growth, and development. Twenty-four-hour-old larvae were stocked at the above densities in 500-mL flasks containing seawater at 25 ppt and 20°C. All treatments received a daily food ration of 100,000 cells/mL of Tahitian strain *Isochrysis* sp., with complete water exchange per flask every 2 days. Three replicates per treatment were subsampled (n = 5), on days 1, 5, 9, 15, and 27. No significant differences ($p = 0.3539$) in survival occurred between stocking densities at day 27, with percent survival ranging from 56% for the 10 larvae/mL to 35% for the 50 larvae/mL treatments. Larval size (in micrometers) was significantly different for all treatments on day 9 ($p < 0.0001$: 50, $\bar{x} = 88.4$ μm < 20, $\bar{x} = 95.0$ μm < 30, $\bar{x} = 101.8$ μm < 10, $\bar{x} = 117.0$ μm) and day 27 ($p < 0.0001$: 50, $\bar{x} = 145.8$ μm < 30, $\bar{x} = 175.6$ μm < 20, $\bar{x} = 195.7$ μm < 10, $\bar{x} = 263.0$ μm). A higher percentage of animals had completed metamorphosis at day 27 in the lower stocking density treatment of 10 (87%) than in the higher stocking density treatments of 20, 30, and 50 larvae/mL (10, 3.0, and 0%, respectively). Optimal stocking densities for cultured *S. s. similis* larvae are equal to or less than 10 larvae/mL.

KEY WORDS: *Spisula*, larvae, culture, metamorphosis, survival, growth, development

INTRODUCTION

The Atlantic surfclam, *Spisula solidissima solidissima* (Dillwyn), which comprises the second largest clam fishery in U.S. waters (O'Bannon 1994), is primarily harvested from natural populations located in the nearshore to offshore waters of the northeastern United States (Ropes 1980). Because of the economic importance of the Atlantic surfclam, much information pertaining to the natural history and culturing of this clam is available (Ropes 1980). In contrast to *S. s. solidissima*, little information is available relating to the natural history and culturing of the southern Atlantic surfclam, *Spisula solidissima similis*. *S. s. similis* is not harvested currently; however, it has demonstrated an aquacultural potential in the coastal waters of the southeastern United States (Walker and Heffernan 1994, Kanti et al. 1993).

The southern Atlantic surfclam, *S. s. similis*, which occurs from Massachusetts to Florida and around the Gulf of Mexico to Texas (Abbott 1974), is found predominately in an oceanic environment; however, it also inhabits the shallow, high-energy, sandy environment of Sounds bordering the estuarine areas in coastal Georgia (Kanti et al. 1993, Walker and Heffernan 1994). Peak gametogenic maturity of *S. s. similis* in coastal Georgia occurs in early April, and the clam undergoes staggered spawnings from April until late June, depending on water temperature and salinity (Kanti et al. 1994). Longevity averages 1.5 y, and clams obtain a mean maximum size of 48 mm in coastal Georgia (Walker and Heffernan 1994). The rapid growth of the clam to a harvestable size and the attainment of sexual maturity by the age of 1 y demonstrate desirable culturing qualities, thus making *S. s. similis* a good aquacultural candidate.

Before larviculture studies are conducted, optimal laboratory conditions related to the gametogenic conditioning of a bivalve species must be documented, because of the dependent nature of egg viability and larval survival on the egg quality (Eversole 1988). Recent broodstock-conditioning studies of *S. s. similis* indicated that 25°C was the optimal temperature for the greatest female gamete release in spawning trials (Walker and Hurley 1995). Fecundity estimates for *S. s. similis*, based on the total number of eggs released over a spawning season in laboratory-kept animals, ranged from 0.14×10^6 to 13×10^6 eggs per female (Walker et al. 1996). The optimal temperature and salinity for larval culture of *S. s. similis* have been determined as 20°C and 25 ppt, respectively (Hurley and Walker in progress).

Knowledge of basic biologic requirements, as related to laboratory-rearing conditions, is key in the establishment or improvement of a commercially reared bivalve species. Factors of ration quality and quantity (Riisgard 1988, Riisgard 1991, Tan Tui et al. 1989, Walne 1970), salinity (Loosanoff and Davis 1963, Walker and Hurley 1995), rearing temperatures (Roosenberg et al. 1984), and stocking densities (Loosanoff and Davis 1963), and the effect that these factors have on growth and survival, play important roles in establishing the optimal rearing protocol to a particular life stage of a cultured bivalve species. The objective of this study was to define the optimal stocking density for laboratory-reared *S. s. similis* larvae for the greatest survival and growth from D-stage to metamorphosis.

MATERIALS AND METHODS

Broodstock obtained on March 22, 1995, from a population of *S. s. similis* located in Saint Catherines Sound, GA, were injected with 0.2 mL (2 mM solution) of serotonin on March 29, 1995, to induce gamete release. Gametes from all participating females (n = 17) and males (n = 26) were pooled, and eggs were fertilized at a 1/10 ratio of egg to sperm on the basis of light microscope count estimates of egg and sperm suspensions. Subsequent embryos were reared for 24 h in a 500-L culturing tank at 25 ppt salinity and 20°C before placement in stocking density treatments. Eighteen 1-L flask replicates filled to 500 mL with filtered (5 μm pore size) seawater were assigned to each treatment of 10, 20, 30, and 50 larvae/mL. All treatments were placed in temperature-controlled room held constant at 20 ± 1°C. Salinity was maintained at 25 ppt

for all treatments throughout the experiment. Each treatment received a food ration of 1.0×10^5 cells of Tahitian strain *Isochrysis* sp. administered once daily, with a complete water change on alternate days. Three replicate flasks from each treatment were subsampled and terminated on sample days 1, 5, 9, 15, and 27. Each subsample (n = 15) consisted of a 1-mL sample fixed in an equal volume of solution containing 10% neutral buffered formalin solution with Rose Bengal stain for later size and number examination by light microscopy. Size estimates for each sample date were based on recording the size (in micrometers) of 10 individuals per subsample per replicate, if possible. Survival estimates were based on the total number of larvae per 1 mL of each 2-mL fixed subsample suspension. Survival estimates were made by light microscopy examination of each 1-mL subsample placed on a Sedgewick-Rafter slide, at which time size estimates were also made with a calibrated ocular micrometer. Termination of the experiment was based on 50% metamorphosis (foot probing) of the larvae in a treatment.

Differences in growth and survival between treatments were determined by nested analysis of variance ($\alpha = 0.05$) and Tukey's studentized range test (SRT) ($\alpha = 0.05$). All percentage survival data were arcsine transformed before statistical analysis. Developmental data confidence intervals are based on table values (Rohlf and Sokal 1981). Statistical analysis was performed on SAS for PC (SAS Institute Inc. 1989).

RESULTS

Larval survival data for comparisons between stocking densities of *S. s. similis* are summarized in Table 1. Nested analysis of variance and Tukey's SRT showed no significant differences in survival existing between any of the stocking density treatments at any sample period throughout the 27-day duration of the experiment.

Larval growth data for comparisons between stocking density treatments are summarized in Table 2. Analysis of variance and Tukey's SRT showed that significant differences occurred in larval size between treatments at all sample periods. Significant differences in all treatments ($p < 0.0001$) by day 27 showed that larval size varied inversely with larval stocking density (Fig. 1).

Larval development to metamorphosis between treatments is summarized in Table 3. A greater percentage of metamorphosed larvae occurred in the lowest stocking density treatment of 10 larvae/mL (87%) as compared with the higher density treatments of 20 (10%), 30 (3%), and 50 (0%) larvae/mL. The failure of overlap of 95% confidence intervals with any of the other treatments indicates significantly greater metamorphosis in the lowest stocking density as compared with the higher stocking density (Table 3).

TABLE 1.

Percent mean survival (larvae/mL) of *S. s. similis* larvae by stocking density treatment and examination period (day).[a]

	Treatment: Stocking Density (Larvae/mL)			
	10	20	30	50
Sample Day and Percent Survival				
Day 0 ($p = 0.3109$)	106.6	86.8	93.7	89.4
Day 5 ($p = 0.7673$)	55.0	57.2	53.9	58.1
Day 9 ($p = 0.1698$)	46.2	78.6	64.4	61.9
Day 15 ($p = 0.1116$)	56.3	88.0	58.0	84.9
Day 27 ($p = 0.3539$)	56.3	50.3	44.1	35.4

[a] No significant differences in percent mean survival occurred, as determined by analysis of variance.

TABLE 2.

Mean larval size (μm) of *S. s. similis* by sample period (days).[a]

	Treatment: Stocking Density (Larvae/mL)			
	10	20	30	50
Sample Day and Tukey's Ranking				
Day 1 ($p < 0.0001$)	70.7c	72.4b	74.1a	73.6ab
Day 5 ($p < 0.0001$)	86.6a	80.4b	80.3b	80.7b
Day 9 ($p < 0.0001$)	117.0a	95.0c	101.75b	88.4d
Day 15 ($p < 0.0001$)	174.1a	123.0b	118.1bc	112.4c
Day 27 ($p < 0.0001$)	263.0a	195.7b	175.6c	145.8d

[a] Letters indicate significant differences, as determined by nested analysis of variance and Tukey's SRT ($\alpha = 0.05$).

DISCUSSION

The results of this study show that the optimal stocking density of those tested for *S. s. similis* is 10 larvae/mL as compared with the higher density treatments. Although larval survival differences between treatments were not significant at any sample period (Table 1), the lowest stocking density of 10 larvae/mL demonstrated significantly greater growth and development. A decrease in the rate of larval development and growth is detrimental from the biologic perspective of cohort survival (Loosanoff and Davis 1963), thus causing negative effects on the potential productivity of the aquaculturist.

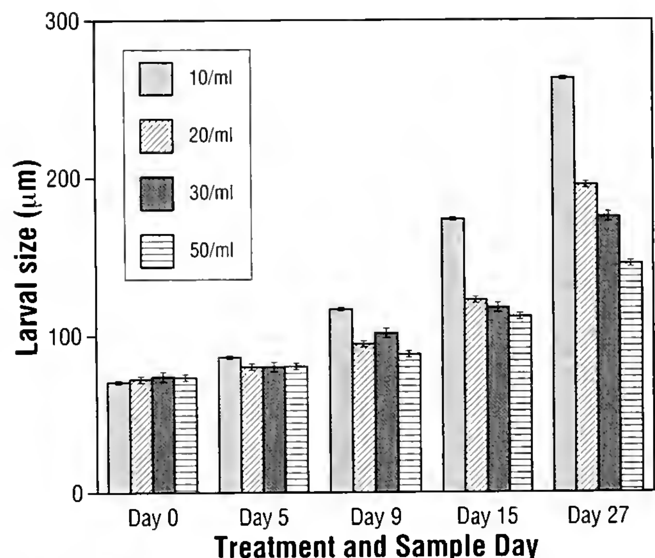

Figure 1. Mean larval size of *S. s. similis* by stocking density treatment (larvae/mL) and sample day.

TABLE 3.

Percent larval development of *S. s. similis* to metamorphosis on day 27 based on the examination of 30 animals per treatment with 95% confidence interval (C.I.).

Treatment (larvae/mL)	% Metamorphosis	95% C.I.
10	87	56.03–90.10
20	10	2.11–26.53
30	3	0.08–17.23
50	0	0.00–9.50

The inverse relationship between growth and larval stocking density is not unique to this study. Loosanoff and Davis (1963) found that stocking densities for bivalve larvae in excess of 20 larvae/mL can result in both increased mortality and reduced growth rates. The observed growth differences between treatments may be attributable to other factors, which, when coupled with stocking density effects, synergistically affect larval size.

Stocking density had a pronounced negative effect on larval development time requirements. The lowest stocking density of 10 larvae/mL had approximately an eightfold increase in metamorphosed animals (86%), as compared with the 20 larvae/mL treatment (10%) and the slowest developing treatments of 30 and 50 larvae/mL (3 and 0%, respectively). These proportional developmental differences between treatments, however, may be confounded by two primary factors. First, the standardized, fixed food ration was disproportional to larval abundance between treatments. Hence, larvae at greater densities could have been developmentally retarded by an effect of starvation (His et al. 1989) because suboptimal food rations have been shown to decrease larval developmental rates (Loosanoff and Davis 1963). In contrast, excessive ration quantity has been associated with high larval mortality due to a decrease in larval feeding activity (Riisgard 1991) and an increase in deleterious biomass production, which in turn increases undesirable microbial pathogens and bacterial contamination (Gulliard 1959, Tubiash 1972). Second, the completion of larval metamorphosis from pediveliger stage into fully metamorphosed animals can occur rapidly. However, this transition is not necessarily associated with increased larval size (Loosanoff and Davis 1963), but more likely is associated with the presence of certain environmental conditions (Wilson 1948, Tetrault and Rice 1994, Chevolot et al. 1991). The termination of the experiment, based on 50% complete metamorphosis of one or more treatments, was sufficient in duration for metamorphosis of the 10 larvae/mL treatment only, leaving the time requirement for the completion of metamorphosis in the remaining stocking density treatments unknown. However, on the basis of observed larval development, the stocking density treatment of 10 larvae/mL was clearly superior in reducing the time necessary for complete larval metamorphosis (Table 3). The time required for 50% complete larval metamorphosis in this study (27 days) is, however, prolonged in comparison to that required by *S. solidissima* (21 days) under similar rearing conditions (Goldberg 1989). The increase in time required for complete metamorphosis in *S. s. similis* as compared with *S. s. solidissima* may be the result of biologic developmental differences or the relative nutritional quality of the ration.

Algal suitability for bivalve larval rearing is determined by the algal species' ability to fulfill specific nutritional requirements of a particular bivalve species on the basis of the algae's biochemical composition (Tan Tui et al. 1989, Ewart and Epifano 1981), in addition to larval ingestion efficiency on the basis of algal cell size (Nelson et al. 1992) and relative algal abundance (Jaspersen and Olson 1982). The algae used in this study, Tahitian strain *Isochrysis* sp., are considered an excellent unicellular diet for clam bivalve larvae (Tan Tui et al. 1989, Ewart and Epifano 1981) and have been found to support complete larval metamorphosis in *Mercenaria mercenaria* and *Cytropleura costata* (Tan Tui et al. 1989), *Tapes semidecussata* (Helm and Laing 1987), *S. solidissima* (Goldberg 1989), and *S. s. similis* in this study.

Tolerance to crowding by bivalve larvae is species specific and accounts for only a portion of the total factors contributing to the establishment of an optimal stocking density. Loosanoff and Davis (1963) found that *M. mercenaria* larvae could be reared to complete metamorphosis in densities of 50 larvae/mL; however, growth rate was considerably reduced and disease susceptibility was found to increase. Helm and Laing (1987) suggested that the optimal stocking density for the growth and development of *Ostrea edulis* was 11–12 larvae/mL fed 4.0×10^4 to 8.0×10^4 cells/mL *Isochrysis galbana*. The highest larval growth in this study was associated with the lowest density treatment (10 larvae/mL) (Fig. 1) and more closely approaches the findings of Riisgard (1988) for 3- to 8-day-old *M. mercenaria* larvae fed a ration of 4×10^4 to 6×10^4 cells/mL of *I. galbana*. Riisgard did not attempt to quantify optimal larval stocking density to metamorphosis, but rather optimal cell stocking ration for maximal hourly clearance rates of larvae stocked at a density of 11/mL.

Our study did not address the effect that increasing larval size (i.e., growth) had on the increasing nutritional demands of the larvae, but feeding demands are assumed to increase with an increase in larval size. The ration quantity of 10×10^5 cells/mL of *I. galbana* was held constant for all treatments throughout the experiment in order to eliminate the uncontrolled effects that ration quantity may have had on larval growth and development if survival between treatments had been significantly different. The combined effects of an increase in filtering efficiency (Riisgard 1991), reduced competition for food (His et al. 1989), and reduced stress associated with the mechanical interactions of swimming larvae (Loosanoff and Davis 1963) in the lowest stocking density of 10 larvae/mL may be responsible for the significantly greater larval growth in the treatments from day 3 to day 27 of the study, as compared with the highest stocking densities.

This study clearly demonstrates that lower stocking densities of 10 larvae/mL are superior for growth and development in laboratory-cultured *S. s. similis* larvae compared with higher stocking densities. Factors contributing to growth and developmental differences between stocking densities need further investigation if questions concerning processes contributing to increased production in lower stocking densities of this subspecies are to be elucidated. From an aquacultural perspective, this study reinforces the biologic feasibility of hatchery-reared southern Atlantic surfclams as a future alternative bivalve resource.

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POPULATION DYNAMICS OF THE BIVALVES *VENUS ANTIQUA*, *TAGELUS DOMBEII*, AND *ENSIS MACHA* FROM CHILE AT 36°S[1]

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ABSTRACT *Venus antiqua* (King and Broderip, 1832), *Tagelus dombeii* (Lamarck, 1818), and *Ensis macha* (Molina, 1782) are dominant members of the shallow-water, soft-bottom communities of the Chilean coastal zone. The reproduction, growth, mortality, and production of these species were studied in a small bay in Chile at 36°S. All of these species had an annual reproductive cycle with a short spawning season in summer. The growth parameters of the von Bertalanffy growth function were: *V. antiqua:* L_∞ = 73.9 mm, K = 0.218/y, t_0 = −0.161/y; *T. dombeii:* L_∞ = 88.5 mm, K = 0.232/y, t_0 = −0.658/y; *E. macha:* L_∞ = 189.9 mm, K = 0.210/y, t_0 = −0.578/y. Somatic 1−, gonad production (grams of ash free dry weight per square meter per year) and the P/$\overline{B}$-ratio of the population were 22.0, 270.3, and 0.180 (*V. antiqua*), 7.8, 22.9, and 0.292 (*T. dombeii*), and 9.7, 98.5, and 0.222 (*E. macha*), respectively. Total mortality (Z) and natural mortality (M) were 1.084 and 0.275 (*V. antiqua*), 0.839 and 0.191 (*T. dombeii*), and 1.089 and 0.216/y (*E. macha*). Production estimates agree very well with data of the same superfamilies taken from the literature.

KEY WORDS: *Venus antiqua, Tagelus dombeii, Ensis macha,* bivalvia, reproduction, growth, mortality, production, Chile

INTRODUCTION

Upwelling of the Peruvian-Chilean ecosystem fuels high primary and secondary production, and bivalve species are dominant members of the benthic community forming valuable resources for artisanal fisheries. Besides their importance, little published information regarding their biology and ecology is available. The ecology of eight commercially important species, which are all distributed in a small bay (Bay of Dichato, Chile, 36°S), was studied recently by Urban (1992). Urban (1994a) discussed the morphological adaptation and niches of the six infaunal species and found that sediment type was the principal physical factor separating their niches and explaining certain morphological adaptations such as shell form, shell weight, and orientation of body axes. Urban and Campos (1994) studied the population dynamics of three of these infaunal species (*Gari solida, Semele solida,* and *Protothaca thaca*) and provided the first time estimates of somatic production and P/$\overline{B}$ ratios.

This article describes the population dynamics of the three other dominant infaunal species from the Bay of Dichato: *Venus antiqua* (King & Broderip, 1832), *Tagelus dombeii* (Lamarck, 1818), and *Ensis macha* (Molina, 1782). The objective is to compare the reproduction cycles, growth, mortality, and production estimates with those of the other bivalves from Dichato Bay and from the published literature. For the first time, a comparative investigation on the population dynamics of nearly all of the infaunal commercially important shallow-water bivalve species that are distributed in the Peruvian-Chilean upwelling system is presented, with a discussion of the fisheries of the exploited bivalve populations from the Bay of Dichato.

MATERIALS AND METHODS

Study Area and Field Sampling

The investigations were undertaken in the Bay of Dichato (36°32′S, 73°57′W), a small bay of approximately 12 km^2, described in Urban (1994a) (Fig. 1). Water temperatures range from 12°C in winter to 16°C in summer. *T. dombeii* and *E. macha* were collected in silty sediments at the northeastern entrance, and *V. antiqua* were collected in silty-stony sediments in the southwestern part of the bay from January 1991 to May 1992 (Fig. 1). Diver hand samples were taken at monthly intervals in 2- to 10-m depths, and all bivalves present in 5–10 randomly selected squares (0.25 m^2) were collected. In addition, every 2 mo, two to three sediment cores of 25 × 25-cm area and 20-cm depth were dug out and sieved through 1-mm-pore-size mesh to collect animals smaller than 5 mm shell length (SL). Sea surface temperature was recorded at each sampling.

Maximum length on the anterior-posterior axis (SL) was recorded from all individuals with vernier calipers. Subsamples of 30–40 individuals were taken from the monthly samples, and total wet weight (shell and body wet weight) was recorded. All soft parts were removed and dried at 60°C to constant weight to determine shell free dry weights (SFDW). Ash free dry weight (AFDW) was obtained by the ignition of dried soft parts at 550°C for 5 h. Valves were also used to age animals from external shell ring readings. Body weight cycles and the production of the population were calculated for the period April 1991 to March 1992 from pooled monthly length-frequency distributions.

Body Weight Cycles as an Indicator of Spawning Cycles

The parameters of the relationship between SL and SFDW (Eq. 1) were estimated by linear regression analysis on log-transformed data. Annual weight cycles for a standard individual of 50 g total wet weight were calculated as

$$SFDW = a\,SL^b \qquad (1)$$

where SFDW is in grams and SL is in millimeters. Analysis of variance was used to test for significant differences ($p < 0.05$) of regression lines of the length-weight relationships between successive months.

Body weight cycles were used to identify the reproductive cycle, with a decrease in weights between two successive months indicating a spawning event. Gonad histology was not available to

[1]Contribution no. 1111 of the Alfred-Wegener-Institut für Polar und Meeresforschung.

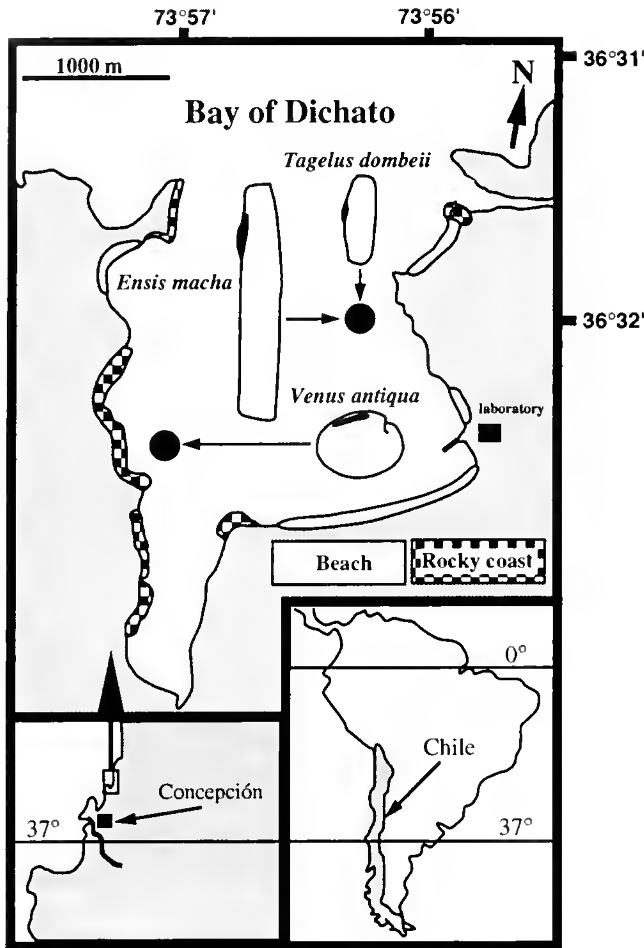

Figure 1. Location of sampling stations in the Bay of Dichato, Chile.

corroborate the indirect evidence of the spawning cycle. However, Urban and Campos (1994) found that the changes in the body weight cycles for all three of their species studied (*G. solida, S. solida, P. thaca*), were a good indicator of the spawning cycle on the basis of the histologic information available for *G. solida*.

Growth Rate Estimates

Annual shell growth rings on the surface of the valves of 30–40 individuals (of each species) were measured with vernier calipers. Disturbance rings were easily distinguished from annual rings because of the stronger and clearer appearance of the latter. Two data sets were obtained: (1) Individual growth increment data arranged as tagging-recapture data with constant time intervals ($t = 1$ y), and (2) mean length for each age was calculated from all individuals to obtain age-length data. The data were fit to the von Bertalanffy growth function, VBGF (von Bertalanffy 1938):

$$L_t = L_\infty(1 - e^{-K(t-t_0)}) \quad (2)$$

where L_∞ is the asymptotic length (in millimeters), K is the growth constant (per year), t is the age (in years), and t_0 is the age at zero length. Growth parameters were estimated by the use of Fabens' method (Fabens 1965), by fitting a rearranged function of Eq. 2 to size-increment data pairs (i.e., tagging-recapture data) by an iterative nonlinear least-square method (Simplex algorithm; Press et al. 1986):

$$L_2 = L_1 + (L_\infty - L_1)(1 - e^{-K(t_2-t_1)}) \quad (3)$$

where L_1 is the length at the beginning and L_2 is the length at the end of the time interval $t_2 - t_1$. t_0 was estimated by fitting the VBGF (Eq. 2) to age-length data with the Simplex algorithm.

In large individuals, it is difficult to separate and count the last few growth rings because they are very close together or overlapping because of reduced growth. Therefore, it is not unusual to obtain data that underestimate L_∞ and overestimate K because these two parameters are inversely related (Pauly 1979). These parameters were verified by an alternative method (modified Wetherall method) that estimates L_∞ from length-frequency data (Wetherall 1986, modified by Pauly 1986), where Beverton and Holt's (1956) Z-equation based on length data is rearranged to a linear aggression equation:

$$\bar{L} - L' = a + b L';\ Z/K = -(1 + b)/b;\ L_\infty = -a/b \quad (4)$$

$\bar{L}$ is the mean length of individuals of length L' and longer, and L' is the length for which all individuals of that length and longer are under full exploitation. In order to obtain correct growth parameters, the growth routine was rerun with fixed values of L_∞, calculated as above.

Mortality Rate Estimates

Total mortality Z was calculated with the single negative exponential model:

$$N_t = N_0 e^{-Zt} \quad (5)$$

(where t is the time and N_0 is the number of individuals at $t = 0$) and the length converted catch curve (Pauly 1983). Thereby, with the parameters of the VBGF, lengths of pooled length-frequency samples are converted into ages by the use of Eq. 6:

$$(N_i/\Delta t_i) = N_0' e^{-Zt_i} \quad (6)$$

where N_i is the number of individuals in length class i, Δt_i is the time required to grow through this size class, and t_i is the age of the middle-length class i. Mortality Z is calculated by linear regression analysis:

$$\mathrm{Ln}(N_i/\Delta t_i) = a + b\,t_i;\ Z = -b \quad (7)$$

Natural mortality M was estimated on an empirical basis with the relationship between the $P/\bar{B}$ ratio and maximum age, A_{max} (Eq. 9). Allen (1971) showed that in a steady-state population, the somatic $P/\bar{B}$ ratio equals Z, if mortality can be described by the single negative exponential model and growth follows the von Bertalanffy growth model. Thus, M can be estimated from empirical relations between the $P/\bar{B}$ ratio and maximum age, because in unexploited populations, Z equals M. Maximum age, A_{max}, was calculated with the inverse VBGF (Eq. 8); the VBGF parameters and maximum length, L_{max}, were taken from the growth estimates and pooled length-frequency samples.

$$A_{max} = t_0 - 1/K\ \mathrm{Ln}(1 - L_{max}/L_\infty) \quad (8)$$

Natural mortality was estimated with two empirical relationships (Eq. 9) taken from the literature: Hoenig (1983) and Etim and Brey (1994). The mean value was taken from these estimates.

$$\mathrm{Log}(P/\bar{B}) = a + b\ \mathrm{Log}(A_{max}) \quad (9)$$

Fishing mortality F (per year) was calculated after Eq. 10, and exploitation rate E (per year) was calculated after Eq. 11:

$$F = Z - M \quad (10)$$

$$E = F/Z \quad (11)$$

Production Estimates

On the basis of the results of the annual body weight cycle, the gonad production of the population P_{gon} (AFDW, in grams per square meter per year) was calculated from length-weight relations (Eq. 1) of the lowest body weight before and the highest body weight during the spawning season from monthly samples:

$$P_{gon} = \Sigma N_i (W_{i_{gon\ dur}} - W_{i_{gon\ bef}}) \quad (12)$$

where $W_{i_{gon\ dur}}$ and $W_{i_{gon.bef}}$ are body weights during and before the spawning season in length class i. The length of first maturity was calculated after Eversole (1989) as 25% of the maximum length (L_{max}) present in the pooled length-frequency data.

The somatic production of the population P (AFDW, in grams per square meter per year) was calculated by the weight-specific growth rate method (Crisp 1984; Eq. 13) from the mean of quantitative samples, pooled length-frequency data, the VBGF-parameters, and the length-weight relation:

$$P = \Sigma N_i W_i G_i \quad (13)$$

where N_i is the mean number of individuals (N per square meter), W_i is the mean body weight (AFDW, in grams) in length class i, and G_i (per year) is the weight-specific growth rate:

$$G_i = b K ((L_\infty/L_i) - 1) \quad (14)$$

where b is the exponent of the length-weight relation (Eq. 1), L_∞ and K are VBGF parameters, and L_i is the mean length in length class i. Individual somatic production P_{ind} (grams of AFDW per square meter per year) was calculated as follows:

$$P_{ind} = \Sigma W_i G_i \quad (15)$$

and the mean biomass of the population $\overline{B}$ (grams of AFDW per square meter per year) was calculated as:

$$\overline{B} = \Sigma N_i W_i \quad (16)$$

The P/$\overline{B}$ ratio of the population was calculated from somatic production P and mean biomass ©B.

RESULTS

Reproduction

Figure 2A shows the reproductive cycle of *G. solida* based on histologic sections from Urban and Campos (1994), and Figure 2B and C overlay the SFDW and sea surface temperature cycles for *G. solida*, *S. solida*, and *P. thaca*. Comparing the reproductive cycle (Fig. 2A) with the body weight cycle (Fig. 2B) of *G. solida* reveals that the high body weights in summer are principally caused by reproductive activities possibly triggered by low temperatures in winter (Urban and Campos 1994).

Figure 2D and E show the overlay of SFDW and sea surface temperature cycles for the bivalves *V. antiqua*, *T. dombeii*, and *E. macha* from this study. As observed in Figure 2B and C, the general body weight cycle follows the temperature cycle. Temperatures and body weights begin to rise from their lowest values in September/October and reach their highest values in January (the highest body weight of *V. antiqua* was observed in February; that of *E. macha* was seen in March). All three species had non-significant body weight changes in winter (May/June and August/September). It can therefore be assumed that the reproductive periods of *V. antiqua*, *T. dombeii*, and *E. macha* follow a pattern similar to that observed by Urban and Campos (1994) and that on the basis of the body weight cycles, the gonad production of the population can be estimated as the difference between the lowest annual body weight in winter and the highest body weight during the summer period by linear regression analysis for each class, accordingly. The linear regression parameters of the length weight relationships for the lowest and highest body weights are given in Table 1.

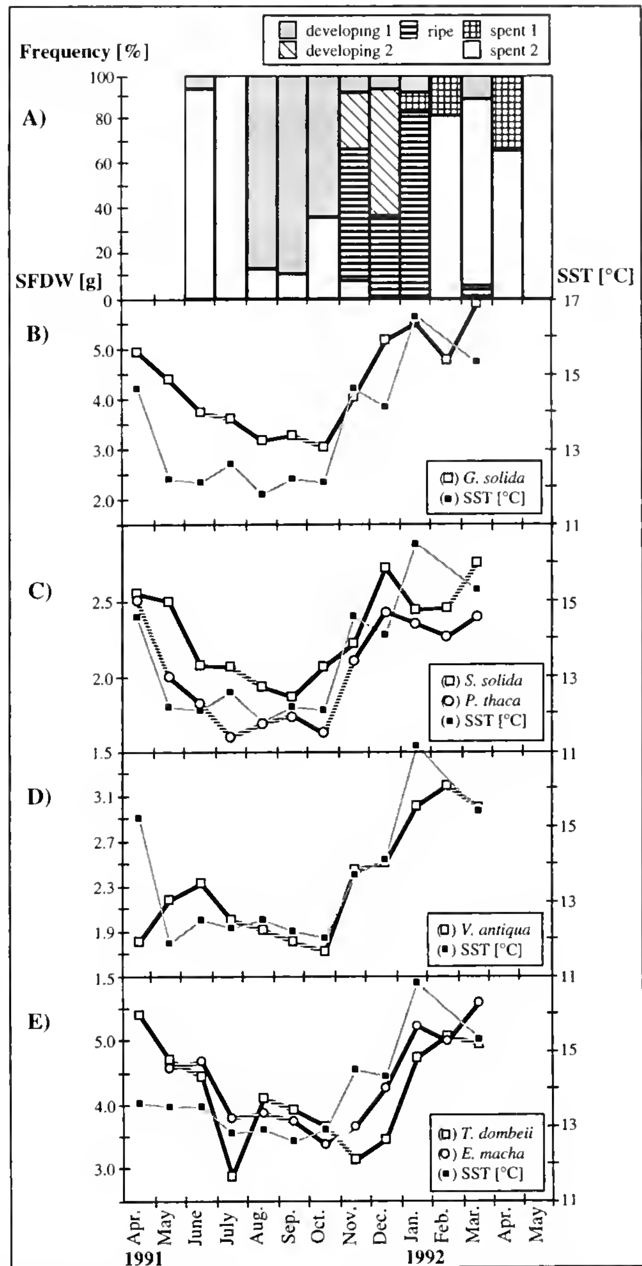

Figure 2. (A) Reproductive cycle, based on histologic sections of *G. solida*, for the period from June 1991 to May 1992. (B and C) Overlay of SFDW cycle for a 50-g total wet weight standard individual and the sea surface temperature (SST) cycle for the period from April 1991 to March 1992 for three Chilean bivalves, *G. salida*, *S. salida*, and *P. thaca*. (Fig. 2A–C taken from Urban and Campos 1994). (D and E) As for Figure 2B and C, but for the Chilean bivalves *V. antiqua* (D) and *T. dombeii* and *E. macha* (E). Solid lines (weight cycles): significant differences ($p \leq 0.05$) between successive months; broken lines: non-significant differences.

TABLE 1.

Parameters of the SL (mm)—SFDW (g) relationship of the Chilean bivalves *V. antiqua*, *T. dombeii*, and *E. macha* before and during the spawning season.[a]

Species	Month	a	b	r^2	n
V. antiqua	Oct 1991	−4.167	2.590	0.77	30
	Feb 1992	−3.839	2.748	0.66	30
T. dombeii	Nov 1991	−4.688	2.621	0.97	36
	Feb 1992	−5.135	3.048	0.98	35
E. macha	Oct 1991	−5.725	2.888	0.88	30
	Mar 1992	−4.893	2.793	0.68	29

[a] Log(SFDW) = a + b Log(SL).

Growth

The asymptotic lengths, L_∞, of *T. dombeii* and *E. macha* estimated with tagging-recapture and age-length data (Table 2) are much lower than the maximum lengths, L_{max} (Table 3), from the pooled length-frequency distributions, which indicates that L_∞ is underestimated, and accordingly, K is overestimated. The L_∞ values estimated with the Wetherall method (Table 2) are therefore better estimates of the parameter. The VBGF parameters K and t_0 shown in Table 2 were all estimated with the fixed L_∞ of the Wetherall method. The corresponding growth curves based on the mean K and t_0 values from tagging-recapture and age-length data are plotted together with age-length data in Figure 3. The curve fitting for *V. antiqua* and *T. dombeii* is better than that for *E. macha*.

Mortality

Length converted catch curves are shown in Figure 4. Total mortality Z values obtained from these regressions are given in Table 3; values are about $Z \approx 1/y$. Maximum age (A_{max}) and natural mortality values (M) of *T. dombeii* and *E. macha* are very similar: $A_{max} \approx 14$ y, $M \approx 0.3/y$ (Table 3). The natural mortality of *V. antiqua* was not computable because $L_{max} > L_\infty$. In this case, maximum age, A_{max}, cannot be calculated from L_{max} with the inverse VBGF (Eq. 8).

TABLE 3.

Total mortality Z, estimated with the catch curve-method and natural mortality M, estimated from two empirical relationships (between maximum age and the P/$\overline{B}$ ratio), of three Chilean bivalves, *V. antiqua*, *T. dombeii*, and *E. macha*.[a]

Species	Z	L_{max}	A_{max}	M[a]	M[b]	©M
Venus antiqua	1.084	74	Not computable $L_{max} > L_\infty$			
Tagelus dombeii	0.839	86	14.7	0.230	0.301	0.266
Ensis macha	1.089	180	13.5	0.254	0.328	0.291

[a] VBGF parameters, necessary for the catch curve-method and to calculate maximum age, A_{max}, were taken from Figure 3. Maximum length, L_{max} used to calculate A_{max}, was taken from the pooled length-frequency distribution. a, estimated with the empirical relationship of Hoenig (1983): $Log(P/\overline{B}) = 0.625 + 0.982\ Log(A_{max})$; b, estimated with the empirical relationship of Etim and Brey (1994): $Log(P/\overline{B}) = 0.682 + 1.130\ Log(A_{max})$.

Production

The parameters of the linear regression analysis of the length-weight relationship used for the calculation of somatic production are given in Table 4. Individual somatic production curves (Fig. 5) have a similar pattern. In *V. antiqua*, they reach their highest value with 0.50 g of AFDW per individual per year at 45 mm SL and decrease thereafter, whereas for *T. dombeii*, it is 0.35 g at 60 mm, and for *E. macha*, it is 0.90 g at 130 mm. Individual gonad production is reflected by exponential curves beginning at 20 mm SL in *V. antiqua*, 25 mm in *T. dombeii*, and 45 mm in *E. macha*.

The length-frequency distributions for the three species show that they are normally distributed (Fig. 6). In *V. antiqua*, the distribution is unimodal, with a size range from 45 to 80 mm, with no small specimens or recruits present. *T. dombeii* and *E. macha* each have a major mode with two smaller modes (*T. dombeii*, between 15 and 50 mm; *E. macha*, between 45 and 105 mm).

The results for the somatic production of the population and mean biomass estimates are given in Table 5. The highest values were recorded for *V. antiqua* (22.0 and 122.0 g of AFDW/m^2 per year), followed by *E. macha* (9.7 and 43.6 g of AFDW/m^2 per

TABLE 2.

VBGF parameters of three Chilean bivalves, *V. antiqua*, *T. dombeii*, and *E. macha*, estimated from two data sets: tagging-recapture data (with Fabens method) and age-length data (with the VBGF).[a]

Species	L_∞	K	t_0	r^2	L_∞[a]	K[b]	t_0[b]	r^{2b}	n
Tagging-recapture data									
V. antiqua	74.6	0.219	—	0.99	73.9	0.223	—	0.99	35
T. dombeii	83.5	0.275	—	0.99	88.5	0.242	—	0.99	44
E. macha	163.8	0.321	—	0.99	189.9	0.222	—	0.97	51
Age-length data									
V. antiqua	73.6	0.225	0	0.99	73.9	0.212	−0.161	0.99	6
T. dombeii	81.0	0.323	0	0.99	88.5	0.221	−0.658	0.97	10
E. macha	163.7	0.318	0	0.99	189.9	0.198	−0.578	0.96	9

[a] a, estimated after Wetherall with pooled length-frequency distribution data; b, estimated with fixed L_∞ (after Wetherall); L_∞, asymptotic length (mm); K, growth constant (per y); t_0, age at zero length; n (for tagging-recapture data), number of growth increments; n (for age-length data), number of ages (= y) corresponding to a mean length, which was calculated from the growth ring data; $r^2 = 1 - RSS/TSS$; RSS, residual sum of squares; TSS, total sum of squares.

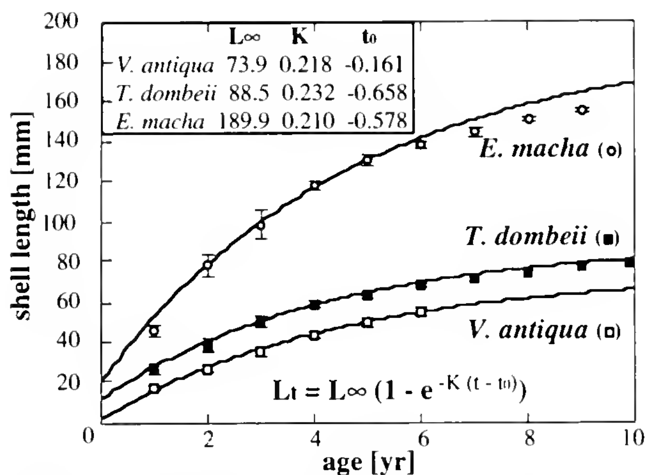

Figure 3. Growth curves of the VBGF and age-length data of three Chilean bivalves, *V. antiqua*, *T. dombeii*, and *E. macha*. VBGF parameters (asymptotic length, L_∞ [mm]; growth constant, K [per y]; age at zero length, t_0) are given in the inset. Vertical bars indicate standard deviations of the mean lengths of the age-length data.

year), and finally, *T. dombeii* (7.8 and 26.7 g of AFDW/m^2 per year). The gonad production of the population was much higher than the somatic production. The following P/$\overline{B}$ ratios were calculated: *T. dombeii* = 0.292, *E. macha* = 0.222, *V. antiqua* = 0.180.

DISCUSSION

Reproduction

All three species of this study exhibited annual reproductive cycles with a short summer spawning period. The influence of latitudinal gradients on the reproductive strategies of *G. solida*, *S. solida*, and *P. thaca* are discussed in Urban and Campos (1994). At 36°S, all three species have annual reproductive cycles with longer resting periods in winter, whereas at lower latitudes, *G. solida* from 14°S, Independence Bay, Peru (Ishiyama and Chávez 1990), *S. solida* from 30°S, Tongoy, Chile (Campos et al. 1993), and *P. thaca* from 23°S, San Jorge Bay, Chile (Henríquez et al. 1981), have biannual reproductive cycles, probably with shorter resting periods or continuous gonad activities throughout the year. Except for *V. antiqua*, no other examples of reproductive studies of the same species studied here were found in the literature. *Tagelus divisus* from 25°N, Biscayne Bay, FL (Fraser 1967), has a biannual body weight cycle with strong increases (followed by spawning events) from September to December and a second smaller increase from March to June. *Ensis minor* from 41°N, Gulf of Manfredonia, Italy (Casavola et al. 1985), on the other hand, has a very short spawning period in March and April followed by a long resting period. All of these results confirm the well-known dependency of the reproductive strategy on latitudinal-dependent factors (e.g., different temperature regimens): with increasing latitudes (in a northern or southern direction), reproductive strategies seem to change from continuous to biannual to annual cycles.

There are, however, certain discrepancies, such as those of *V. antiqua* from the Bay of Ancud, Chile (Lozada and Bustos 1984), which has a continuous biannual reproductive cycle, although located further south (at 43°S) than the Bay of Dichato of this study.

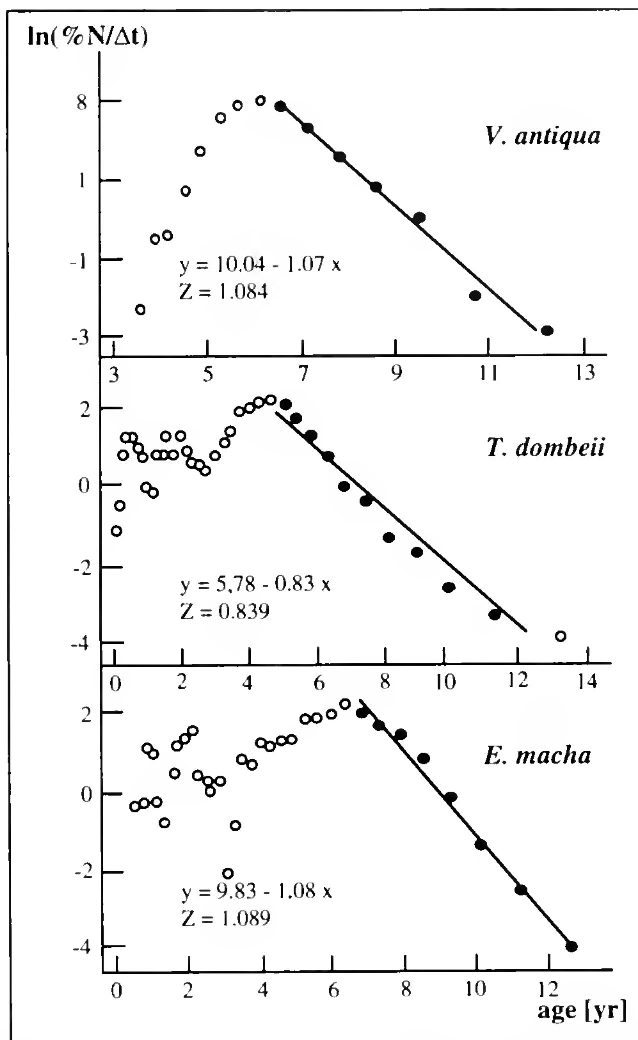

Figure 4. Length converted catch curves of three Chilean bivalves, *V. antiqua*, *T. dombeii*, and *E. macha* based on pooled length-frequency samples (from April 1991 to March 1992). The VBGF parameters L_∞, K, and t_0 required for this method are given in Figure 3. Solid symbols: used for calculations of total mortality (Z); open symbols: excluded from calculations. Linear regression equation and estimated Z values are given.

This could be because of local specific ambient conditions (e.g., currents, upwelling, and nutrients) of the Bay of Ancud that may make it a more productive region than the Bay of Dichato (which is a rather protected bay). Another reason could be that *V. antiqua*

TABLE 4.

Parameters of the SL (mm) AFDW (g) relationship for three Chilean bivalves, *V. antiqua*, *T. dombeii*, and *E. macha*.[a]

Species	a	b	r^2	n
V. antiqua	−4.297	2.689	0.93	35
T. dombeii	−5.277	2.978	0.99	29
E. macha	−6.494	3.285	0.96	33

[a] Log(AFDW) = a + b Log(SL).

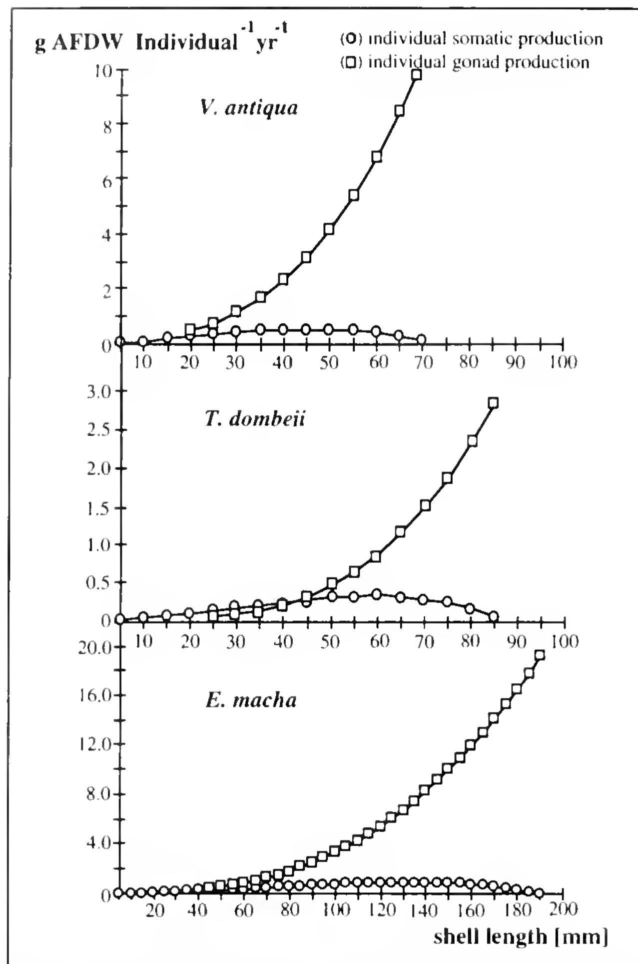

Figure 5. Individual production of somatic tissues and individual gonad production for different length classes of three Chilean bivalves, *V. antiqua*, *T. dombeii*, and *E. macha*.

is better adapted to the thermal regimen because it has a more southern distribution limit than *G. solida*, *S. solida*, and *P. thaca* (Urban 1994b). *V. antiqua* is found up to the Magellan Strait, south of Chile, 55°S (Urban and Tesch 1996).

TABLE 5.

Summary of production estimation for three Chilean bivalves, *V. antiqua*, *T. dombeii*, and *E. macha*.

Parameter	Species		
	V. antiqua	*T. dombeii*	*E. macha*
Population gonad production ($g\ AFDW/m^2$ per y)	270.3	22.9	98.5
Population somatic production ($g\ AFDW/m^2$ per y)	22.0	7.8	9.7
Population mean biomass ($g\ AFDW/m^2$ per y)	122.0	26.7	43.6
Population P/$\overline{B}$ ratio	0.180	0.292	0.222
Mean abundance ($g\ AFDW/m^2$)	47.5	26.2	12.6
Mean body weight (g AFDW)	2.57	1.02	3.46

Growth

There are no published growth rates for the species of this study, other than for *V. antiqua* from the Bay of Yaldad, Chile 43°S (Clasing et al. 1994). Their initial VBGF parameters (L_∞ = 71.2 mm and K = 0.224/y) are almost identical to those of the population studied in Bay of Dichato (L_∞ = 73.9 mm and K = 0.218/y). According to Knight (1968) and Theisen (1973), very misleading species-specific estimates of L_∞ can be obtained with data that does not cover most of a species growth range. Consequently, Clasing et al. (1994) did estimate a second set of VBGF parameters based on the maximum length of their population (L_{max} = 80 mm) that resulted in K = 0.183/y. Fortunately, the *V. antiqua* population from Dichato had an estimated L_∞ and an actual observed maximum length that were almost identical (L_∞ = 73.9 mm, L_{max} = 74 mm), which could be explained by commercial fishing activities.

The VBGF parameters L_∞ and K are inversely related; consequently, it is not advisable to use only one of them, for example K, for growth comparisons. An index that considers the relationship between L_∞ and K was developed by Munro and Pauly [1983; $\Phi' = \mathrm{Log}(K) + 2\,\mathrm{Log}(L_\infty)$]. Comparing the Φ' values (*V. antiqua*, Dichato = 3.076; *V. antiqua*, Yaldad = 3.069; *T. dombeii* = 3.259; *E. macha* = 3.879) gives almost identical growth for the two *V. antiqua* populations, a slightly higher value for *T. dombeii*, and the highest growth performance for *E. macha*.

Mortality

The mortality data of all six infaunal bivalve species from the Bay of Dichato are summarized in Table 6 (this study and Urban and Campos 1994). The information reveals some similar features. Maximum age ranges between 13 and 17 y; natural mortality, M, lies around 0.3 and is much lower than the fishing mortality, leading to high exploitation rates, E, around 0.7.

Little comparative information exists in the literature. Saldivia (1981) estimated the mortality of *V. antiqua* from the Bay of Ancud, Chile (43°S). He obtained Z = 0.43, M = 0.38, F = 0.05, and E = 0.12. As can be seen by the very low E value, in

TABLE 6.

Summary of mortality data for the six Chilean bivalve species obtained from Urban and Campos (1994) and from this study.[a]

Species	A_{max}	Z	$\overline{M}$	F	E
G. solida	13.5	0.846	0.291	0.555	0.656
S. solida	12.7	0.916	0.310	0.606	0.662
P. thaca	17.2	0.628	0.226	0.402	0.640
V. antiqua[b]	11.7	1.084	0.337	0.747	0.689
T. dombeii	14.7	0.839	0.266	0.573	0.683
E. macha	13.5	1.089	0.291	0.798	0.733

[a] Z is total mortality, M is mean natural mortality, F is fishing mortality, and E is exploitation rate. Also given is A_{max} (y), the maximum age.
[b] Calculated with L_∞ = 80.0 mm, taken from Clasing et al. (1994), because L_∞ found in this study is smaller than L_{max}.

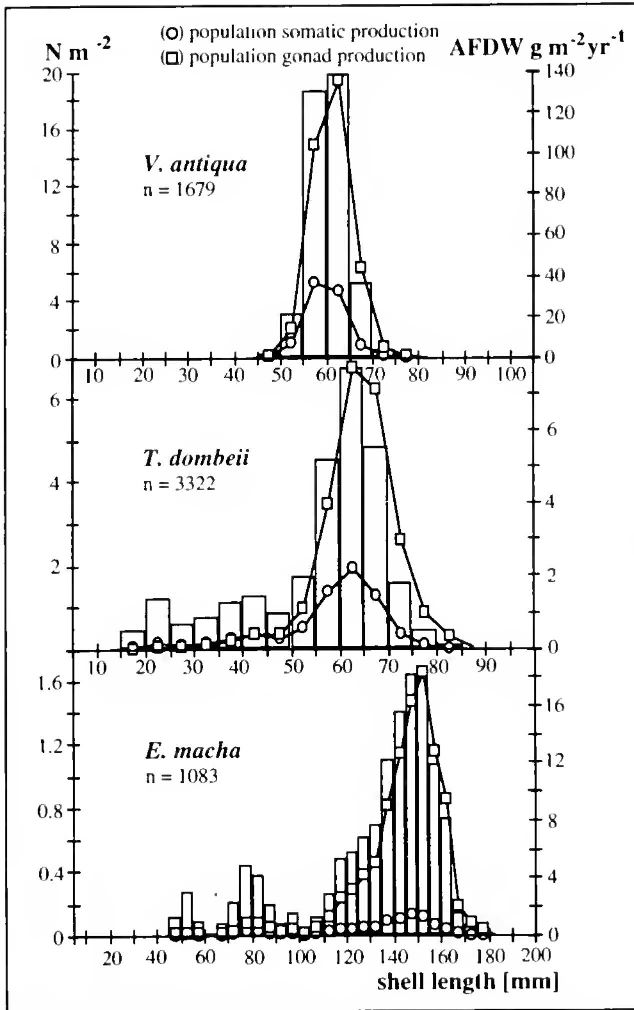

Figure 6. Distribution of somatic production and gonad production of the population as well as mean abundance for different length classes of three Chilean bivalves, *V. antiqua*, *T. dombeii*, and *E. macha*.

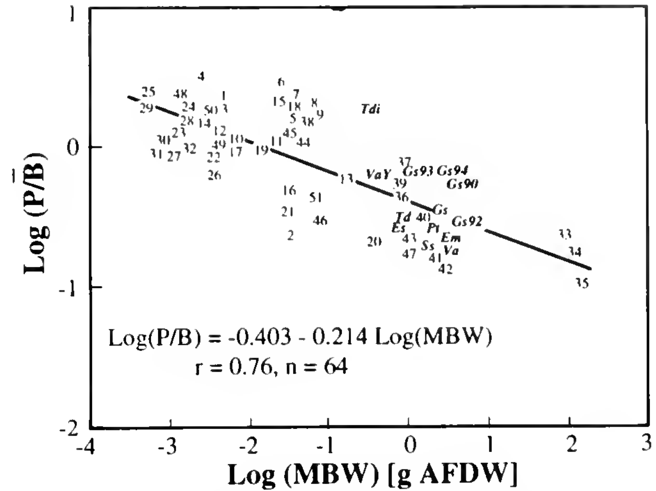

Figure 7. Comparison of somatic production of three Chilean bivalve species with other bivalve species of the same superfamilies (taken from the literature) by plotting the P/$\bar{B}$ ratio against the mean body weight (MBW). Below, name of species, location of study area, and literature source. (I) same orders from other areas or related species from the same area—Va: *V. antiqua*, Td: *T. dombeii*, Em: *E. macha*, Bay of Dichato, Chile, 36°S (this study); Gs: *G. solida*, Ss: *S. solida*, Pt: *P. thaca*, Bay of Dichato, Chile, 36°S (Urban and Campos 1994); *Gs90*, *Gs92*, *Gs93*, *Gs94*: *G. solida* from four different years, Independence Bay, Peru, 14°S (Urban and Tarazona 1996); VaY: *V. antiqua*, Bay of Yaldad, Chile, 43°S (Clasing et al. 1994); Tdi: *T. divisus*, Florida (Fraser 1967); Es: *Ensis siligua*, South Wales, United Kingdom (UK) (Warwick et al. 1978); (II) superfamily *Tellinacea*—1: *Donax vittatus*, South Wales, UK (Warwick et al. 1978); 2: *Scrobicularia plana*, Cornwall, UK (Warwick and Price 1975); 3, 4: *Abra alba*, Kiel Bight, Germany (Rainer 1985); 5–9: *A. alba*, Manche, France (Dauvin 1986); 10: *Abra nitida*, Northumberland, UK (Buchanan and Warwick 1974); 11, 12: *A. nitida*, North Sea, Sweden (Josefson 1982); 13: *Pharus legumen*, South Wales, UK (Warwick et al. 1978); 14: *Macoma balthica*, Nova Scotia, Canada (Burke and Mann 1974); 15, 16: *M. balthica*, North Sea, Denmark (Madsen and Jensen 1987); 17: *M. balthica*, Cornwall, UK (Warwick and Price 1975); 18, 19: *M. balthica*, North Sea, Netherlands (Wolff and de Wolf 1977); 20, 21: *Macoma calcarea*, Arctic Sea, Greenland (Petersen 1978); 22–29: *Tellina fabula*, German Bight, Germany (Salzwedel 1980); 30, 31: *T. fabula*, Yorkshire, UK (Rees 1983); 32: *T. fabula*, South Wales, UK (Warwick et al. 1978); (III) superfamily *Veneracea*—33–35: *Callista brevisiphonata*, Sea of Japan, Russia (Selin and Selina 1988); 36, 37: *Chione cancellata*, Florida, USA (Moore and Lopez 1969); 38: *Dasinia elegans*, Florida, USA (Moore and Lopez 1970); 39–41: *Mercenaria mercenaria*, Southampton (Hibbert 1976); 42, 43: *M. mercenaria*, Georgia, (Walker and Tenore 1984); 44, 45: *Venerupis aurea*, Southampton, UK (Hibbert 1976); 46: *Venerupis decussata*, Southampton, UK (Hibbert 1976); 47: *Venerupis pullastra*, North Sea, Norway (Johannessen 1973); 48–50: *Venus ovata*, Manche, France (Dauvin 1985); 51: *Venus striatula*, South Wales, UK (Warwick et al. 1978).

1981, this particular population must have been exploited at a very low level. Clasing et al. (1994), who studied the population dynamics of *V. antiqua* from the Bay of Yaldad, reported Z = 0.66, M = 0.33, F = 0.33, and E = 0.50, which are in accordance with the data obtained in this study.

According to Beddington and Cooke (1983), Caddy and Csirke (1983), and Francis (1974), an exploitation rate of E = 0.5 is above the optimal rate of exploitation for a fishery. Therefore, it can be assumed that all six bivalve species from the Bay of Dichato are severely overexploited. Indeed, fisherman from Dichato reported that catches in the Bay of Dichato have declined considerably during the last few years, so now, most of the fishermen exploit other banks or other resources. During the study period, little fishing activity was observed in the Bay of Dichato.

The methods used here to calculate total and natural mortality are only valid under the assumption of a steady-state population. Unexploited populations under nonchanging biotic and abiotic conditions or populations exploited with the same exploitation rate for some time can be assumed to fulfill this assumption. The statements that catches have declined during the last years (with seemingly similar effort levels by commercial fishers) and that fishermen now exploit other resources (see previous paragraph) appear at first to suggest that the steady-state assumption is not valid anymore. However, the steady-state assumption may still be true for the following reasons: the exploited species from Dichato are rather long-lived individuals (13–17 y; Table 6); changes in fishing pressure/effort, on the other hand, are only a very recent (3–4 y) development (personal information of local fishermen). Therefore, it is deemed acceptable to use the standard methods for the estimation of Z, M, F, and E for the Dichato populations

because they consisted mainly of old (and large) specimens (Fig. 6 in Urban and Campos 1994 and Fig. 6 in this study) when the study was conducted.

Production

The P/©B values of the six Chilean bivalve species from the Bay of Dichato (studied here and taken from Urban and Campos 1994) are plotted together with literature data on related bivalve species over their corresponding mean body weights (Fig. 7). The values of *V. antiqua*, *T. dombeii*, and *E. macha* are very similar to those of *G. solida*, *S. solida*, and *P. thaca*. No other production estimates exist in the literature except for *V. antiqua* from the Bay of Yaldad, Chile, 43°S (Clasing et al. 1994), and for *G. solida* from Independence Bay, Peru, 14°S (Urban and Tarazona 1996). Comparatively, the latter two populations have higher P/©B values (Fig. 7). A possible explanation for the higher somatic production of the populations from the Bay of Yaldad and Independence Bay is that Clasing et al. (1994) and Urban and Tarazona (1996) described their investigation areas as being very productive, with a high level of secondary production. Interestingly, Clasing et al. (1994) and Urban and Tarazona (1996) also reported strong fishing activities within their study areas. According to Urban and Campos (1994) SCUBA-diving fisherman select the larger bivalves in order to obtain better market prices. Thereby, the size-frequency distribution of the population is shifted toward smaller individuals with higher somatic production. Thus, if—because of recruitment—biomass remains constant, such populations have higher $P/\bar{B}$ ratios (Hall et al. 1970).

The $P/\bar{B}$ values of the Peruvian-Chilean populations seem to form a separate cluster with low productivity and high mean biomass values (Fig. 7). Length-frequency distributions show that all populations consist of old and large individuals (Fig. 6, this study; Fig. 6, Urban and Campos 1994). Hence, these individuals devote most of their surplus energy to gonad production rather than to somatic production (growth), leading to low $P/\bar{B}$ values. The reason is not clear; it could be, however, an adaptation to the high productivity of the upwelling system: the bivalves studied here are well adapted, such that they form dense populations all over their distribution range, dominating the benthic ecosystem (Urban 1994a). Most likely, recruitment to the population is space-habitat limited.

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SHELL LENGTH–MEAT WEIGHT RELATIONSHIPS OF OCEAN QUAHOG, *ARCTICA ISLANDICA* (LINNAEUS, 1767), FROM ICELANDIC WATERS

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ABSTRACT Shell length–meat weight relationships were analyzed for the ocean quahog, *Arctica islandica*, for three geographic areas in Iceland. The allometric growth curves for the three areas were: northwest area, $W = 0.0000567\ L^{3.08}$; north area, $W = 0.000173\ L^{2.75}$; east area, $W = 0.0000929\ L^{2.92}$. However, when the allometric growth model was used, the three condition factors were not found to be significantly different from each other Only one condition factor was therefore estimated for all areas but three different slopes: northwest area, $W = 0.000637\ L^{3.05}$; north area, $W = 0.000637\ L^{2.98}$; east area, $W = 0.000637\ L^{3.01}$. When assuming isometric growth for the three areas (i.e., the slope = 3.0), the condition factors were found to be significantly different from each other; however, this model did not fit the data as well as the allometric one. The results from assuming either isometric or allometric growth indicated that the greatest relative meat weights for similar-sized quahogs were observed in the northwestern area. This may be due to the higher temperature and productivity of the northwestern area compared with the others.

KEY WORDS: *Arctica islandica*, ocean quahog, shell length–meat weight relationship

INTRODUCTION

The ocean quahog, *Arctica islandica*, is one of the largest commercially fished bivalve molluscs inhabiting the marine waters of Europe and North America. It occurs in the North Atlantic along the East Coast of North America from Newfoundland to Cape Hatteras; on the coasts of Iceland, Faroes, the Shetlands, and the British Isles; and along the European Coast from the White and Barrents Seas to the Bay of Cadiz in Spain (Merrill and Ropes 1969). It is found in waters as shallow as 4 m to as deep as 256 m, but the commercial fisheries occur on the continental shelf in waters from about 25 to 60 m deep (Merrill and Ropes 1969). Icelandic fisheries have only just begun, but investigations on distribution and abundance indicate a major resource that could support a commercial fishery (Thórarinsdóttir and Einarsson 1996 in press).

Much is known about the reproduction, growth, and age of the ocean quahog in North America (Rowell et al. 1990; Fritz 1991, Kraus et al. 1991, Kennish et al. 1994), and some recent information about recruitment and mortality rates also exists (Anonymous 1993, Weinberg 1993, Anonymous 1995, Kennish and Lutz 1995). Aspects of ocean quahog density and distribution along the eastern coast of North America have been reviewed by Merrill and Ropes (1969, 1970), Fogarty (1981), Rowell and Chaisson (1983), and Chaisson and Rowell (1985), whereas the shell length–meat weight relationships of ocean quahog were examined by Murawski and Serchuk (1979), Murawski et al. (1982), and Fritz (1991). Little is known about populations of this species in the vicinity of Iceland. The objective of this study was to determine the shell length–meat weight relationship of *A. islandica* at the northwestern, northern, and eastern coasts of Iceland and to determine if there were significant differences in the mean weights calculated from direct observations and mean meat weights calculated by using the length-weight relationship.

MATERIALS AND METHODS

Ocean quahog samples for the length-weight analysis were collected from Icelandic waters during assessment surveys conducted in January to March 1994 and May to June 1994 in the three principal areas, as shown in Figure 1. A commercial hydraulic clam dredge was used for the sampling (1.5-m-wide cutting blade). The spacing between bars in the dredge was 34 mm. Sampling sites in each area were identified on sea charts according to the bottom topography and depths. Tows were then made from water depths ranging from 5 to 50 m.

Individuals were randomly sampled for shell length measurements at each site and measured directly from dredge catches on the site (on-site measurements). The length-frequency distributions for the three areas were compared by use of the Kolmogorov-Smirnoff two-sample test (see Thórarinsdóttir and Einarsson 1996 in press).

Subsamples from each area were frozen and later thawed to determine shell length and meat weight relationships. Shell length was measured with vernier calipers to the nearest 0.1 mm, and the wet meat weight was determined to the nearest 0.1 g.

The relationship between meat weight and shell length was assumed to be of the form:

$$W = c\ L^{p} \quad (1)$$

where W is the wet meat weight (in grams), L is the shell length (in millimeters), and c and p are coefficients to be estimated, i.e.,

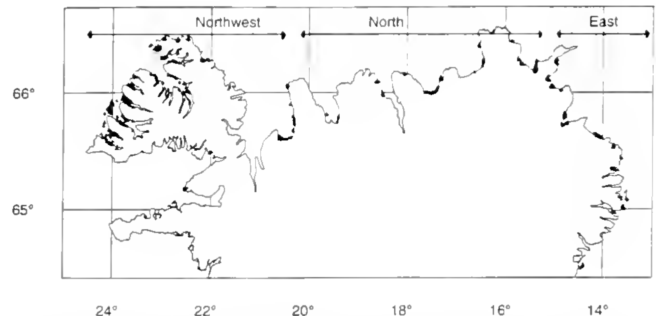

Figure 1. The locations of sample sites (black triangles) for *A. islandica*, in northwest, north, and east sampling areas of Iceland, surveyed from January to June 1994.

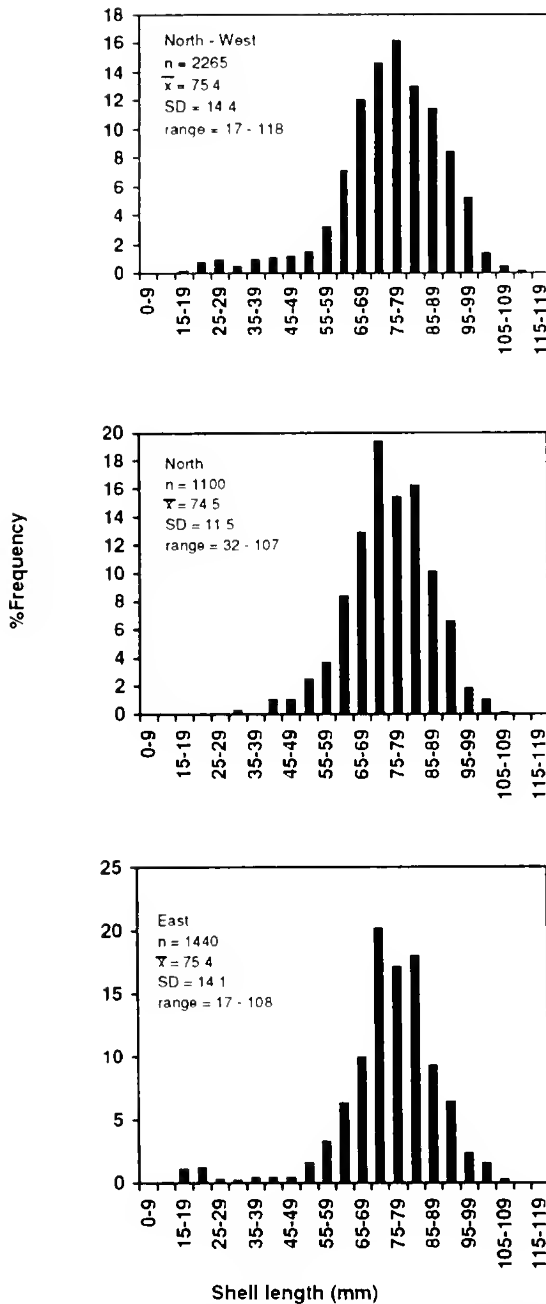

Figure 2. Shell length–frequency distribution for *A. islandica* (on-site measurements) from northwest, north, and east sampling areas of Iceland. Also shown are the mean shell length, standard deviation, and range.

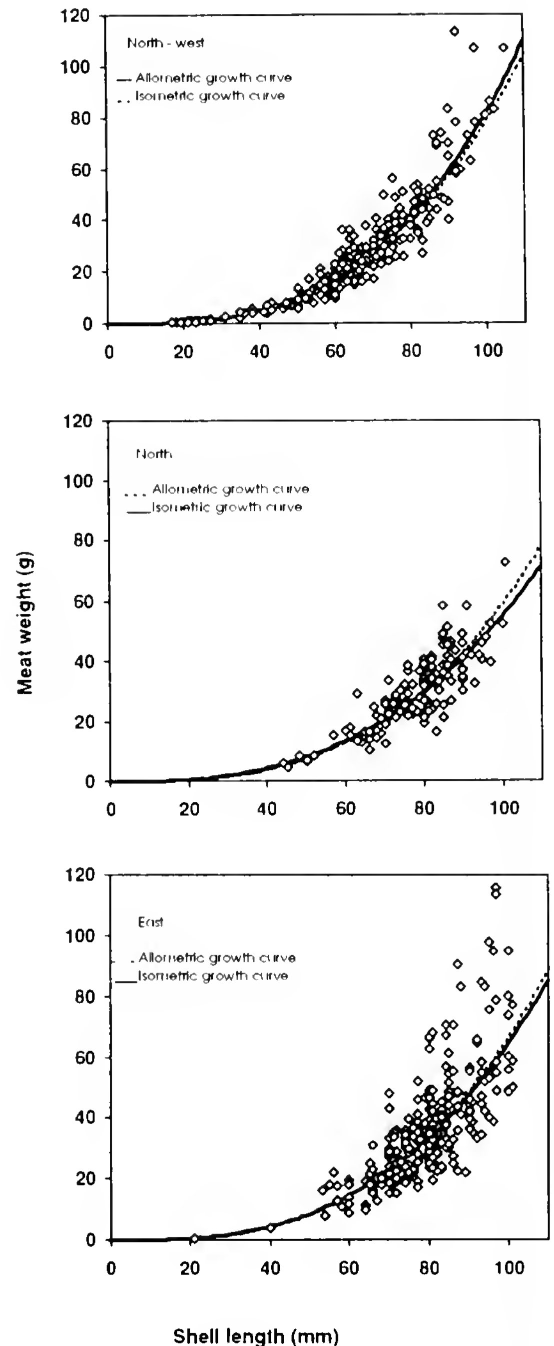

Figure 3. Estimated shell length–meat weight relationship for *A. islandica* from northwest, north, and east sampling areas of Iceland. The model estimates allometric and isometric growth curves with different condition factors and slopes for each of the three areas.

the condition factor and the slope. To estimate c and p, Eq. 1 was log transformed, making the estimation a matter of a simple linear regression:

$$\log(W) = \log(c) + p \log(L) \quad (2)$$

An interesting special case of Eq. 2 is when the slope (p) is fixed equal to 3.0—isometric growth (implying unchanged ratios of linear measurements as the organism grows).

Analysis of covariance (ANCOVA) was applied to compare the length-weight relationship between the three areas defined in Figure 1 and to explore deviation from the isometric relationship ($p = 3.0$).

The analysis was performed with *S-plus* (Becker et al. 1988, Chambers and Hastie 1992, Venables and Ripley 1994).

RESULTS

The shell length frequency distributions and statistical summaries of length data for the ocean quahogs from the three areas are presented in Figure 2. No significant differences ($p > 0.05$) were

TABLE 1.

The estimated shell length–meat weight relationship: type of relationship, ln of the condition factor (Cond.) ± s.e. in brackets, the condition factor (Cond.), the slope ± s.e., r^2 total SSE (sum of squared errors), and p value from an F test indicating the significance of using an allometric relationship.

Area	Type	ln Cond. (±s.e.)	Cond.	Slope	r2	n	SSE	p Value
	Shell Length–Meat Weight Relationship							
NW	Isometric	−9.45 (0.014)	0.0790E-3	3.00 (Fixed)	0.966	277	14.20	
—	Allometric	−9.78 (0.14)	0.0567E-3	3.08 (0.034)	0.967		13.91	0.018
N	Isometric	−9.74 (0.020)	0.0588E-3	3.00 (Fixed)	0.773	134	7.17	
—	Allometric	−8.66 (0.55)	0.173E-3	2.75 (0.13)	0.779		6.87	0.054
E	Isometric	−9.61 (0.018)	0.0667E-3	3.00 (Fixed)	0.725	259	21.66	
—	Allometric	−9.28 (0.49)	0.0929E-3	2.92 (0.11)	0.725		21.62	0.50
All Areas	Isometric	−9.57 (0.011)	0.0698E-3	3.00 (Fixed)	0.914	670	51.64	
—	Allometric	−9.11 (0.15)	0.0110E-3	2.89 (0.34)	0.915		50.87	0.002

observed for the mean shell lengths from the northwestern (75.4 mm), northern (74.7 mm), and eastern (74.5 mm) areas. The shell length frequency distribution from the northwestern area was significantly different ($p < 0.05$) from the others (Thórarinsdóttir and Einarsson 1996 in press).

An ANCOVA was carried out for the model:

$$\log(W_{ia}) = \log(c_a) + p_a \log(L_{ia}) + \epsilon_{ia} \quad (3)$$

Where W_{ia} and L_{ia} are the i-th measurements from area a, c_a and p_a are area-specific condition factors and slopes, and ϵ_{ia} is a normal error term (classic multiple regression). The results showed that the estimated slope (p) was highest in the northwestern area and the lowest in the northern area. In contrast, the condition factor (c) was highest in the north, followed by the east and then the northwest (Table 1, Fig. 3). Reducing the model in Eq. 3 to a model that estimates only one condition factor and slope, $\log(c) + p\log(L_{ia})$ was not significant: for $\log(c) + p\log(L_{ia})$, the d.f. was 668; RSS (residual sum of squares) was 50.874; and R^2 was 0.9148. For $\log(c_a) + p_a \log(Li_{ia})$, the d.f. was 664; RSS was 42.405; R^3 was 0.9290; F value was 33.151; and $p < 0.01$.

Similarly, reducing the full model in Eq. 3 to a model that estimates only one slope and three different condition factors, $\log(c_a) + p\log(L_{ia})$, was not significant ($R^2 = 0.9283$ and p value for differences in RSS was 0.036). On the other hand, reducing Eq. 3 to a model that estimates one condition factor and three different slopes, $\log(c) + p_a \log(L_{ia})$, was significant ($R^2 = 0.9286$ and p value for differences in RSS was 0.139) (Fig. 4). For the special case of Eq. 3, when the slopes were fixed equal to 3.0 (isometric growth), the three condition factors were significantly different from each other: for $\log(c) + 3.0\log(L_{ia})$, the d.f. was 699 and the RSS was 51.637; for $\log(c_a) + 3.0\log(L_{ia})$, the d.f. was 667; RSS was 42.926; F value was 67.679; and $p < 0.01$. However, the model with one condition factor and three different slopes was significantly better than the isometric growth model: for $\log(c_a) + 3.0\log(L_{ia})$, the d.f. was 667 and RSS was 42.926; for $\log(c) + p_a\log(L_{ia})$, the d.f. was 666; RSS was 42.658; F value was 4.177; and $p < 0.041$.

The estimated slopes from the model with the same condition factor for all of the areas were 3.053, 2.981, and 3.010 for the northwest, north, and east areas, respectively, and they were all significant different from each other (Fig. 4).

The results indicate that the quahog from the northwestern area generally contained more meat per unit shell length for the range of lengths considered than did individuals from the other areas (Figs. 3 and 4; Table 2). The mean meat weight of quahog in each area was estimated by using the length-weight relationship along with the observed length frequency distributions and compared with the mean weight from empirical data, as shown in Table 2. The two estimates were very similar, with the second method always within 2 s.e. from the first method (95% confidence interval). The average meat weights of quahogs from the northwestern, eastern, and northern areas were 38.5, 30.2, and 26.1 g, respectively.

DISCUSSION

In this study, the estimated length-weight relationships were significantly different among the three main areas. The meat weight for similar-sized quahogs were highest in the northwestern area, followed by the east; the lowest weight was observed in the northern area. No significant differences ($p > 0.05$) were observed among the average lengths of quahogs from the three areas, but there were significant differences among length-frequency distributions. Factors that are known to influence the relative condition of quahogs include physical and biologic variables such as water depth (Kraus et al. 1991), temperature (Lutz et al. 1983), and food supply (Lutz et al. 1983, Grizzle and Lutz 1989, Kraus et al. 1991).

Murawski and Serchuk (1979) calculated the length-weight re-

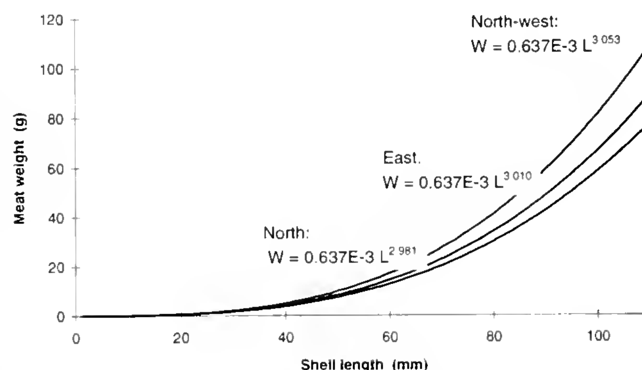

Figure 4. Estimated shell length–meat weight relationship for *A. islandica* from northwest, north, and east sampling areas of Iceland. The model estimates the same condition factor for all three areas but three different slopes.

TABLE 2.

MMW of ocean quahogs from the three areas off Iceland and in the total area calculated by using (1) the observed meat weights in the subsample, (2) the shell length distribution and the estimated length-weight relationship, both from the subsample, and (3) the on-site length distribution with the estimated length-weight relationship from the on-site measurements.

	MMW (g)			
Meat Weight	NW	N	E	Overall Average
Observed Subsample	26.5	30.2	37.0	31.3
	(s.e. = 1.1)	(s.e. = 1.6)	(s.e. = 1.2)	(s.e. = 0.74)
Estimated	25.8	29.5	35.3	30.2
On-Site	38.5	26.1	30.2	31.7

lationships in ocean quahogs from the Middle Atlantic shelf and confirmed allometric growth in two out of three areas. They observed that size-specific meat weights in the quahogs increased from north to south. This was attributed to be the stability of the thermal environment or related to density-dependent factors, but different stages in reproduction development could probably have affected their conclusion.

Knowledge of both the physical and the biologic oceanography of inshore waters around the Icelandic Coast, unfortunately, is rather sparse to further explain the observed differences. Temperature data from 1908 to 1973 for stations within the three study areas show that the temperature range is 2.2–7.4°C at about 70-m depth in the northwestern area, 1.5–6.7°C in the north area, and 1.2–6.9°C in the eastern area (Stefánsson and Jónsdóttir 1974). The primary production is also highest in the northwestern area (mean 184 g/cm^2 per year), followed by the eastern area (150 g/cm^2 per year) and the northern area (90 g/cm^2 per year), respectively (Thórdardóttir 1976).

A more favorable thermal environment and higher primary productivity are important factors governing metabolic processes and partially explain the observed higher relative meat yields in quahogs from the northwest area. Murawski et al. (1982) observed small difference (4–11%) when comparing length-weight equations from February and August for quahogs (65–115 mm) from the Middle Atlantic Bight. Winter samples were heavier in meat weight at a given shell length than summer samples. These differences were explained as related to weight changes associated with sexual development or statistical artifact. In ocean quahogs from New Jersey, size-specific somatic weight changed little through the year, which was suggested to be site differences in growth rate and reproductive cycling, and/or lack of synchrony of reproductive cycles of individuals at a given site (Fritz 1991). In this study, the samples from the northwestern area were taken from January to March, whereas the samples from north and east were taken from May to June. Further studies are therefore necessary to determine the existence of seasonal changes in these length-weight relationships and the effects of sexual maturity. The weight of the soft tissue of the quahog from the northwest area increases from April until June and then falls in July and August, which is considered to be the main spawning time (Auđunsson and Gunnarsson 1995).

Inverse relationships have been observed between growth and intraspecific density in quahogs, which may be the result of competition for food or space (Eversole et al. 1990, Hurley and Walker 1994, Kraus et al. 1992, Rice et al. 1989). Beal and Kraus (1989) observed depressed growth in quahogs when the local density was over 600 individuals/m^2 ($\bar{x}$ = 49 mm); at lower densities (130–323/m^2), no difference in growth was observed. In this study, the mean density was only 25, 24, and 39 individuals/m^2 ($\bar{x}$ = 75 mm) in the northwest, north, and east areas, respectively (Thórarinsdóttir and Einarsson 1996 in press); therefore, density-dependent factors have hardly affected the different growth curves observed in these areas.

The length-specific meat weight in this study observed from the shell length–meat weight relationship was greater in all three areas than reported for quahogs from the eastern coast of the United States. In this study, calculated meat wet weight (MWW) for an individual of 95-mm shell length from the northwestern area sampled in winter was 70 g, and for the eastern and northern areas sampled in summer, it was 55.5 and 47.6 g, respectively. By use of the shell length–meat weight relationships reported by Murawski and Serchuk (1979) for quahogs collected in winter off New Jersey, MWW of 36 g was calculated for an individual of 95-mm shell length. Murawski et al. (1982) reported MWW of 38 and 36 g in winter and summer samples, respectively, for 95-mm quahogs collected off New York, which is almost the same as the 41 and 39 g MWW calculated for quahogs of the same size collected at the same time of the year off New Jersey (Fritz 1991).

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TEMPORAL VARIATION AND TISSUE LOCALIZATION OF PARALYTIC SHELLFISH TOXINS IN THE NEW ZEALAND TUATUA (SURFCLAM), *PAPHIES SUBTRIANGULATA*

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ABSTRACT Changes in the paralytic shellfish poison (PSP)-toxin profiles in populations of Tuatua (*Paphies subtriangulata*) inhabiting beaches in the Bay of Plenty were analyzed by high-performance liquid chromatography during the contamination phase caused by a bloom of *Alexandrium minutum* in January 1993 and over a 6-mo period 1 yr later, when low-level toxin residues persisted within these shellfish. During the peak of toxicity ($\leq$412 μg of saxitoxin [STX] equivalents/100 g), the toxin profiles consisted of various proportions of the carbamate (gonyautoxin) derivatives GTX_1, GTX_2, GTX_3, GTX_4, and neoSTX, and STX, with some traces of the decarbamoyl derivative dc-STX. These profiles resembled those produced by the toxic dinoflagellate itself. One year later, when the toxicity had declined to a stable level of about 40 μg/100 g, only traces of derivatives other than STX remained and almost all of this toxin was sequestered within the siphons. The considerable length of time that toxin residues are retained, the tissue localization, and change with time in the spectrum of toxin derivatives in tuatua are very similar to those observed in other surfclam species elsewhere in the world. Analysis of toxin profiles in these shellfish provides a means of determining whether the observed PSP toxicity is the result of recent or long-past contamination episodes.

KEY WORDS: Tuatua, surfclam, *Paphies subtriangulata*, paralytic shellfish poisoning, *Alexandrium minutum*, Bay of Plenty, New Zealand

INTRODUCTION

During mid-summer (December to January) 1992–1993, the first documented case of paralytic shellfish poison (PSP) contamination of shellfish within New Zealand occurred when PSP toxins were detected by mouse bioassay in a variety of shellfish species from the Bay of Plenty. There was no human illness associated with the event, and the maximum recorded toxicity was a moderate 412 μg of STX equivalents/100 g of shellfish flesh weight. It was quickly discovered (Chang et al. 1995) that the source of this contamination was a bloom of the toxic dinoflagellate *Alexandrium minutum* apparently associated with a local upwelling event (Chang et al. 1996). As a result of this and other associated incidents (MacKenzie 1995, MacKenzie et al. 1995), a nationwide shellfish biotoxin-monitoring programme, administered by the New Zealand Marine Biotoxin Management Board (N.Z.M.B.M.B.), was established in early 1993. This programme has involved the weekly sampling and testing of a variety of shellfish species for aqueous and lipid soluble toxins from between 100 and 150 locations around the entire New Zealand coast. From September 1993 to June 1994, a weekly plankton-monitoring programme, incorporating the water column sampling of 17 sites along the north/east coast of North Island (including three sites in the Bay of Plenty), was also carried out. After the cessation of the 1993 *A. minutum* bloom, the PSP toxicity of most shellfish species within the Bay of Plenty declined rapidly. However, in tuatua, the PSP toxicity only gradually declined and it was not until 22 mo after the initial contamination event that this toxicity of these shellfish became consistently undetectable by mouse bioassay. At the time of the peak in toxicity in January 1993, Bay of Plenty tuatua were analyzed by high-performance liquid chromatography (HPLC) to verify the results of the mouse bioassays and to examine the specific toxin composition. Between late 1993 and mid-1994, when a stable base level of toxicity within these shellfish had been reached, a further series of analyses were done to compare with the original tests and to identify the reason for this continued contamination. The results of this investigation are presented here.

METHODS

Tuatua were collected within the surf zone from Papamoa and Waihi beaches in the Bay of Plenty (Fig. 1) and transported live to the respective laboratories for mouse assays and HPLC analyses. Samples for mouse assays, carried out as part of the official marine biotoxin-monitoring programme were collected weekly from both of these beaches until August 1994, after which only Papamoa

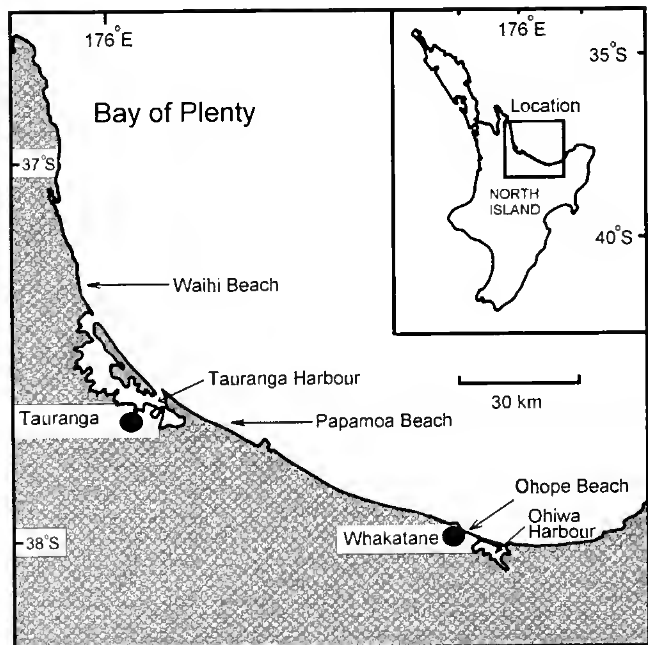

Figure 1. Map of the North Island, New Zealand, showing the location of shellfish-sampling sites in the Bay of Plenty.

Beach was sampled. The mouse assays were performed at the Communicable Diseases Centre (Environmental Science Research Ltd.), Porirua, by use of the standard A.O.A.C. method (A.O.A.C. 1990). Between mid-January and mid-February 1993, the interim U.S.F.D.A. modification to the official A.O.A.C. extraction method (Hall 1991) involving the use of 1.0N HCl was used. Thereafter, 0.1N HCl was used according to the original standard method.

Two samples (January 27 and 29, 1993) of tuatua collected from Papamoa Beach during the height of the bloom were analyzed by HPLC, as were six samples (December 6, 1993; January 17, 1994; February 8, 1994; March 6, 1994; May 5, 1994; and July 4, 1994) from Waihi Beach. Extracts of whole shellfish and dissected body parts (from 10 shellfish) for HPLC analysis were prepared by boiling homogenized tissue in 0.1N HCl (equivalent tissue weight/HCL volume), cooling, and adjusting the pH to 3. Extracts were cleaned up by passage through a "Sep Pak" (Waters) C18 cartridge and by centrifugation through a 10,000 MW ultrafiltration membrane (Ultra Free C3-GC; Millipore); 10 μL of this extract was injected into the HPLC system. The toxin spectra were resolved by use of a slight modification of the method described by Oshima et al. (1989b). These modifications involved the use of a GL Sciences Inc. Intersil C8 silica reversed-phase column and the following mobile phases for the different groups of toxin: (a) 2 mM tetrabutyl ammonium dihydrogen phosphate in acetate buffer (pH 5.8) for the C_1–C_4 toxins; (b) 2 mM heptane sulfonate in 10 mM ammonium phosphate buffer (pH 7.1) for gonyautoxins (GTX) 1–5; (c) 2 mM heptane sulfonate in 30 mM ammonium phosphate buffer (pH 7.1):acetonitrile, 100:5, for the saxitoxin (STX)-neosaxitoxin (neoSTX) group. The oxidizing agent was 7.0 mM periodic acid and 10 mM potassium phosphate buffer (pH 9.0). The fluorescent derivatives were measured on a Hitachi F-1000 fluorescence spectrophotometer at excitation and emission wavelengths of 330 and 390 nm, respectively. The standards used for the calibration of the analysis were pure toxin standards prepared by the Laboratory of Food Hygiene (Department of Food Chemistry, Tohoku University, Sendai, Japan). The STX group standard mixture contained STX, neoSTX, and decarbamoyl saxitoxin (dcSTX). The GTX group standard mixture contained GTX 1–5 (GTX_1–GTX_5), and the N-sulfocarbamoyl mixture contained toxins C_1–C_4. The calculation of the total toxicity of shellfish tissues (micrograms of STX equivalents per gram) from the HPLC data was accomplished with the conversion factors of Oshima (1995).

On January 17, 1993, water samples at 3-m-depth intervals along three transects were sampled between Tauranga Harbour entrance and Waihi Beach. Between September 1, 1993, and June 30, 1994, weekly phytoplankton samples from 3-m-depth intervals were collected from three sites within the Bay of Plenty—off Waihi Beach, Tauranga Harbour, entrance, and Ohiwa Harbour. Subsamples (10 mL) of Lugol's iodine–preserved samples were settled in Utermöhl chambers and examined under an inverted microscope. All dinoflagellate species were documented and counted during whole-chamber scans.

RESULTS

The highest cell numbers of *A. minutum* observed during the 1992–1993 bloom were 1.2×10^5 cells/L at a site off Tauranga Harbour on January 17, 1994. The peak in *A. minutum* abundance was coincident with the highest toxicity scores in tuatua from Bay of Plenty beaches. The *A. minutum* bloom in the Bay of Plenty declined soon after the maximum toxicity scores in shellfish within the bay were recorded, disappeared entirely within a few weeks, and was not observed within the phytoplankton in this area again between June 1993 and June 1994.

A slow and somewhat erratic decline in toxicity within tuatua was observed after the disappearance of *A. minutum* from the plankton (Fig. 2), and shellfish toxicity did not consistently descend below the official action level of 80 μg of STX equivalents/100 g until December 1993, 10 months after the initial contamination event. Consistently negative mouse bioassay results from Papamoa Beach were not obtained until November 1994.

Analysis of toxin profiles within whole-body extracts of tuatua (Fig. 3), collected from Papamoa Beach when toxicity was near its maximum (Fig. 3A), showed that STX and neoSTX (comprising 36 and 19% of total toxin body burden, respectively) were the major toxins, followed by various amounts of GTX_1–GTX_4. No N-sulfocarbamoyl derivatives were observed, although small amounts of the decarbamoyl derivative dcSTX were present. Profiles from whole-shellfish extracts of six samples collected between December 6, 1993, and July 3, 1994 (Fig. 3B), showed that

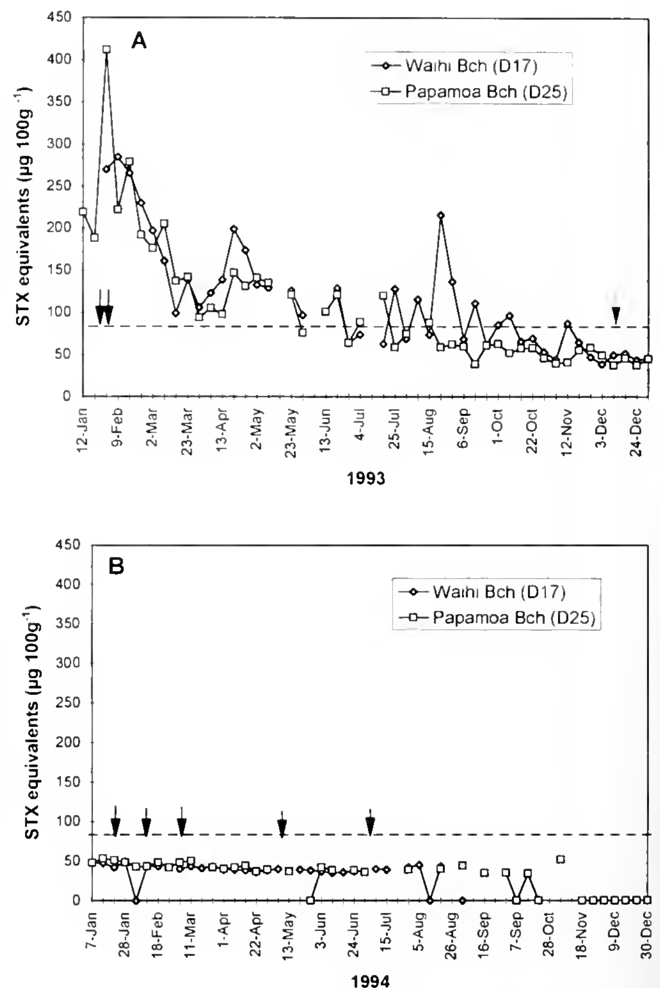

Figure 2. PSP toxicity of tuatua, determined by mouse bioassay, from Papamoa and Waihi Beaches, Bay of Plenty, during 1993 (A) and 1994 (B). The arrows indicate the dates on which samples of tuatua for HPLC-toxin analysis were collected.

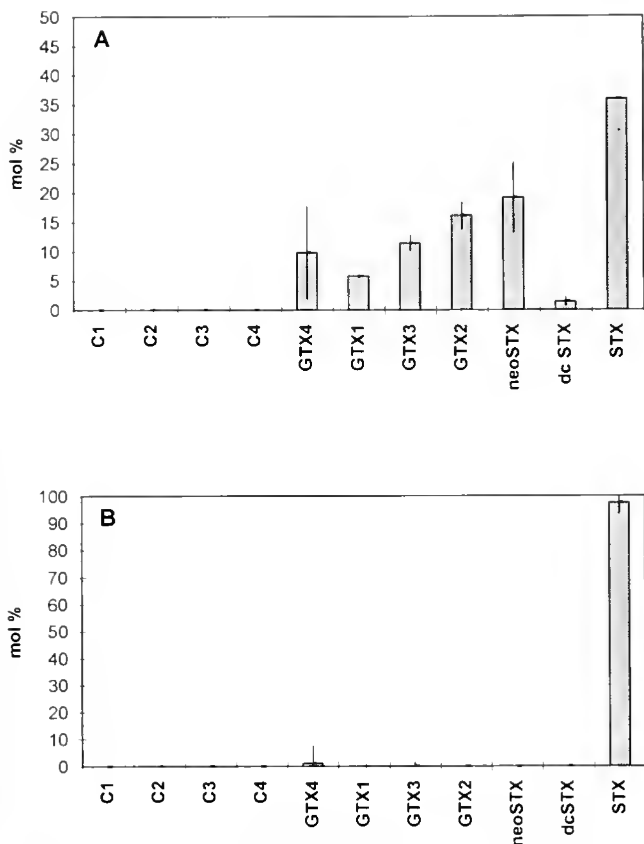

Figure 3. PSP-toxin profiles (mol%) in whole tuatua from Papamoa and Waihi beaches, 1993–1994. (A) Analysis of profiles in tuatua from Papamoa Beach collected on January 27 and 29, 1993 (n = 2). (B) Toxin composition in tuatua collected from Waihi Beach between December 6, 1993, and July 3, 1994 (n = 6). Vertical lines indicate the range of values.

STX was the overwhelmingly dominant derivative (94–100%), with only small traces (up to 7%) of GTX_4 present in some samples. No N-sulfocarbamoyl derivatives were observed in whole-body extracts of these shellfish.

Analysis of individual tissues within the tuatua (Fig. 4) revealed the presence of trace amounts of other toxins (including some N-sulfocarbamoyl derivatives) in various parts of the shellfish; however, the quantities of these and their contribution to the total toxicity of the shellfish were small in comparison to the predominance of STX residues within most tissues. Although only comprising a small proportion of the total body mass of the tuatua (1.3–2.5% by weight), the two siphons clearly contained the highest concentrations of toxin residues (Fig. 5). The toxin was almost exclusively in the form of STX (Fig. 4), and high levels (up to 6.2 μg of STX/g) were retained in these parts (Fig. 5A). Together the siphons contained, on average, 71% of the total toxin body burdens in these shellfish (Fig. 5B).

DISCUSSION

Since the initial discovery of PSP toxicity in the Bay of Plenty shellfish (Chang et al. 1995), the New Zealand shellfish biotoxin-monitoring programme has revealed several other instances of PSP-toxin contamination due to this dinoflagellate, the most important of which occurred within the Marlborough Sounds in January 1994 (MacKenzie 1994). With the exception of a recent event within the eastern Bay of Plenty that has been associated with *Alexandrium catenella* (MacKenzie unpublished), all cases of PSP-toxin contamination in New Zealand to date (where concurrent phytoplankton data are available to substantiate this) have been attributable to blooms of *A. minutum*. The absence of *A. minutum* in the phytoplankton at the monitoring sites off Waihi Beach and Tauranga Harbour between September 1993 and June 1994 provides good evidence that the continued presence of PSP toxins within the tuatua in this region was not the result of continuing cryptic contamination. The absence of PSP toxicity in other shellfish species (mussels, cockles, oysters) in this region over this period (M.B.M.B. records) provides further evidence that this was not the case.

Because of the long retention time of PSP by tuatua, this species figures disproportionately in the New Zealand shellfish biotoxin contamination statistics. Between January 7, 1994, and January 6, 1995, 5,327 mouse bioassays for PSP were carried out (M.B.M.B. records) on a variety of shellfish species (of which 10% were tuatua) from an average of 102 sites per week distributed around the entire coast of New Zealand. Only 2.9% of these bioassays returned positive results, of which 79% were in tuatua and all but one were the result of low-level residues in these shellfish from the Bay of Plenty and Northland regions. None of the PSP-toxin bioassay results in tuatua during this period exceeded the 80 μg of STX equivalents/100 g action level.

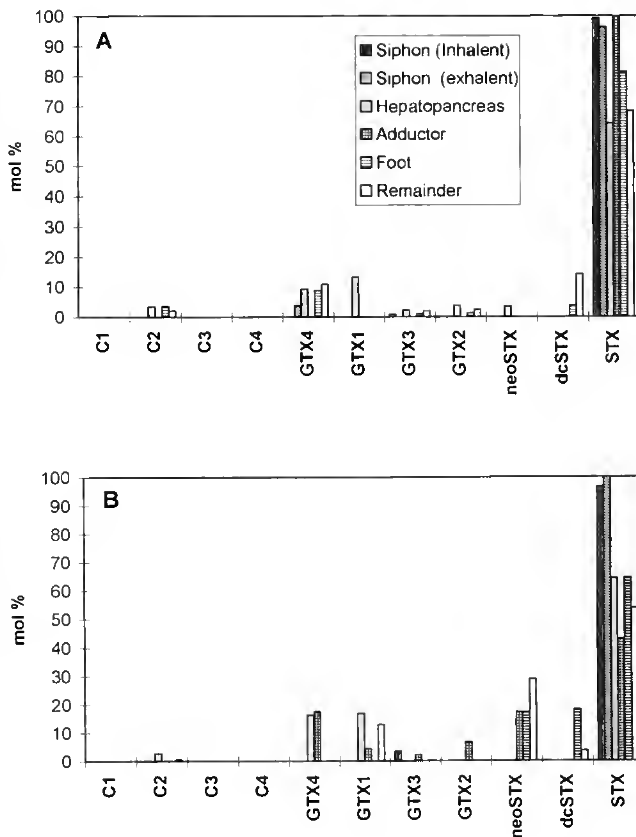

Figure 4. PSP-toxin profiles (mol%) in partitioned tissues of tuatua. (A) Samples collected on December 6, 1993. (B) Samples collected on June 4, 1994.

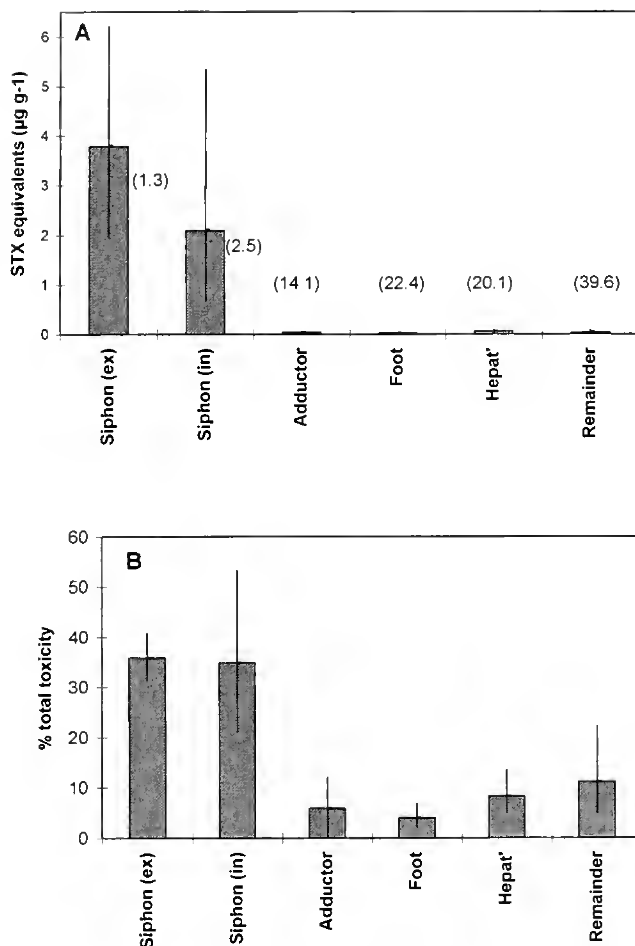

Figure 5. Mean toxin burdens in partitioned tissues of tuatua collected from Waihi Beach between December 6, 1993, and July 3, 1994 (n = 6). (A) Specific toxicity (µg of STX equivalents/g) in various tissue types; the numbers in parentheses are the mean proportion (%) of each tissue type to total body mass. (B) The proportion that each tissue type contributed to total toxin body burden. Vertical lines indicate the range of values.

The analysis of the PSP-toxin profile in an *A. minutum* isolate, established in culture from specimens collected during the 1993 Bay of Plenty bloom, was composed predominantly of neoSTX and STX, with lesser amounts of GTX_{1-4} and GTX_{2-3} (Chang et al. 1996). The PSP-toxin profile produced by this isolate is rather different from the toxin profiles exhibited by *A. minutum* in other parts of the world. French isolates of *A. minutum* (Ledoux et al. 1993) have been shown to produce predominantly GTX_2 and GTX_3, with only trace amounts of STX, whereas Spanish isolates of *A. minutum* have been shown (Franco et al. 1994) to produce mainly GTX_4 (80–90%), with lesser amounts of GTX_1 (10–15%) and only small amounts (3%) of GTX_3 and GTX_2. Australian *A. minutum* (Oshima et al. 1989a, Franco et al. 1995) produces predominantly GTX_4 (77%) and GTX_1 (23%), with only small amounts ($\leq$10%) of GTX_2 and GTX_3 and no evidence of any of the other 14 toxins known to occur naturally. The toxin profiles in tuatua collected at the time of the January bloom (Fig. 3A) were very similar to the toxin profiles observed in the dinoflagellate. The very small amounts of N-sulfocarbamoyl (C_{1-4}) derivatives in the tuatua profiles do not provide unequivocal evidence that these toxins did not play a role in the contamination because samples were extracted with 0.1N HCl. It is likely that during this process, any N-sulfocarbamoyl derivatives that may have been present in these shellfish would have been hydrolyzed to their corresponding carbamate (GTX_{1-4}) analogues (Cembella et al. 1993). The presence of small amounts of dcSTX in the tuatua, but apparently not in the dinoflagellate (Chang pers. comm.), is consistent with a high capacity for in vivo PSP carbamate to decarbamoyl analogue conversion in surfclams (Cembella et al. 1993).

The magnitude and nature of PSP toxin retention by shellfish vary depending on the shellfish species (Cembella et al. 1993, Cembella et al. 1994), and it has been known for many years (e.g., Medcof et al. 1947), and recently reaffirmed (Shumway et al. 1994), that surfclams are particularly prone to the retention of PSP toxins for considerable periods of time. The predominance of STX in the tuatua containing residual toxicity (Figs. 3 and 4) is typical of residues observed in other surfclam species elsewhere. Cembella and Shumway (1995) found that STX was the dominant toxin in most tissues of the surfclam *Spisula solidissima* in the Gulf of Maine, although there were substantial differences in the toxicity of different tissues as a proportion of total body toxin burden (digestive gland > mantle = gill > siphon = foot > adductor muscle). The butter clam (*Saxidomus giganteus*) likewise is known to remain toxic for years after a contamination event (Quayle 1969) and to almost exclusively retain toxins in the siphon, primarily in the form of STX, thus strongly resembling the nature of toxin retention by the New Zealand tuatua reported here. The predominance of STX as a long-term toxin residue within surfclams is due to either the accumulation and preferential retention of STX over other derivatives acquired from the toxic dinoflagellates, or the conversion of other derivatives (GTXs) or precursors to STX within the shellfish tissues themselves. The experimental contamination of *S. giganteus* with a strain of *A. catenella*, which does not produce STX, suggests that the latter alternative is most likely in this species (Beitler and Liston 1990). Those investigators found that toxin derivatives rapidly accumulated throughout the shellfish tissues in proportions similar to those produced by the dinoflagellate; however, over a subsequent 58-day depuration period, the GTX derivatives declined as STX accumulated within the siphon. Bricelj and Cembella (1995), on the other hand, found no evidence of the de novo appearance of STX in juvenile *S. solidissima* after experimental contamination with a strain of *A. minutum* rich in GTX. They hypothesized that the predominance of STX in wild *S. solidissima* populations was due to the exposure of these shellfish to STX-producing dinoflagellates. Because the strain of *A. minutum* responsible for the initial contamination of the Bay of Plenty tuatua does produce STX as a major part of its toxin profile (Chang et al. 1996), both mechanisms of STX residue retention are possible in these shellfish.

The long-term retention of STX by tuatua is a nuisance for public health management because it only takes additional trace level contamination to raise the toxicity above the 80 µg of STX equivalents/100 g action level. On the other hand, where there is a suitable habitat for these shellfish, they do provide a means of mapping PSP contamination and the analysis of their toxin profiles gives an estimate of the frequency of PSP-toxin contamination events around the New Zealand coast. The results of the last 3.5 y of the N.Z. biotoxin-monitoring programme (January 1993 to June 1996) indicate that tuatua containing PSP-toxin residues are confined to the north/east coast of the North Island, although this species is routinely sampled on a few surf beaches elsewhere

(North Island west coast and the South Island). This provides evidence for a low incidence of PSP causing blooms in these latter regions. Other New Zealand surfclam species (e.g., the Pipi; *Paphies australis*) may be equally good indicators for past intoxication events, and some preliminary HPLC analyses (MacKenzie unpublished) have indicated the presence of trace amounts of PSP toxins in these shellfish at levels undetectable by the mouse bioassay.

All studies of the anatomical localization and retention of PSP toxin residues in surfclams have so far been carried out on northern hemisphere species, mainly from the northern North American continent. This investigation demonstrates that similar, although taxonomically and geographically very distant, bivalve molluscs occupying the same surf zone habitat in the southern hemisphere have similar characteristics in this regard and suggests that an ecological advantage may be gained by this capability. This lends weight to the theory (Kvitek 1993) that surfclams have evolved to use the retention of PSP toxins as a chemical defense mechanism against predation. Furthermore, the observations made here that toxin residues are sequestered in high concentration in the siphons support the hypothesis that this chemical defense strategy is specifically aimed at protection against siphon-nipping fish. Tuatua are numerous on surf beaches throughout New Zealand and, because of their low intertidal zonation, are vulnerable to predation by sea birds, fish, and crustaceans and may provide a significant part of the diet of some of these species. If it is assumed that the ability to retain PSP toxins has evolved independently as a chemical defense device in indigenous surfclams, it leads to the conclusion that although the PSP contamination of New Zealand shellfish is a recently discovered phenomenon, it has in fact occurred for a long time and no doubt will continue to do so in the future.

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REPRODUCTIVE CYCLE OF *LAEVICARDIUM ELATUM* (SOWERBY, 1833) (BIVALVIA: CARDIIDAE) IN BAHIA CONCEPCION, BAJA CALIFORNIA SUR, MEXICO

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ABSTRACT The reproductive cycle of the cockle *Laevicardium elatum* in Bahía Concepción, B.C.S. Mexico, was studied from October 1988 to October 1990. Microscopic analysis established that *L. elatum* is a functional hermaphrodite with male and female follicles intermingled in the gonad. The development and spawning of male and female gametes were synchronous. Spawning occurred between October and April (18–23°C) and was related to a high food availability and to a lower condition of the organisms.

KEY WORDS: reproductive cycle, bivalves, *Laevicardium*, histology

INTRODUCTION

The cockle *Laevicardium elatum* (Sowerby 1833) is distributed from southern California to Panama and lives in sandy bottoms; it has a length to 150 mm and is the largest of the living species of the family *Cardiidae* (Keen 1971). This species is considered a potential fishery in Baja California Sur (B.C.S.) (Baqueiro et al. 1982). *Argopecten circularis* and *Megapitaria squalida* are the principal species of economic importance of Bahía Concepción. When the stocks of *A. circularis* and *M. squalida* are depleted, *L. elatum* is harvested and has a good acceptance because of its white meat. It is mostly harvested for its shell for ornamental purposes. Despite its biologic and economical importance, there are no published records on the biology of this species from Bahía Concepción waters. In contrast, *Cardium edule*, *Cardium glaucum*, and *Cardium hauniense* have been well studied in Europe (Boyden 1971, Kingston 1974, and Wolowicz 1987). There are also a small number of studies on the reproductive biology of some Cardiidae species, e.g., *Clinocardium nuttallii* at Isla San Juan, United States, and *Laevicardium laevigatum* from Venezuela (Gallucci and Gallucci 1982, Penchaszadeh and Salaya 1983). This study was carried out to describe the reproductive cycle and the spawning season of *L. elatum* in Bahía Concepción, B.C.S., Mexico and its relation to the temperature, food availability, and condition (fatness) of the organisms.

MATERIALS AND METHODS

Between October 1988 and October 1990, in Bahía Concepción, B.C.S. (26°55′–26°30′ N and 112°–111°40′ W) (Fig. 1), 30–40 adult specimens of *L. elatum* were collected by skin diving between 2- and 6-m depth. The surface water temperature was recorded at the time of sampling. Shell heights and total and soft body wet weights were recorded for each clam.

The clams were fixed in a neutral formalin solution. Tissue sections were taken at a standard point halfway between the digestive diverticula and the foot. These tissue sections were dehydrated in alcohol and embedded in paraffin. Section (7μm) were placed on slides and stained with hematoxylin-eosin (Humason 1979). A modification of the developmental stages established by Gallucci and Gallucci (1982) for *C. nuttallii* was used to categorize follicles.

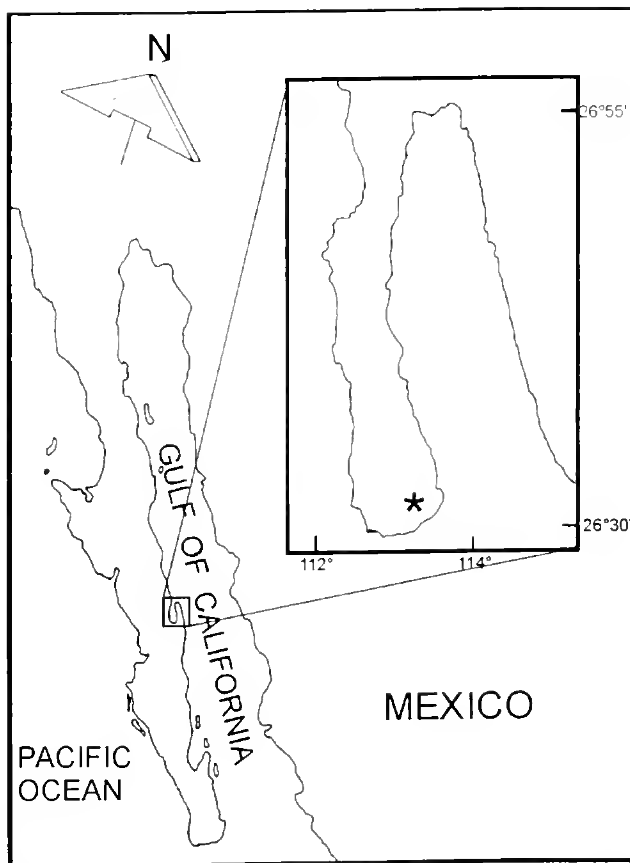

Figure 1. Location of Bahia Concepción, B.C.S., Mexico. *Sampling area.

Indifferent Stage

This stage is characterized by a total absence of gametes; therefore, it is not possible to distinguish the sex. The connective tissue occupies almost all of the space (Fig. 2A).

Developing Stage

In the female, there were rounded oocytes along with oocytes attached to the follicle wall. Some detached oocytes occurred. In

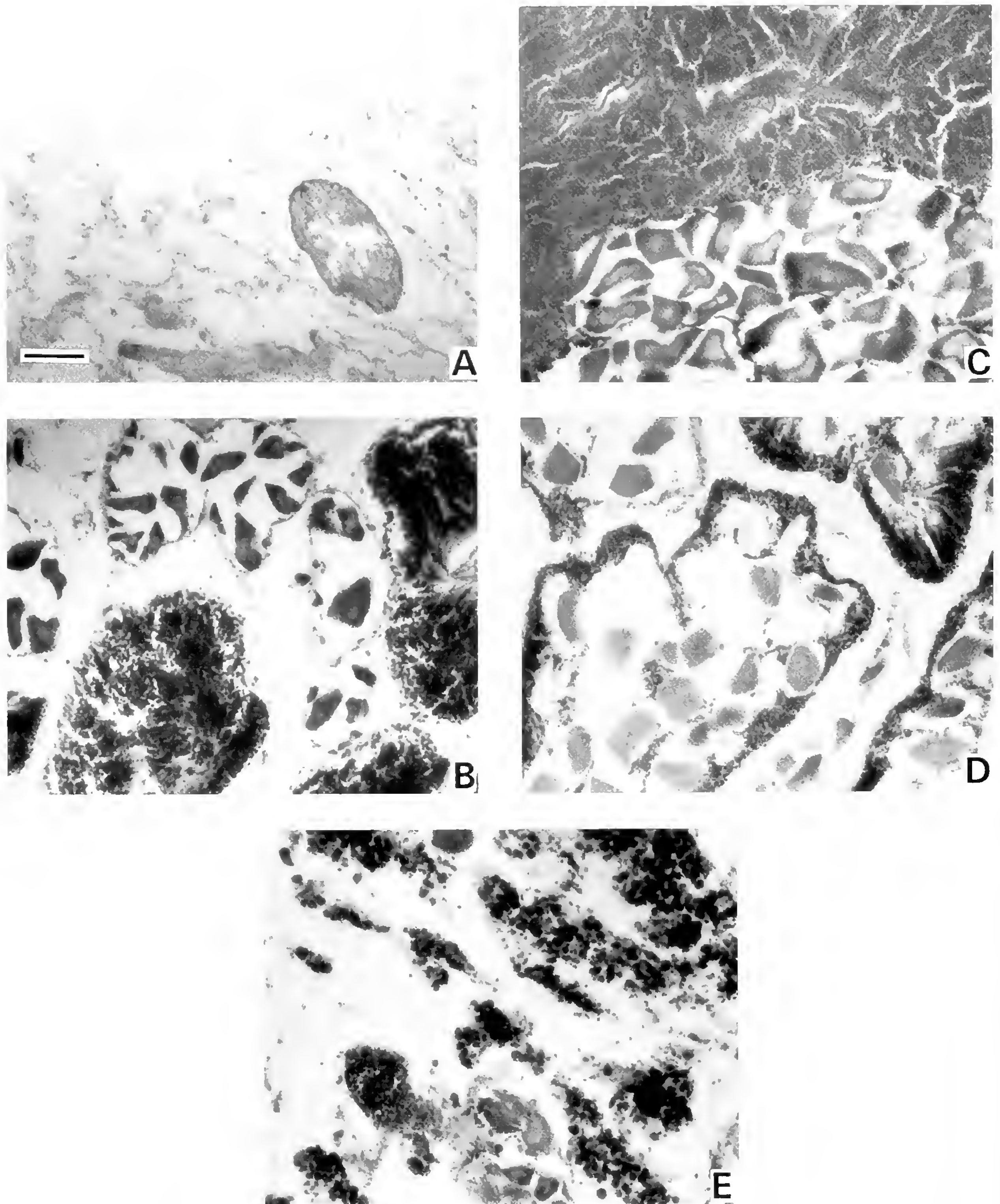

Figure 2. Photomicrographs of gonadal stages of *L. elatum*. (A) Indifferent. (B) Developing. (C) Ripe. (D) Partially spawned. (E) Spent. Bar = 55 μm.

the male, varying quantities of spermatogenic cells were present (Fig. 2B).

Ripe Stage

In the female, most oocytes were free within the follicles, but some oocytes remained attached to the follicle wall. In the male, follicles filled by spermatozoa arranged in characteristic bands (Fig. 2C).

Partially Spawned

In the female, large spaces inside the follicles and between free oocytes were present. Some follicles were completely devoid of

oocytes. In the male, a marked decrease in the quantity of spermatozoa was observed. Large spaces inside the follicles occurred. In some follicles, only a few residual spermatozoa were present (Fig. 2D).

Spent

At this stage, some unspawned oocytes and spermatozoa were observed within follicles. The gametes were being phagocyted by amebocytes (Fig. 2E). The diameter of at least 100 oocytes, in each of six randomly selected females, was measured with an eyepiece graticule calibrated with a stage micrometer. The measurements were made along the longest axis of the oocyte, sectioned through the nucleus. From these data, mean oocyte size was obtained. Individuals with few measurable oocytes and extensive phagocytosis were not considered, following the criteria of Grant and Tyler (1983a and b).

The condition was estimated by the use of Fulton's equation (Hile 1936):

$$CF = \frac{W}{H^3}$$

where CF is condition factor, W is soft body wet weight (in grams), and H is shell height (in millimeters).

RESULTS

Clams ranged in size from 65 to 155 mm in shell height, with the mode at 125 mm in height (96% of the organisms fell between 95 and 145 mm in height). *L. elatum* is a functional hermaphrodite. In the male and female acinis, the gametes were in the same developmental stage. Male and female follicles were intermingled in the gonad; therefore, the gonad did not have well-differentiated male and female glandular areas.

The reproductive cycle of *L. elatum* from Bahía Concepción, B.C.S., is summarized in Figure 3. From June to August, most cockles were inactive, as determined by the presence of indifferent and spent stages. The developing stage was minimal in this period. The ripe stage was present from October to April 1988 and from January to March and October 1990. The partially spawned stage, which is the indicator of the period of spawning, was present from February to April and October 1989 and January to March and October 1990.

The measurements of oocyte diameters (Fig. 4) shows that gametogenesis and maturity are rapid processes. Oocyte development was faster from October to November 1988, when the highest frequencies of mature individuals occurred. The oocytes were fully developed (56.4 μm, mean diameter; s.d. = 9.9) from February to April 1989 and January to March 1990, when the highest frequencies of partially spawned stage were present.

The higher values of condition factor were found in April and August 1989 and in October 1990, whereas the lower values of condition factor occurred in November 1988, October 1989, and June 1990. There were two periods of recovery—November to April 1988 and June to October 1990 (Fig. 4). The minimum water

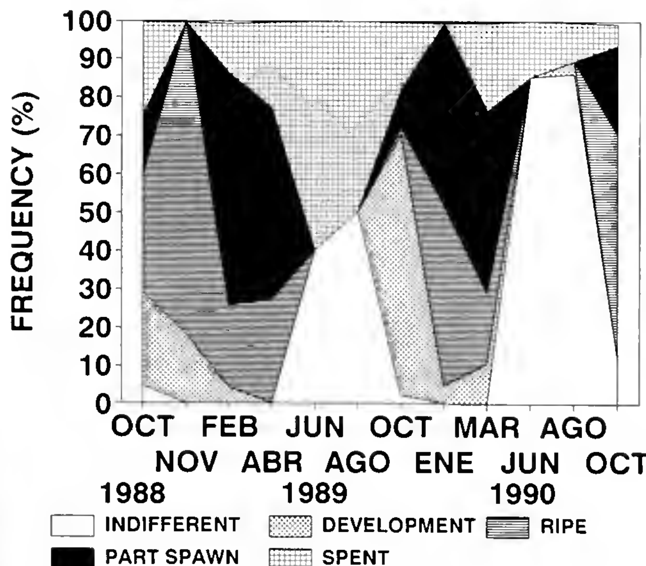

Figure 3. Reproductive cycle of *L. elatum*. Relative frequency of gonadal stages between October 1988 and October 1990.

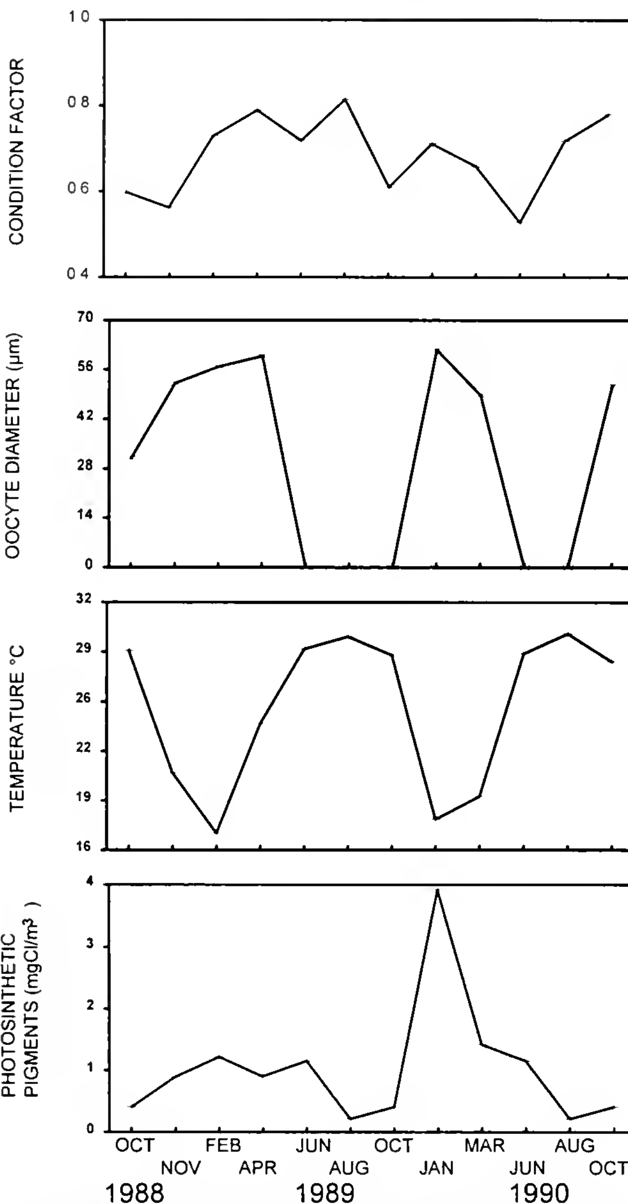

Figure 4. Mean condition factor values of *L. elatum* of Bahía Concepción, mean oocyte diameters of female, water temperature, and photosynthetic pigment concentration.

temperature (18°C) was recorded in February, and the maximum (30°C) was recorded in August (Fig. 4).

The photosynthetic pigment concentration mg of chlorophyll per cubic meter) from Bahía Concepción, B.C.S., obtained from satellite-derived information (Tran et al. 1993), was greater in cold months than in warmer months. There was a marked winter bloom that reached the maximum value in January (3.92 mgCl/m^3). The minimum value was found in August (0.22 mgCl/m^3) (Fig. 4).

DISCUSSION

The characteristics of gametogenesis in *L. elatum* were similar to those described for *C. nuttallii* (Gallucci and Gallucci 1982). In both species, male and female follicles were shown to develop in phase with each other and the gametes of both sexes were spawned at about the same time. Among the majority of the hermaphroditic bivalve species, the condition of having intermingled male and female follicles is anomalous and they are reported as unusual cases (Penchaszadeh and Salaya 1983). Nevertheless, other functional hermaphrodite bivalve species, such as *C. nuttallii*, exist (Gallucci and Gallucci 1982). In Bahía Concepción, the only other hermaphrodite bivalve species that has been studied is *A. circularis* (Villalejo-Fuerte and Ochoa-Báez 1993).

The mean diameter of oocytes of *L. elatum* is similar to that of other bivalves found in Bahía Concepción, *A. circularis* and *M. squalida* (Villalejo-Fuerte and Ochoa-Báez 1993, Villalejo-Fuerte et al. unpublished data). Comparing oocyte diameters with gonadal stages in *L. elatum*, it is clear that minimum diameters coincide with the developing stage and maximum diameters coincide with the mature and partially spawned stages. Therefore, the oocyte diameters are reflective of the gametogenic cycle. Similarly, in *Mercenaria* spp. from Florida and in *Glycymeris gigantea* from Bahía Concepción, maximum oocyte diameters were observed in conjunction with the period of maturation and spawning (Hesselman et al. 1989, Villalejo-Fuerte et al. 1995).

Temperature is an important environmental factor in the regulation of bivalve reproduction (Sastry 1979). Differences in the timing of gametogenesis and spawning within a species over a latitudinal range occur because critical temperatures are attained at different times (Hesselman et al. 1989). The reproductive cycle of *L. elatum* in Bahía Concepción shows a clear seasonality related to the water temperature. The inactive period occurs from June to August, with water temperatures of 28–30°C. Gametogenesis and spawning occur from October to April, starting when temperatures are declining and continuing during the cooler months (18–23°C). A similar relationship between the temperature and gonadal activity has been observed for *A. circularis* (Villalejo-Fuerte and Ochoa-Báez 1993), *G. gigantea* (Villalejo-Fuerte et al. 1995) in Bahía Concepción, and *Mercenaria* spp. in Florida (Hesselman et al. 1989). Higher temperature inhibits gametogenesis.

Food availability has been related to the timing of bivalve reproduction (Sastry 1979, Bayne and Newell 1983, MacDonald and Thompson 1985, Jaramillo et al. 1993). The spawning in bivalves might be synchronized to coincide with maximum food availability for larval development (Seed 1976). Martínez-López and Gárate-Lizárraga (1994) found high concentrations of nannoplankton in Bahía Concepción in February; this represents a source of food for larvae of *A. circularis* and other mollusks of commercial importance.

The reproductive cycle of *L. elatum* has a seasonality related to food availability, expressed as the concentration of photosynthetic pigments. This relation is not observed in other bivalves like *Hinnites giganteus* (Malachowski 1988). The spawning season of *L. elatum* coincides with the highest food availability, giving larvae the chance of exploiting the winter phytoplankton bloom. In this context, *L. elatum* is a conservative species under the scheme of Bayne (1976), who classified the reproductive strategy of bivalves according to the relation between the spawning and the storage cycles.

The fluctuations of the condition factor are associated with the reproductive or nutritional condition of the mollusks (Searcy-Bernal 1984). In *L. elatum*, the maximum values of condition factor found in the inactive period (April and August 1989 and October 1990) could be produced by the accumulation of reserve substances during this period, which will be used during ripening. This has been observed for other bivalves (Sastry 1979). However, the fluctuations of the condition factor also may be a consequence of the water content in the soft body or changes in the mass of nutritive tissue (Giese and Pearse 1974). Then, the condition factor is not a reliable indirect indicator of the spawning season and it is necessary to examine gonads microscopically.

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USE OF SIEVES FOR THE RAPID SIZE SELECTION OF *DREISSENA POLYMORPHA* SAMPLES

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ABSTRACT We describe a method that uses sieves of uniform pore size for rapidly size sorting large populations of *Dreissena polymorpha* (zebra mussels) or collecting large numbers within a selected size range. This method may be a valuable tool applicable to a wide range of zebra mussel research projects and possibly also useful in size sorting other bivalve species. Sieves are used in pairs and are repeatedly moved vertically in and out of a water column, with mussels passing lengthwise down through the pores. The upper and lower limits of the size range of mussels collected between the sieves are determined by the pore sizes of the upper and lower sieves, respectively. Sieve pairs with pore sizes of 6.30 and 5.60 mm, 5.60 and 4.75 mm, and 4.75 and 4.00 mm, for example, yielded mussels in size classes of 13.8–10.3, 12.6–8.5, and 10.6–7.0 mm in length, respectively. Sieving in the field eliminated the need to transport mussel-laden rocks and other substrates to the laboratory, reduced the effort required to properly dispose of mussel-contaminated materials, and proved even simpler to perform than in the laboratory. One of the challenges in using this sieving procedure is determining the exact sieve sizes that will retain mussels only within a particular length range. For example, because large numbers of zebra mussels 6–11 mm in length are used in our laboratory's research program, trials were conducted to compare the effectiveness of sieving and hand picking to obtain mussels solely within this size class. With sieves of 5.6 and 2.8 mm pore sizes, the mean yield of mussels was over three times faster by sieving than hand picking (10.2 versus 3.0 mussels/min), but 7.1% of the mussels collected were outside the desired length range of 6–11 mm. In judging the usefulness of this sieving procedure, an error factor such as this is unavoidable and must be weighed in individual research projects against the significant increase in mussel yield.

KEY WORDS: Sieves, zebra mussels, size selection

In our biological control research program (Molloy and Griffin 1992), thousands of 6- to 11-mm-long *Dreissena polymorpha* (zebra mussels) are required for use in laboratory bioassays of potentially lethal microorganisms. Our traditional method of obtaining these mussels has been to collect mussel-encrusted rocks, transport them to the laboratory, and hand select this size class with forceps. The sorting of zebra mussels into specific size classes by hand picking can be tedious and time consuming. As an alternative, we successfully tested the efficiency of using sieves for size selecting large numbers of mussels. In the course of evaluating sieves that would select the 6- to 11-mm size, we generated information on the mussel size ranges that could be collected by other pairs of sieves and present these data here.

This sieving procedure might be useful in a wide variety of *Dreissena* projects, including selecting zebra mussels of a given size class for growth studies (Dorgelo 1993) or characterizing the size structure of populations (Effler and Siegfried 1994). The size selection of other bivalve species might also be practical, based on these techniques.

When suspended in water, a zebra mussel typically passes through a sieve pore lengthwise (Fig. 1) and is restricted by a pore when the mussel's largest cross-sectional dimension exceeds the pore's width. For the zebra mussels used in our study, the maximum cross-sectional dimension was their height, not their width (our unpublished data). Thus, even though our size classes were expressed in terms of shell *length*, the retention of a mussel on a particular sieve was determined primarily by its *height* (Fig. 2). For this reason, we present data defining the length-height relationship in the zebra mussels we used in this study.

MATERIALS AND METHODS

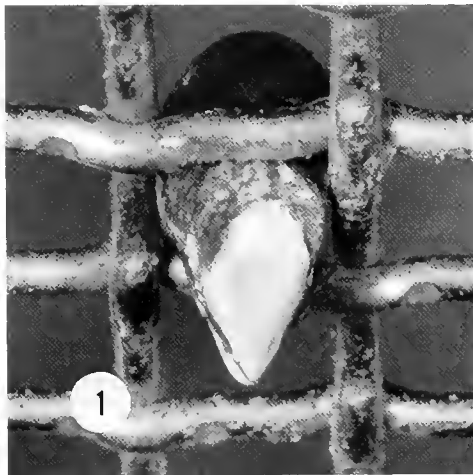
In October 1994, mussel-encrusted rocks were collected from the Mohawk River (Crescent, NY) and were transported under refrigeration to the laboratory. With a Manostat caliper (model 15-100-500), the shell length and height of 1,530 mussels were measured and the interrelationship of these two characters was fitted by power regression.

To test the comparative efficiency of rapidly size selecting hundreds of mussels, we conducted timed trials in the laboratory using both the hand picking and the sieving procedures as outlined below. In the process of selecting our desired size class, i.e., 6–11 mm, all mussels were removed from rock substrates, thereby producing three size classes: <6, 6–11, and >11 mm. Data were tabulated separately on each of these size classes, and means were tested for significance by a t-test.

Mussel mortality resulting from their handling during size-selection procedures was also compared. Immediately after picking or sieving, the mussels were placed in beakers and stored in 80-L aquaria equipped with filters and air stones and filled with unchlorinated tap water (4°C). To retain mussels, the mouth of each beaker was covered with nylon screening held in place by a rubber band. Beakers were stacked on their sides in the aquaria, with ≥10 cm between beaker mouths and aquarium walls for water circulation. After 7 days, mortality was scored. Gaping mussels or those that did not close their shells when gently pried apart were considered dead.

Picking Procedure

Three timed trials (185, 231, and 378 min) were conducted. In each, mussels were detached from rocks by grasping their byssal threads with fine-tipped forceps and gently pulling the byssus from the substrate. A gauge was used for rapidly separating the detached mussels into the three size classes (Fig. 3). When placed in this gauge, if a mussel's length exceeded Slot A, it was counted in the >11-mm size class; if less than or equal to Slot B, it was



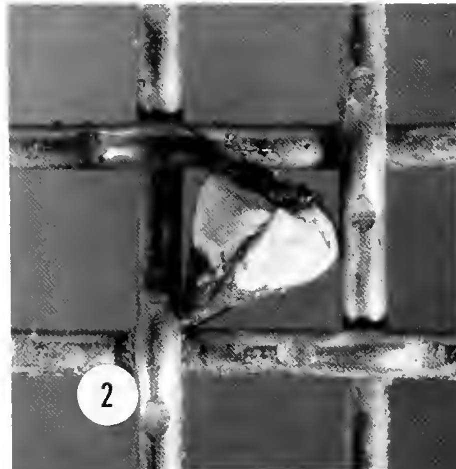


Figure 1. Zebra mussel passing downward through a sieve pore lengthwise.
Figure 2. Zebra mussel being selected in a pore by its largest-cross-sectional diameter, i.e., height.

counted in the <6-mm size class. All other mussels were counted in the 6- to 11-mm size class. After this size separation, mussels of the same size class were placed together in beakers and held as outlined above.

Sieving Procedure

Preliminary trials suggested that sieves with 5.6- and 2.8-mm pores sizes would retain mussels 6–11 mm in length between them. Timed sieving trials (46, 60, and 186 min) were thus conducted with this pair of sieves to test the accuracy of their size selection. With a chisel-tipped Exacto® knife, care was taken to cut the byssal threads and not to pry the mussels off of rocks. Mussels were separated from each other either by gently rubbing them between the fingers or by using the knife to cut byssal threads. They were then layered in the top sieve (i.e., 5.6 mm) to a maximum thickness of ca. 1.5 cm; a thicker layer risked obstructing free passage of individual mussels through pores. For convenience, a third sieve with a 1.0-mm pore size was used to trap mussels passing through both sieves. A sieve cover was placed on top of the three sieves, and a 2-cm-wide elastic band was attached to keep the cover and sieves firmly together. Sieving was performed in a 18-L pail filled with unchlorinated tap water. The sieves were submerged into the pail of water rapidly, thus causing the mussels to be flushed upward into the water column. When air bubbles stopped emerging from around the sieve cover (ca. 10–15 sec), the sieves were lifted completely out of the water. At this point, the mussels were floating in the water column, and as the water drained out, they were drawn lengthwise down through the pores. This submerging process was repeated four times. The mussels still remaining on the 5.6-mm sieve (presumably >11 mm long), those collected between the 5.6- and 2.8-mm sieves (presumably 6–11 mm long), and those that had passed through both sieves (presumably <6 mm long) were transferred into separate beakers and stored as described above. After the 7-day mortality check, the length of each mussel was measured with the gauge (Fig. 3) to check the accuracy of the size class separation.

Additional sieves were used in pairs to examine the size range of zebra mussels for which they would select. The pore sizes of these sieves were: 1.00, 2.00, 2.36, 2.80, 3.35, 4.00, 4.75, 5.60, 6.30, 9.50, and 12.50 mm. The zebra mussels from the Mohawk River that were used in these experiments ranged from 2.1 to 31.2 mm in length.

RESULTS AND DISCUSSION

Regression analysis revealed a strong correlation between shell length and height of *D. polymorpha* from the Mohawk River (height = 0.65 length$^{0.92}$; r^2 = 0.99); for any given length, only a small range existed in height, particularly with smaller mussels (Fig. 4). Although the sieving procedure could be used to separate size classes of any zebra mussel population, the results of our sieving trials are only applicable to mussel populations whose maximum cross-sectional dimension is height (not width) and whose length-height regression line would not significantly differ from that of the Mohawk River population. Because of differences in shell shape, our data are also not directly applicable for *Dreissena bugensis* (quagga mussels), the other exotic dreissenid present in North America. Our length-height regression line, however, is very similar to that described by Prejs et al. (1990) for zebra mussels in Poland (Fig. 4, insert). The near overlap of these regression lines suggests that our sieving data would likely be applicable to at least some European *D. polymorpha* populations.

Sieving yielded more than three times as many 6- to 11-mm-long mussels/min as did hand picking (statistically significant, p = 0.01) (Table 1). Mean yields from sieving and picking were 10.2 and 3.0 mussels/min, respectively, with 0.4% mortality after

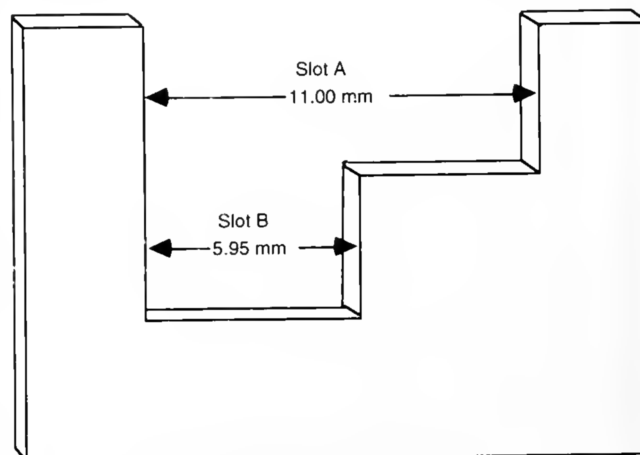


Figure 3. Gauge used to separate zebra mussels into the following three size classes: <6, 6–11, and >11 mm in length. See text for explanation.

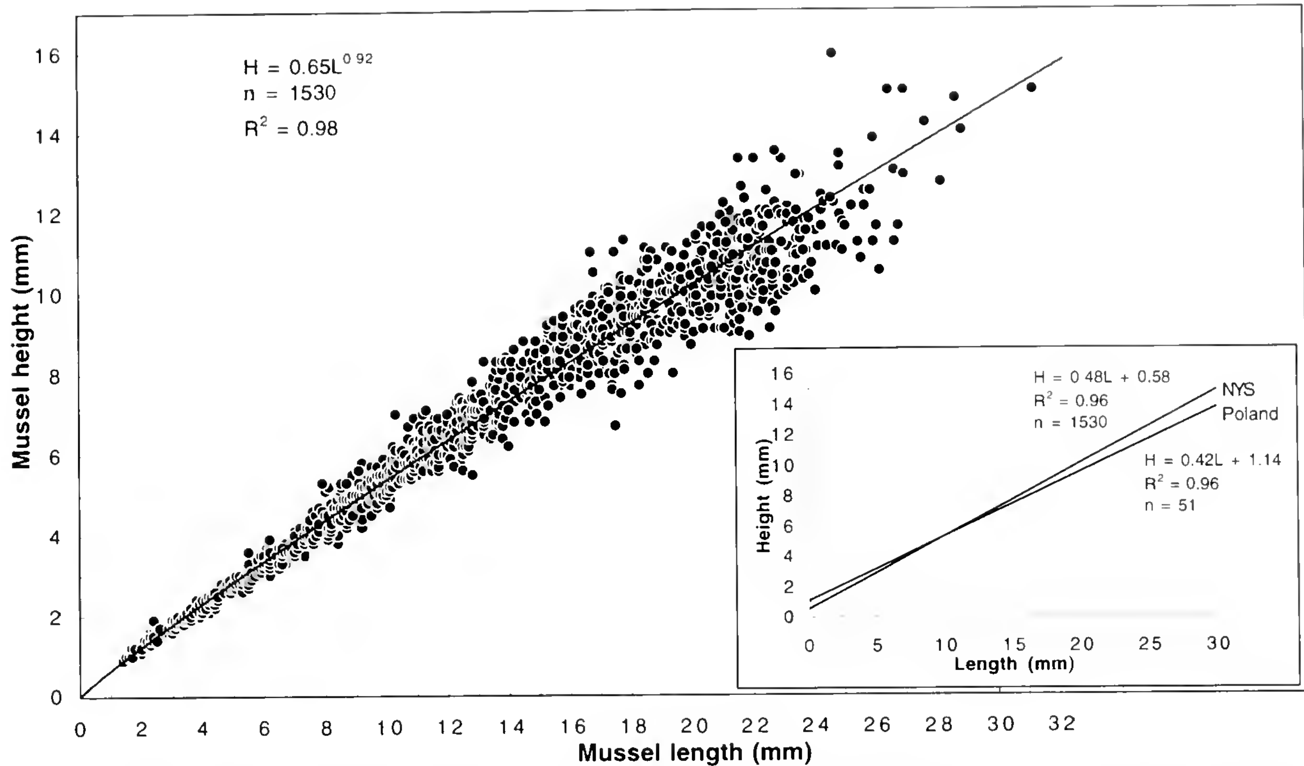


Figure 4. Power equation expressing the relationship between shell length (L) and height (H) of *D. polymorpha* from the Mohawk River in October 1994. Insert: Mohawk River data expressed as a simple linear equation (NYS) in order to compare it with data (Poland) in Prejs et al. (1990).

7 days for both procedures. Sieving also produced greater yields per minute than picking in the other two size classes, i.e., <6 and >11 mm (statistically significant, $p = 0.10$ and $p = 0.05$ respectively) (Table 1).

Sieving, however, did not completely separate the mussels into nonoverlapping size classes, as desired. Individual length measurements revealed that 1.1, 7.1, and 11.1% of mussels that were separated into the "<6, 6–11, and >11 mm" size classes were not within these ranges (Table 1). It became evident that an overlap in the length of the mussels collected by a series of sieves was unavoidable primarily because of natural morphological variation in the zebra mussel population. Not all mussels of the same length were of the same height (Fig. 4), and height was the character a sieve pore selected for as a mussel passed lengthwise through it. Thus, two mussels of the same length, but of different heights, could be retained on different sieves. Furthermore, as was observed in our data, the greater the variation in height for a given length, the more likely mussels of this length would be retained on different sieves. In the Mohawk River population, variation in height increased steadily with mussel length (Fig. 4), and the percent error in size class selection (1.1, 7.1, and 11.1%) also increased steadily with size class length (<6, 6–11, and >11 mm). In judging the usefulness of the sieving procedure versus picking, the inability of this procedure to strictly separate zebra mussel size classes by length has to be weighed in individual research projects against the significant increase in mussel yield.

TABLE 1.

Comparison of Hand Picking and Sieving Zebra Mussels.

Mussel Length (mm)	Mean (±SD)	Hand Picking	Sieving
<6	No. of mussels collected/min	2.1 ± 0.1	6.3 ± 1.9
	% Error in selection	0	1.1 ± 1.7
	% 7-Day mortality	1.2 ± 0.4	3.2 ± 1.7
6–11	No. of mussels collected/min	3.0 ± 0.3	10.2 ± 1.4
	% Error in selection	0	7.1 ± 0.3
	% 7-day mortality	0.4 ± 0.1	0.4 ± 0.5
>11	No. of mussels collected/min	2.8 ± 0.8	10.0 ± 4.4
	% Error in selection	0	11.1 ± 0.2
	% 7-Day mortality	0.4 ± 0.3	0.2 ± 0.4

Performing this sieving procedure in the field (Fig. 5) was actually simpler than in the laboratory. Working directly in the river eliminated the need to transport mussel-laden rocks and other substrates to the laboratory and reduced the effort required to properly dispose of contaminated materials.

When a wide range of sieve pairs was used, mussels were separated into discrete ranges in length (Fig. 6). The upper and lower limits of these size ranges were determined by the pore sizes of the upper and lower sieves, respectively. As expected from natural variation in shell length and height (as discussed above), these size ranges partially overlapped each other and increased with mussel length. The data presented in Figure 6 clearly demonstrate that the larger the difference in pore size between the two sieves being used, the larger the range in the size of the mussels collected between them.

One of the challenges in using this sieving procedure is determining the exact sieve sizes that will retain mussels only within a particular length range. Individuals wanting to use sieving for zebra mussel size separation can use the regression equation, pore size = 0.47 mussel length + 0.29, as a guide for estimating the sieve pore sizes needed to collect mussels in a desired size range.

Figure 5. Sieving under field conditions. The use of 5.6-, 2.8-, and 1.0-mm sieves (top to bottom) in the Mohawk River (Crescent, NY) to collect zebra mussels, respectively, >11, 6–11, and <6 mm long.

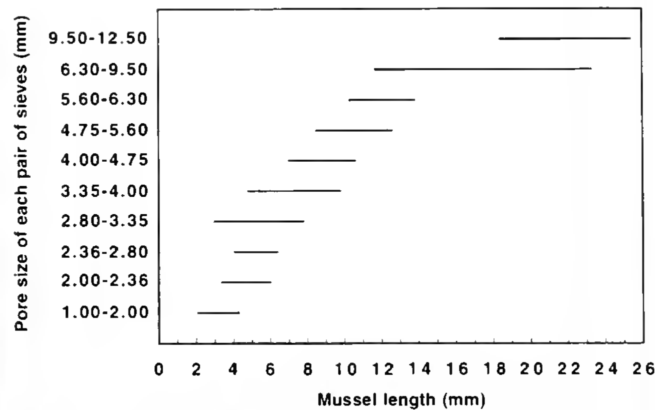


Figure 6. Range in length of *D. polymorpha* selected by various pairs of sieves.

This equation was produced from plotting and analyzing all sieving data produced in this study and assumes a length-height relationship identical to our study population (Fig. 4) and a population whose maximum cross-sectional dimension is height. This equation predicts, for example, that mussel size classes with approximate lengths of 1–5, 5–10, 10–15 and 15–20 mm would result from the use of pairs of sieves with pore sizes, respectively, of 0.8 and 2.6 mm, 2.6 and 5.0 mm, 5.0 and 7.3 mm, and 7.3 and 9.7 mm.

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TRENDS IN BLUE CRAB (*CALLINECTES SAPIDUS* RATHBUN) CATCHES NEAR CALVERT CLIFFS, MARYLAND, FROM 1968 TO 1995 AND THEIR RELATIONSHIP TO THE MARYLAND COMMERCIAL FISHERY

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ABSTRACT In an effort to understand some of the consequences of increased fishing pressure on the Maryland blue crab population, we have analyzed data collected along 12 km of western Chesapeake Bay in Calvert County from 1968 to 1995. Commercial peeler crab pots of 25-mm-pore-size mesh, baited daily with menhaden, were used to sample crab stocks at three locations with up to 60 pots fished during alternate weeks from June through November. Station catches were sorted, measured, and weighed by sex. From 1968 through 1995, 113,002 crabs were caught in 18,106 pots, of which 73% were legal size (127-mm carapace width). Although annual mean catch per unit effort (CPUE) varied considerably, it appeared to be within some normal range. Total CPUE ranged from 0.85 in 1968 to 20.01 in 1981 (legal CPUE ranged from 0.73 to 13.57); Maryland commercial landings ranged from 4.7×10^6 kg to 27.1×10^6 kg during the same years. From 1968 to 1980, legal CPUE averaged 3.60; from 1981 to 1985, it averaged 8.14; and from 1986 to 1995, it was 3.66. Thus, the legal CPUE of the most recent period was nearly identical with that of the earliest period. There are, however, several trends that have become apparent, indicating that increased fishing pressure may be having a deleterious effect on the blue crab population. A significant correlation between this fishery-independent data and Maryland Department of Natural Resources' fishery-dependent data demonstrates the relevance of these trends. From 1968 to 1982, the annual male percentage decreased significantly from 66 to 38% ($r^2 = 0.79$; $p < 0.01$). Since 1983, this percentage has shown greater fluctuation among years, but has shown no further decrease. The mean carapace width and weight of females have not changed significantly over time, but the mean width of males ($r^2 = 0.47$) and the mean weight of males ($r^2 = 0.34$) have both decreased significantly ($p < 0.01$). Percent legal size crabs, which constituted 64–86% of the annual catch between 1968 and 1991, has had its three lowest years (averaging 52%) since just 1992; the percentage of legal males in the catch decreased from 56% in 1968 to 19% in 1995 (13% in 1994) ($r^2 = 0.75$; $p < 0.01$). These downward trends related to the size of males indicate that they are being removed from the population shortly after reaching legal size. With fewer large males available to crabbers, even more pressure may be exerted on females, which could eventually result in further decreases in population size and stability.

KEY WORDS: blue crab, *Callinectes sapidus*, Chesapeake Bay, population trend, harvesting, fishery, fishing pressure, regulations

INTRODUCTION

The annual value of the blue crab (*Callinectes sapidus* Rathbun) landings in the Maryland Chesapeake Bay was less than that of the oyster (*Crassostrea virginica* Gmelin) from the late 1800s until 1983, when 23.8×10^6 kg of crabs valued at $22.6 million surpassed the 3.4×10^6 kg of oyster meats valued at $14.0 million (NMFS 1985). Since then, with the continuing decline of the oyster fishery due primarily to disease (MSX and Dermo), the difference between the values of the two fisheries has continued to widen. The 1993 oyster season yielded 440×10^5 kg of oyster meats worth about $3.2 million compared with 28.0×10^6 kg of crabs (hard and soft) worth $37.4 million (NMFS 1994). The real difference between the size of these two fisheries is actually much greater than indicated by these figures because the recreational harvest of blue crabs is also substantial and far greater than the recreational harvest of oysters. Although the recreational crab harvest adds nothing to the value of the commercial fishery, it does add value to the Maryland economy. The most recent recreational harvest expenditures (1990) were estimated in excess of $110 million (USFWS 1993, Stagg et al. 1994).

The oyster fishery, however, has not been the only commercial fishery to decline in recent years and thus affect the bay's watermen. Regulations on certain finfishes such as American shad and striped bass, which once supported heavy commercial activity, now include closed or tightly regulated seasons, changes in minimum or maximum size, and daily and/or seasonal catch limits. The soft clam fishery has also suffered major stock reductions in recent years, resulting in increased regulations.

With major reductions in the size of finfish, oyster, and clam fisheries, more pressure has been exerted by watermen on the blue crab to make up some revenues lost from other fisheries. Although blue crab landings have often fluctuated widely among years (Pearson 1948, Van Engel 1958, Tagatz 1965, Abbe 1983, Lipcius and Van Engel 1990), catches often recovered rapidly after poor years, and the fishery has generally held up well considering long-term increases in fishing pressure. Because a resource has been a consistent producer, however, is no guarantee that it will remain so forever. The striped bass (*Morone saxatilus*) fishery was once thought to be inexhaustible, but poor recruitment from the early 1970s to the early 1980s, coupled with questionable management practices, necessitated a moratorium on harvesting for several years and changes in the management of the fishery before it began to return to a healthy condition.

Because so many of Maryland's fisheries have declined over the past two decades, it seems obvious why concern has been

shown for the blue crab fishery, which is the healthiest fishery in Maryland at this time. Legislators who saw decreased crab landings in 1992 (14.2×10^6 kg worth $17.6 million; NMFS 1994) were concerned that the crab fishery might also be declining with oysters, clams, and some finfish. As a result, efforts began on a series of conservation-oriented changes in the fishery that were implemented before the 1994 season. These included limited entry to the fishery with a 2-y wait for a new license, limiting a commercial license holder to 300 crab pots with a maximum of 900 per vessel (if two additional allocations were purchased for additional crew), limiting the hours that pots could be fished, and requiring a 59-mm (2 5/16-inch) cull ring in the upper chamber of each pot. New regulations were also set in place for the use of trotlines and crab scrapes. Further emergency restrictions were added during the 1995 season that shortened the workday by several hours, the workweek by a day, and the season by 6 wk.

In the late 1960s, when many of Maryland's fisheries were far healthier than they are now, there was also concern about the possible effects of the thermal discharge from the Calvert Cliffs Nuclear Power Plant (CCNPP) (then under construction) on the blue crab population in the Chesapeake Bay waters adjacent to the plant. This led to a series of studies of population size and structure from 1968 to 1983 (Abbe 1987). Those studies detected no adverse effect of the power plant on numerous parameters of the local crab populations, but after 28 y, there appear to be changes in the population structure that may be undesirable for the fishery. Although there still appear to be no station differences indicative of power plant effect, the observations of change are based on long-term trends. Long-term fishery-independent data sets of at least 10–15 y are important in understanding the dynamics of commercially valuable populations (Lipcius and Van Engel 1990), but such data sets are also uncommon. Changes in a population over 3–5 y may be meaningful, but because of relatively short duration, it is difficult to show significance unless the changes are large. Long-term data sets, however, enable an investigator to attach significance to more subtle changes.

But what do these changes at Calvert Cliffs mean? Do they represent changes in the localized population only or are they reflective of change on a broader baywide scale? To examine this question, it was necessary to compare the long-term fishery-independent data from Calvert Cliffs with the fishery-dependent data of the Maryland Department of Natural Resources (MDNR). We felt that if the Calvert Cliffs and MDNR data compared favorably over many years, then the Calvert Cliffs data might be reflective of the baywide crab population (or at least a large portion of it), and the changes observed at Calvert Cliffs could also be occurring elsewhere in the bay. This article reports some of the changes observed over time that may have resulted from increased fishing pressure as well as descriptive statistics of these populations over the entire 28-y period. Calvert Cliffs and MDNR data are compared where possible.

MATERIALS AND METHODS

Calvert Cliffs Studies

Stations

Sampling sites were located adjacent to and on either side of the CCNPP (Fig. 1). Although the Plant Site station was located within [illegible] m of the plant discharge in 2.5 m of water, it did not receive the full effect of the thermal plume because it was off to one side. The temperature averaged 1–2°C above ambient. Another station was located in 4 m near Kenwood Beach, 8 km northwest of the plant. The third station was southeast of Rocky Point, approximately 4 km from the plant in 4 m of water. Both Kenwood Beach and Rocky Point were outside the predicted area of thermal loading from the CCNPP when they were established in 1968. Plant operation did, however, result in occasional temperature increases of up to 1°C at Rocky Point; Kenwood Beach was unaffected. Crab pots remained on station from June to November; they were not moved to deeper water in the fall as shallow waters cooled, as is done by many commercial crabbers.

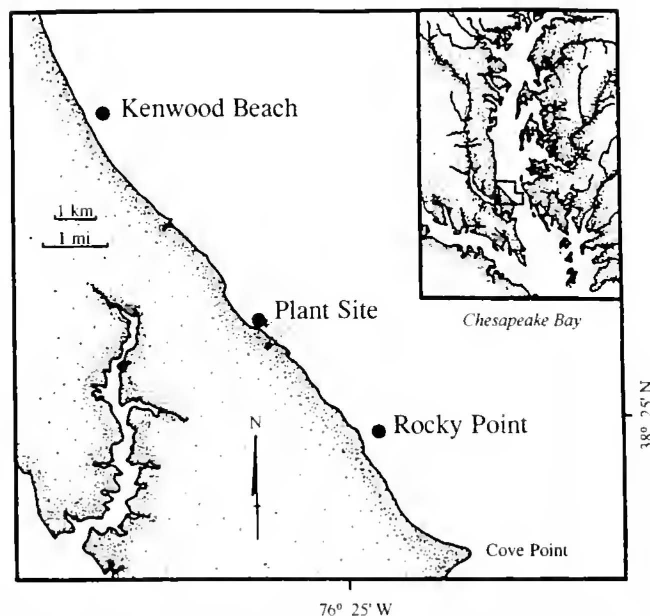

Figure 1. Locations of crab pots in the study area near Calvert Cliffs in central Chesapeake Bay from 1968 to 1995.

Sampling Procedures

Commercial crab pots of 25-mm (1-in) galvanized-wire mesh were used to sample crab stocks at the three stations from spring until late fall, when water temperatures decreased to levels at which crabs would no longer actively enter pots (10–12°C). Because blue crabs enter baited pots as a feeding response, temperatures must be warm enough for them to actively feed. Van Engel (1962) reviewed the development of crab pots and the methods used to fish them. Most commercial pots are constructed of 38-mm (1½-in) mesh and will retain few crabs smaller than 76-mm (3-in) carapace width. With the present requirement for a cull ring, even much larger crabs are able to escape from commercial pots. The cull ring can be closed when catching peeler crabs for the soft crab market because 76-mm peeler crabs are legal in Maryland, in contrast to hard crabs, which must be 127 mm (5 in) across the lateral spines. The smaller meshed peeler pots used in this study (which contained no cull rings) allowed some crabs smaller than 51 mm (2 in) to be caught, although such pots were probably not very efficient at catching crabs this small.

Crab pots provided an excellent means to sample the blue crab population, although they may not be the choice for sampling other crab species that exhibit agonistic behavior. Miller (1980) and Williams and Hill (1982) suggested that such behavior exhib-

ited by *Cancer productus*, *Cancer irroratus*, *Hyas araneus*, and *Scylla serrata* could limit the catch in a trap because the first crab caught might then prevent others from entering. *C. sapidus* does not behave this way; our record for crabs in a pot set for 1 day was 57.

Pots were generally fished every other week from spring until fall. In 1968, three pots were fished at each station for 5 days; from 1969 to 1981, five pots were fished for 4 days; and from 1982 to 1995, 10 pots were fished for 2 days. From 1970 to 1981, sampling began in early May and sometimes continued into December. From 1982 to 1995, sampling was conducted only from early June through November. To help normalize the data, only June to November data are examined here. The crab catch is generally light during both May and December because of cold water temperatures; the elimination of May and December data reduced the total catch by 2,965 crabs or about 5.5% of the 54,006 crabs caught from 1970 to 1981. Pots were baited daily with menhaden, with up to 20 pots fished per station per week (15 per week in 1968), except when losses occurred to storms or boats. Station catches were weighed by sex to the nearest 0.1 kg, and the carapace width (CW) of each crab was measured to the nearest ⅛ in (3 mm) across the lateral spines. Field CW measurements were later converted to metric.

The number of pots fished annually varied considerably, especially during the early years, because of changes in starting dates and differences in ending dates resulting from weather and population fluctuations. Limiting the analyses to the June–November data helped reduce, but not eliminate, the variance in pots sampled.

Bottom temperature and salinity were determined monthly by thermistor probe and titration, respectively, from 1968 to 1978 and daily during the weeks fished from 1979 to 1995, with a Beckman RS5-3 portable salinometer. Dissolved oxygen concentrations were determined monthly through 1974 and daily thereafter, either by Winkler titration or with a YSI Model 57 dissolved-oxygen meter.

Data Analysis

Much of the Calvert Cliffs data were examined graphically for trends and by standard linear regression techniques (Draper and Smith 1966, Kleinbaum and Kupper 1978). If a regression r^2 was significant, then we concluded that the trend most likely had biologic significance.

MDNR Data

Before 1981, data were collected from a census of commercial crabbers. On the basis of an intensive census in 1979, a random stratified sampling procedure was developed to more accurately estimate blue crab catch and effort for the pot fishery and the trotline fishery (Summers et al. 1983a, Summers et al. 1983b). For trotlines, stratification was by month; for pots, it was by month and county of residence. In 1994, MDNR returned to a census approach. Both the sampling and the census approaches have allowed estimates of effort and catch per unit effort (CPUE) in the pot component of the Maryland blue crab fishery since 1981.

Virginia data through 1992 were compiled by the NMFS from voluntary industry reporting programs. Since 1993, the Virginia Marine Fisheries Commission has adopted a mandatory reporting system. Chesapeake Bay values are simply Maryland and Virginia values combined.

Statistical Analysis

Pearson correlation analysis (SAS 1985) was conducted to examine the relationships among the Calvert Cliffs legal CPUE and several fishery-dependent variables. These included the reported Maryland blue crab pot catch and total catch from 1968 to 1995, the reported Virginia pot catch and total catch from 1968 to 1993, the reported Chesapeake Bay pot catch and total catch from 1968 to 1993, and the reported Maryland pot CPUE from 1981 to 1995. Because there appears to be a notable difference in the patterns of blue crab abundance between the period before 1981 and the period from 1981 onward, and because of the change in Maryland's reporting system in 1981, the data were thus divided and examined separately.

RESULTS AND DISCUSSION

MDNR

The fishery-dependent data for Maryland and Virginia used in the correlation analysis are presented in Table 1. Over the entire time period (1968–1995), the highest correlations between Calvert Cliffs CPUE and various reported catch statistics were 0.718 for the Maryland pot harvest and 0.707 for the Chesapeake Bay pot harvest (both $p < 0.0001$; Table 2). Although lower, there was also a significant (0.421; $p < 0.05$) relationship with the Virginia pot fishery. Because Chesapeake Bay numbers are totals of Maryland and Virginia, however, they are not independent confirmations of the representative character of the Calvert Cliffs data. In general, the correlations for the 1968–1980 period were lower and less significant than corresponding correlations for the 1981–1995 period (Table 2). This difference could be the result of the more accurate reporting of harvests (especially for Maryland) in the latter period. In the 1981–1995 period, correlations between Calvert Cliffs CPUE and Maryland pot harvest (0.794) and Maryland total harvest (0.817) were both highly significant (Table 2). The highest correlation was between Calvert Cliffs CPUE and Maryland pot CPUE (0.881; $p < 0.0001$) and was not unexpected. The strong relationship between these data sets demonstrates the representative character of the Calvert Cliffs data. It further suggests that when reasonably accurate measures of commercial harvests and efforts can be estimated, the results are quite similar to fishery-independent measures of abundance.

Calvert Cliffs

Since 1968, a total of 113,002 crabs have been caught in 18,106 pots at Calvert Cliffs (Table 3). The total number of crabs per pot has ranged from 0.85 in 1968 to 20.01 in 1981, whereas the number of legal crabs per pot has ranged from 0.73 to 13.57 for the same years (Table 3). Maryland commercial landings have ranged from a low of 4.68×10^6 kg in 1968 to a high of 27.14×10^6 kg in 1981 (Table 1). From 1968 to 1980, the total number of crabs caught per pot or caught per unit effort (CPUE) and the legal CPUE averaged 4.63 and 3.60 per year, respectively; from 1981 to 1985, these averages increased to 11.40 and 8.14, respectively; and from 1986 to 1995, they averaged 5.33 and 3.66, respectively. Because these figures indicate that the mean legal CPUE of the 1986–1995 period was almost identical with that of the 1968–1980 period, one might assume that other aspects of the populations were similar as well, but this was not so. Wide fluctuations in the size of the sample population based on CPUE continues to be the normal pattern (Fig. 2) and results in part from the fact that crabs

TABLE 1.

Maryland (MD), Virginia (VA), and Chesapeake Bay (CB) blue crab harvests from the pot fishery and the total fishery in millions of kilograms from 1968 to 1995; also included is the Maryland pot fishery CPUE in kilograms per pot per month from 1981 to 1994.

Year	MD Pot Fishery	MD Total Fishery	MD Pot CPUE	VA Pot Fishery	VA Total Fishery	CB Pot Fishery	CB Total Fishery
1968	2.3	4.7		14.2	20.7	16.5	25.4
1969	6.0	11.5		10.9	16.2	16.9	27.7
1970	6.5	12.0		12.9	19.7	19.4	31.7
1971	7.1	12.5		16.2	22.0	23.3	34.5
1972	6.3	11.4		16.5	22.4	22.8	33.8
1973	5.3	9.5		12.9	17.1	18.2	26.6
1974	7.1	12.0		15.1	18.9	22.2	30.9
1975	7.2	11.8		13.9	16.1	21.1	27.9
1976	5.9	9.5		9.1	12.0	15.0	21.5
1977	6.2	9.7		14.3	17.2	20.5	26.9
1978	5.9	7.9		13.5	16.6	19.4	24.5
1979	9.2	11.7		15.0	18.5	24.2	30.2
1980	10.1	12.0		12.9	17.4	23.0	29.4
1981	16.1	27.1	25.9	14.5	19.3	30.6	46.4
1982	11.7	19.8	12.9	16.6	20.3	28.3	40.1
1983	14.3	23.8	14.9	18.3	21.2	32.6	45.0
1984	13.9	22.1	16.4	17.9	22.9	31.8	45.0
1985	17.4	26.5	19.6	15.5	17.6	32.9	44.1
1986	14.1	23.5	16.6	13.6	17.4	27.7	40.9
1987	12.7	20.9	13.9	12.2	14.8	24.9	35.7
1988	12.9	19.5	14.2	13.9	16.9	26.8	36.4
1989	11.7	19.7	13.0	15.6	20.2	27.3	39.9
1990	12.1	21.2	15.9	19.8	23.6	31.9	44.8
1991	12.9	21.8	15.5	16.4	20.4	29.3	42.2
1992	8.6	14.2	10.7	9.0	10.8	17.6	25.0
1993	16.6	26.0	13.1	20.1	24.0	36.7	50.0
1994	10.3	17.7	10.3		21.8		39.5
1995	10.2	17.0					

reach legal size in only their second year (Van Engel 1958). Thus, a poor year (1970) can be followed by a good year (1971), or the reverse can occur (1986 and 1987), based on reproductive and recruitment success. Large annual fluctuations are also apparent in the Maryland and Virginia populations (Pearson 1948, Van Engel 1958, Abbe 1987, Lipcius and Van Engel 1990). Blue crab populations have remained relatively strong over the long term, even though the current estimate of total instantaneous mortality is about 73% and fishing mortality is about 59% (NOAA unpublished data). Other estimates put mortalities much higher (Rothschild et al. 1992). The apparent relative stability of the fishery in spite of high mortalities may be due to high fecundity (the external

TABLE 2.

Correlation coefficients (r) between Calvert Cliffs legal CPUE and various fishery-dependent catches for the pot fishery and the total fishery from Maryland (MD), Virginia (VA), and all of Chesapeake Bay (CB) for 1968–1995, 1968–1980, and 1981–1995; the Calvert Cliffs legal CPUE is also correlated with Maryland CPUE for the most recent period.

CC Legal	MD Pot	MD Total	MD CPUE	VA Pot	VA Total	CB Pot	CB Total
1968–1995							
r	0.718	0.703		0.421	0.283	0.707	0.686
p <	0.0001	0.0001		0.032	0.153	0.0001	0.0001
n	28	28		26	27	26	27
1968–1980							
r	0.691	0.725		0.224	−0.006	.597	0.427
p <	0.009	0.005		0.461	0.984	0.031	0.146
n	13	13		13	13	13	13
1981–1995							
r	0.794	0.817	0.881	0.321	0.298	0.589	0.645
p <	0.0004	0.0002	0.0001	0.285	0.300	0.034	0.013
n	15	15	14	13	14	13	14

TABLE 3.

Catches of blue crabs in Chesapeake Bay near Clavert Cliffs from 1968 to 1995.

Year	Total No.	No. of Males	No. of Females	Percent Males	Legal Males	Legal Females	Total Legal Size (>127 mm)	Total Sublegal	Percent Legal	Total Pots Fished	Total Crabs per Pot	Legal Crabs per Pot	Legal Males per Pot	Legal Females per Pot
1968	239	158	81	66.1	133	73	206	33	86.2	281	0.85	0.73	0.47	0.26
1969	2,833	1,995	838	70.4	1,450	556	2,006	827	70.8	472	6.00	4.25	3.07	1.18
1970	1,318	800	518	60.7	613	400	1,013	305	76.9	504	2.62	2.01	1.22	0.79
1971	4,463	2,461	2,002	55.1	1,811	1,632	3,443	1,020	77.1	590	7.56	5.84	3.07	2.77
1972	2,699	1,611	1,088	59.7	1,160	906	2,066	633	76.5	690	3.91	2.99	1.68	1.31
1973	2,903	1,663	1,240	57.3	1,264	1,026	2,290	613	78.9	727	3.99	3.15	1.74	1.41
1974	3,718	2,225	1,493	59.8	1,658	1,137	2,795	923	75.2	640	5.81	4.37	2.59	1.78
1975	4,467	2,142	2,325	48.0	1,713	2,042	3,755	712	84.1	751	5.95	5.00	2.28	2.72
1976	2,735	1,192	1,543	43.6	743	1,144	1,887	848	69.0	734	3.73	2.57	1.01	1.56
1977	1,998	1,024	974	51.3	883	818	1,701	297	85.1	630	3.17	2.70	1.40	1.30
1978	3,340	1,622	1,718	48.6	1,155	1,356	2,511	829	75.2	740	4.51	3.39	1.56	1.83
1979	5,386	2,839	2,547	52.7	2,109	2,129	4,238	1,148	78.7	699	7.71	6.06	3.02	3.05
1980	3,206	1,361	1,845	42.5	1,089	1,653	2,742	464	85.5	741	4.33	3.70	1.47	2.23
1981	14,809	6,691	8,118	45.2	3,385	6,657	10,042	4,767	67.8	740	20.01	13.57	4.57	9.00
1982	3,797	1,449	2,348	38.2	981	1,920	2,901	896	76.4	657	5.78	4.42	1.49	2.92
1983	5,400	2,640	2,760	48.9	1,780	2,446	4,226	1,174	78.3	682	7.92	6.20	2.61	3.59
1984	7,347	3,470	3,877	47.2	1,736	3,190	4,926	2,421	67.0	653	11.25	7.54	2.66	4.89
1985	7,373	2,870	4,503	38.9	1,497	4,015	5,512	1,861	74.8	613	12.03	8.99	2.44	6.55
1986	3,823	1,548	2,275	40.5	1,004	2,088	3,092	731	80.9	620	6.17	4.99	1.62	3.37
1987	1,573	880	693	55.9	570	547	1,117	456	71.0	687	2.29	1.63	0.83	0.80
1988	3,380	1,620	1,760	47.9	983	1,435	2,418	962	71.5	655	5.16	3.69	1.50	2.19
1989	4,287	1,790	2,497	41.8	759	1,991	2,750	1,537	64.1	684	6.27	4.02	1.11	2.91
1990	3,474	1,458	2,016	42.0	1,080	1,824	2,904	570	83.6	662	5.25	4.39	1.63	2.76
1991	4,073	1,260	2,813	30.9	770	2,627	3,397	676	83.4	678	6.01	5.01	1.14	3.87
1992	2,802	1,418	1,384	50.6	535	1,042	1,577	1,225	56.3	618	4.53	2.55	0.87	1.69
1993	5,703	2,621	3,082	46.0	1,367	2,586	3,953	1,750	69.3	687	8.30	5.75	1.99	3.76
1994	3,521	1,693	1,828	48.1	466	1,170	1,636	1,885	46.5	612	5.75	2.67	0.76	1.91
1995	2,335	1,271	1,064	54.4	452	809	1,261	1,074	54.0	659	3.54	1.91	0.69	1.23
Total	113,002	53,772	59,230		33,146	49,219	82,365	30,637		18,106				
Mean	4036	1920	2115	47.6	1184	1758	2942	1094	72.9	647	6.24	4.55	1.83	2.72

egg mass of a female may contain from 750,000 to 8 million eggs, depending on her size; Prager et al. 1990) and a short life span of 2–3 y (Hay 1905, Churchill 1919, Van Engel 1958). Although recent evidence based on tag returns (McConaugha 1991) indicates that blue crabs may live to be 7 to 8 y old, most probably do not live longer than 2 or 3 y because they are exploited by the fishery. Ultimate age may be critical in a mathematical population model, but it is less critical to the crab population itself because blue crabs are capable of spawning in their second or third year. If there was considerable reproductive output from 4- to 7-y-old blue crabs in the Chesapeake Bay, then the harvest of 2 and 3 y olds could have a major effect on population size, but this is probably not the case.

The 53,760 males caught between 1968 and 1995 accounted for slightly less than half of the catch (47.6%), or 49.5% if averaged across equally weighted years. The trend for male percents has been downward throughout much of this study, and the decline is significant across all years from 1968 to 1995 ($r^2 = 0.476$; $p <$

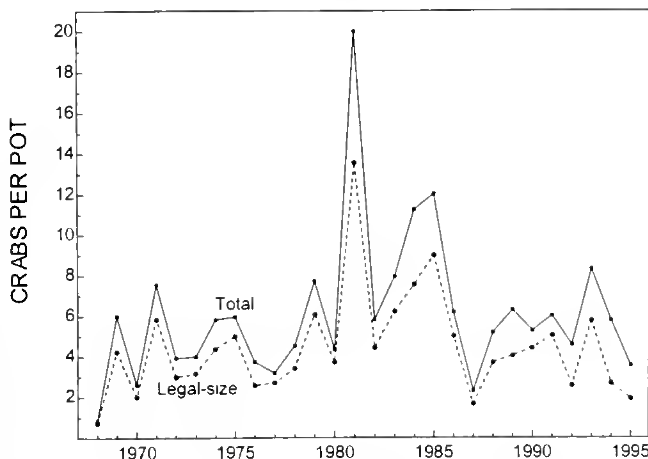

Figure 2. Total and legal-size CPUE near Calvert Cliffs from 1968 to 1995.

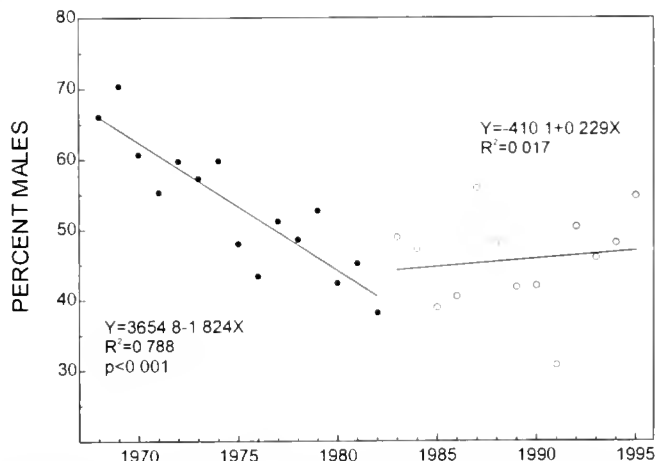

Figure 3. Annual percentage of total catch near Calvert Cliffs consisting of males showing the decline from the late 1960s through the early 1980s and the subsequent leveling off.

0.001). It was most evident, however, between 1968 and 1982 (Fig. 3), when males decreased from 66.1 to 38.2% of the total catch ($r^2 = 0.788$; $p < 0.001$). Because the ratio of females to males is higher at higher salinities, salinity data from 1968 to 1981 were analyzed to determine if there had been an increase in salinity over time that would explain the decline in males, but no increase in salinity was apparent (Abbe 1983). From 1983 to 1995, the percentage of males fluctuated more than it had during earlier years, but there was no further decline ($r^2 = 0.017$).

When percent legal males was examined, however, the decline was continuous over time, and this is vital to the fishery because legal crabs are the ones that comprise the fishery. The three highest years were in 1968–1970 (as with total males), with a significant decrease until 1995 ($r^2 = 0.753$; $p < 0.001$); however, unlike percent total males, which began to level off in the early 1980s (Fig. 3), percent legal males continued to decline, reaching a low of just 13% in 1994 (Fig. 4). In contrast to legal males, however, legal females have shown a significant increase ($r^2 = 0.233$; $p < 0.01$) over the entire period (Fig. 4), but it was far less significant than the decrease of legal males. Legal females, however, have shown lower percents during the last 4 y than during many earlier years, and this may be the result of added fishing pressure as well.

The 82,365 legal crabs accounted for 72.9% of the total, 73.7% when averaged across equally weighted years. From 1968 to 1991, legal crab percents formed a fairly tight group, ranging from 64 to 86% of the annual catch. During 7 of these years, at least 80% of the catch was legal size, and during 15 y, at least 75% was legal. A regression line fit to the 1968–1991 data is flat ($r^2 = 0.018$). However, from 1992 to 1995, legal crabs fell to 56, 69, 46, and 54% of total catch, respectively. When these years are included in the data set, the regression becomes significant ($r^2 = 0.263$; $p < 0.01$). Although the regression is significant, a smooth curve illustrates the changes better than a straight line (Fig. 5). The fact that the three lowest percentages occurred during the last 4 y is another indication that fishing pressure may be greater than the legal portion of the population can withstand.

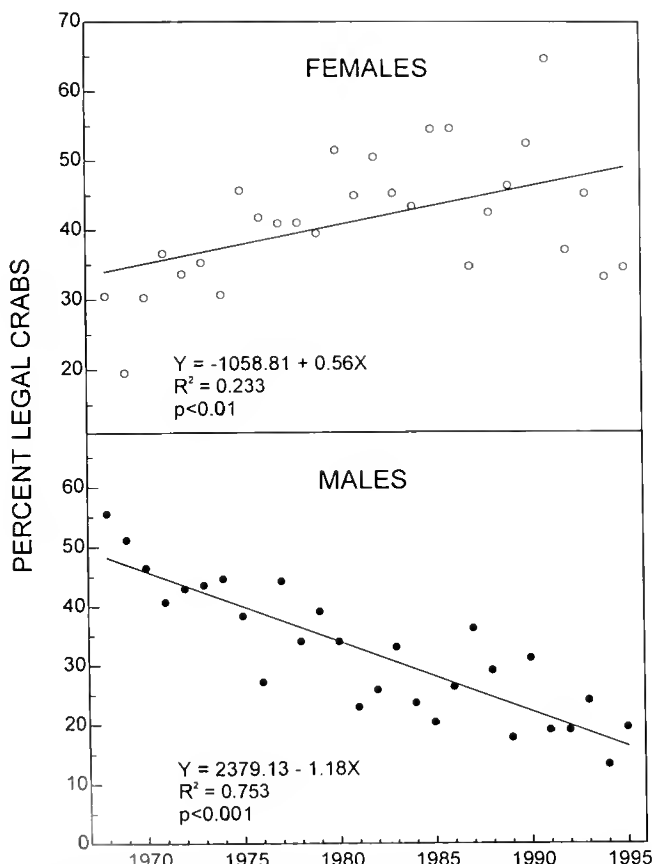

Figure 4. Percentages of legal males and females relative to the total catch near Calvert Cliffs from 1968 to 1995.

During the 28 y, male crabs averaged 134-mm CW and 154 g while females averaged 146-mm CW and 149 g. Females have longer lateral spines than males and thus are lighter per unit width than males, as indicated in Figure 6 and by others (Newcombe et al. 1949, Tagatz 1965, Pullen and Trent 1970). These means indicate only that males were smaller and heavier than females, but they do not illustrate what has happened to the male populations. Male crabs have shown a decrease in mean size (both width and weight) over time in contrast to females. The regression line for female width is almost flat ($r^2 = 0.021$), although annual means have been below average the last 4 y (Fig. 7). Males, however, decreased significantly ($r^2 = 0.471$; $p < 0.001$), and 7 of the last 8 y have been below the long-term average (Fig. 7). Females showed no decrease in weight over time ($r^2 = 0.015$), but males decreased significantly ($r^2 = 0.344$; $p < 0.01$). Because width and weight are highly correlated ($p < 0.001$ for both sexes; Fig. 6), it is not surprising that both width and weight show similar trends by sex.

The decrease in male size led to analysis of just the legal sector of the population because the decline could have resulted simply from increased numbers of sublegal males, which could have driven down the mean size of the entire male population. If this were so, there might be little effect on the mean size of the legal males, which make up a major part of the fishery. The regression of the mean width of legal males did show a significant downward trend ($r^2 = 0.356$; $p < 0.001$) (Fig. 8), similar to that for the entire male population (Fig. 7). The regression for legal females was almost flat (Fig. 8), as it was for the total female population (Fig. 7).

The annual mean widths of females were always greater than for males, but weights were not. The annual mean weights of males were greater than those of females from 1968 to 1980 and during 17 of the 19 y from 1968 to 1986. Since 1987, however, males have been heavier than females only once (1988), further

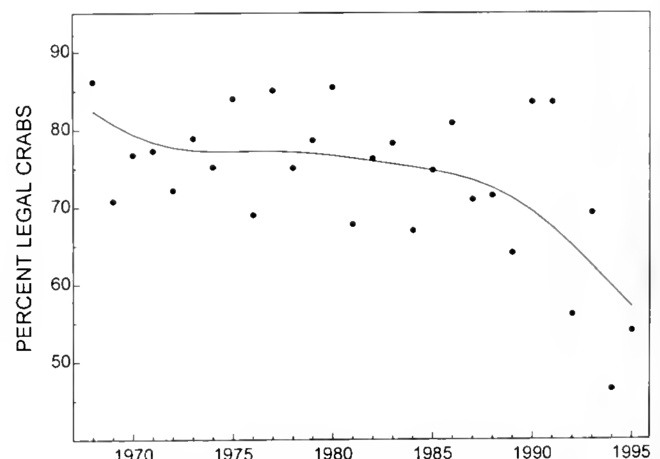

Figure 5. Annual mean percentage of legal crabs relative to the total catch, showing the stable period from 1968 to 1991 and the low levels during 3 of the last 4 y.

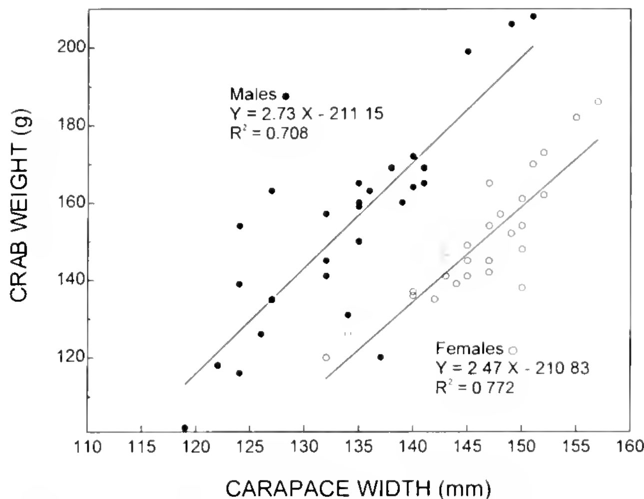

Figure 6. Relationship between annual mean CW and weight for males and females caught near Calvert Cliffs from 1968 to 1995.

supporting the decrease in average male size. The cause of this decline in male size is not certain, but it may well be associated with increased fishing effort. The more crabs that are removed from the population shortly after reaching minimum legal size, the fewer there are that can attain larger size. As fishing pressure increases, the situation only gets worse, although there is a lower limit that can be reached because of the 127-mm minimum legal size limit. Females may be somewhat immune to this because they often initiate their terminal molt at a sublegal size of about 115-mm CW (Knotts 1989) and average 155-mm CW when finished (Knotts 1989, Hines et al. 1987), although many get much larger. The ultimate size of females is probably influenced more by events occurring earlier in development than by environmental conditions at the time of the terminal molt (Haefner and Shuster 1964). This jump to larger size made by maturing females is fortuitous for the overall population because fecundity is directly related to CW (Prager et al. 1990). Thus, if the mean size of females was decreasing as is occurring with males, the number of eggs produced by females would be decreasing also.

There has been some concern that the size of the fall population of females (those migrating down bay to spawn the following spring) may be decreasing because of more intense fishing pres-

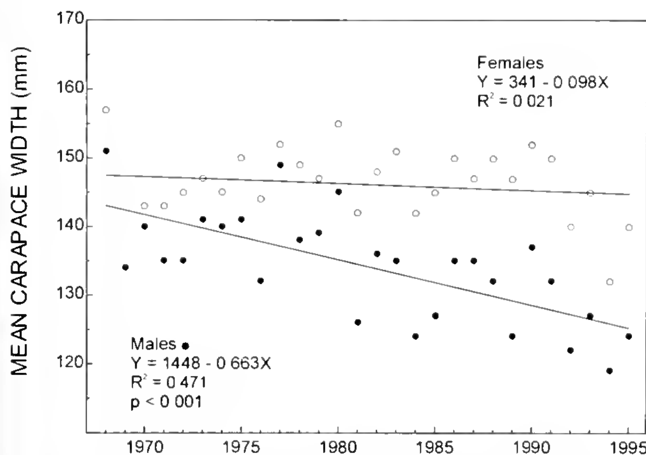

Figure 7. Annual mean CW for all crabs, showing the relative stability of females and the declining size of males.

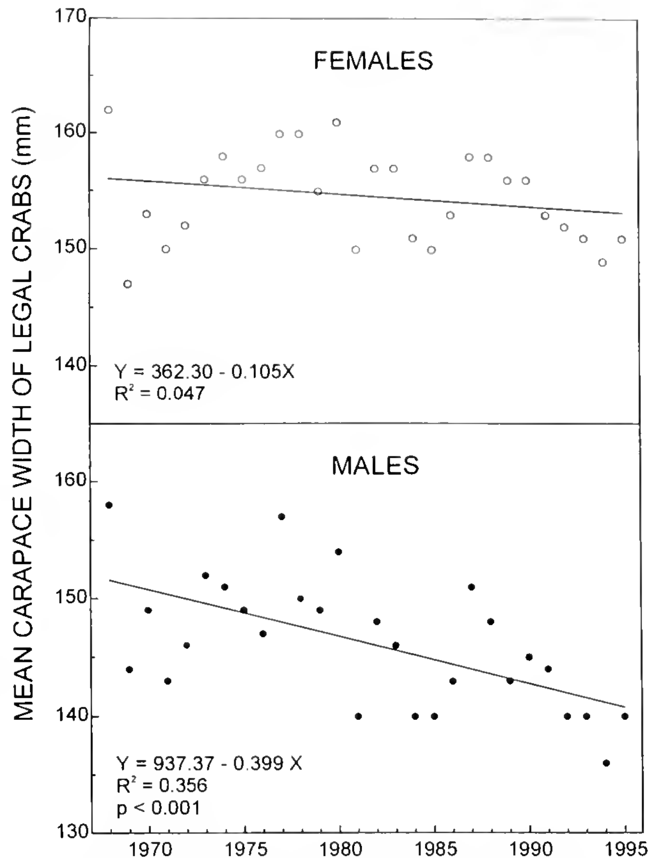

Figure 8. Annual mean CW of legal males and females.

sure and because fishing continues later into the year than it once did because of the collapse of other fisheries (personal observations). When the oyster fishery was healthy, many crabbers began oystering as early as September or October. With the collapse of the oyster fishery, however, some crabbers worked far into December because little fishery activity remained for them after crab season, although this was not possible in 1995 when emergency restrictions ended the season in mid-November. If the size of the fall population of females has been relatively stable across years, then CPUE should have decreased if effort by commercial crabbers has increased. If the fall population size of females has been decreasing, then the CPUE should have decreased at an even greater rate. Data on female CPUE during October and November do not, however, present overwhelming evidence that population sizes of fall females have been declining (Fig. 9), although 1992, 1994, and 1995 were three of the lowest years since 1988. Figure 9 represents legal females only, but the same pattern exists when all females are included because 92% of the females caught in October and November have been legal size.

The facts that the numbers and sizes of male crabs have declined in relation to females and that the percentage of the catch composed of legal crabs has declined during a time of increased fishing pressure indicate a certain amount of stress on the population, but these data do not point to a collapse of the Maryland blue crab fishery. Some of the above data may even point to a fishery in fairly good condition. There are, however, enough warning signs of fishery instability that monitoring of certain population parameters should be continued, and acted on if necessary, to prevent further deterioration of the fishery, as has occurred with other fisheries in the Chesapeake Bay.

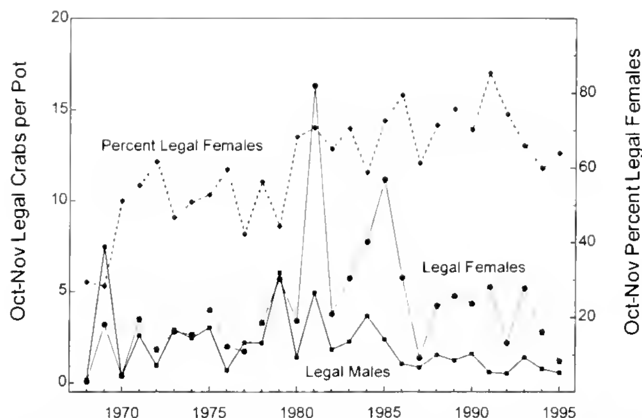

Figure 9. Legal CPUE for males and females and the percentage of legal females relative to the total catch during October and November 1968–1995.

Many watermen who depend on blue crabs for a livelihood are reluctant to admit that changes have occurred in the structure of the populations and therefore see no need for further regulations, although it may be regulations that extend their careers. Many crabbers also feel that there is little relationship between localized scientific data and baywide crab populations, even though samples have been collected from the same populations that they are fishing. The relationship exists and has been demonstrated here.

A rebound in some of these population parameters during the next year or two might be viewed as a sign of recovery that could force managers to relax regulations. However, the trends exhibited here did not develop over 1 or 2 y; rather, they developed over many years and will need to be monitored for many more years before we can be assured that downward trends have leveled or been reversed. If downward trends continue, additional regulations may be required.

ACKNOWLEDGMENTS

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EFFECT OF NITRITE ON GROWTH OF JUVENILE RED SWAMP CRAWFISH, *PROCAMBARUS CLARKII*

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Crawfish is a traditional culture species in Louisiana, dating back to the 1870s (de la Bretonne 1970). Crawfish aquaculture is one of the most important cultured crustaceans in the United States, with annual production near 60 million pounds (USDA 1993). The major culture species are red swamp crawfish, *Procambarus clarkii*, and white river crawfish, *Procambarus zonangulus*. Crawfish production is affected by water quality, forage type and abundance, and stocking density (Lutz and Wolters 1986, de la Bretonne and Romaire 1987, de la Bretonne and Romaire 1989, Avault and Brunson 1990). Nitrite is a limiting factor in aquaculture production (Colt and Armstrong 1981). High concentrations of nitrite are lethal to culture species, and a sublethal concentration may affect growth (Colt and Armstrong 1981, Chien 1992). Hymel (1985) and Gutzmer and Tomasso (1985) investigated the acute toxicity (96-h/LC_{50}) of nitrite to red swamp crawfish. Our preliminary study evaluated the chronic effect of nitrite on juvenile red swamp crawfish.

Juvenile red swamp crawfish were obtained from a drainage canal at the Aquaculture Research Laboratory, LSU Agricultural Center, Baton Rouge, LA. Crawfish were held in a recirculating system that contained dechlorinated Baton Rouge city water (also used as dilution water) for at least 1 wk before the test. Water hardness was adjusted to approximately 100 mg/L as $CaCO_3$ with calcium chloride. The chloride concentration of dilution water was 78.9 mg/L, which might slightly increase the tolerance of the crawfish to nitrite. The laboratory temperature was maintained at 22.0 ± 2.0°C, and the photoperiod was 16-h light and 8-h dark with fluorescent light.

A 1,000 mg/L stock solution of nitrite was made by dissolving 4.93 g of reagent grade sodium nitrite in a 1-L volumetric flask and then bringing it up to volume with distilled water. Test solutions (0.59, 2.97, and 4.75 mg/L) were prepared by adding an appropriate amount of stock solution to a 24-L tank and then bringing it up to volume with dilution water. Water quality (temperature, dissolved oxygen, pH, and ammonia) was monitored before the test solution was distributed randomly to triplicate tanks and during the test. Test containers were 40.5-L plastic tanks. Crawfish were measured, weighed, and placed separately into 9-cm polyvinyl chloride pipe chambers. One end was covered with plastic mesh netting to prevent cannibalism. There were 8 crawfish per replicate, 24 crawfish per treatment. A control, which did not contain any test substance, was maintained concurrently with the three test solutions. Tanks were continuously aerated, and test solutions were renewed daily to maintain proper concentrations. Crawfish were fed with a commercial trout diet (40% protein) once daily at 1–2% of body weight. Dead crawfish, exuviae, and uneaten feed were removed daily when the test solution was renewed. Nitrite concentrations were monitored twice per week with an ORION 960 ion-electrode analyzer (HNU Systems, Inc.). The test lasted 30 days.

Wet weight and total length were recorded at the initiation of the test and thereafter every 15 days. Mortality and observed sublethal effects were also recorded. An analysis of variance was used to evaluate the chronic effects of nitrite among different concentrations. Growth differences in concentration means were declared significant at $p < 0.05$ with Duncan's new multiple range test. A Statistical Analysis Software (SAS) program was used for data computation and analysis (SAS 1992).

The growth (mean wet weight and total length) of the crawfish exposed to each test solution is shown in Table 1. Mean crawfish growth rates were 1.81, 1.55, 1.08, and 1.23 g in weight or 8.7, 7.1, 4.0, and 5.8 mm in length at concentrations of 0, 0.59, 2.97, and 4.75 mg/L of nitrite-N, respectively, over the 30-day period. The percent weight gain and percent length increase of juvenile crawfish exposed to each test solution are presented in Table 2. No statistical difference in weight and length gain was observed on day 15, but after 30 days, the weight and length gains of crawfish exposed to 2.97 and 4.75 mg/L of nitrite-N were significantly lower than controls and the 0.59 mg/L treatment ($p < 0.05$). Nitrite reduced the growth of the crawfish. Crawfish mortality among different concentrations was not significantly different ($p > 0.05$). However, more crawfish were observed dead in test solutions during molting than from cannibalism (Table 3). This might have been caused by nitrite toxicity. Nitrite concentrations were maintained within 75–125% of the nominal test concentrations (Table 4). Dissolved oxygen, pH, temperature, and ammonia were monitored within suitable ranges for crawfish growing during the experiment.

Nitrite is known to be toxic to freshwater crawfish; however, literature about the acute and chronic effects of nitrite to crawfish is limited. Gutzmer and Tomasso (1985) found that the 96-h LC_{50} of nitrite to adult red swamp crawfish was 28 mg/L of nitrite, and Hymel (1985) reported that the acute toxicity of nitrite (LC_{50}) to juveniles was 5.9 mg/L of nitrite-N. Hymel (1985) also suggested that nitrite concentrations less than 1.0 mg/L of nitrite-N should have no negative effect on the production of crawfish. Beitinger and Huey (1981) reported the 96-h LC_{50} for *Procambarus simulans* to be 1.9 mg/L of nitrite-N, and Johnson (1983) reported the 48-h LC_{50} for *Procambarus acutus* to be 600 mg/L. Our study showed that high concentrations of nitrite (>0.59 mg/L) may significantly affect the growth of juvenile red swamp crawfish. Compared with penaeid shrimp and freshwater prawns, red swamp crawfish are relatively sensitive to nitrite (Armstrong et al. 1976, Wickins 1976, Jayasankar and Muthu 1983, Chen and Chin 1988,

TABLE 1.

Mean weight and length (standard deviation) of juvenile red swamp crawfish, *P. clarkii*, exposed to different concentrations of nitrite for 30 days.

Nitrite-N (mg/L)	Weight (g)			Length (mm)		
	0 day	15 day	30 day	0 day	15 day	30 day
0.00	1.45	2.11	3.26	40.1	43.4	48.8
	(0.46)	(0.64)	(0.90)	(4.5)	(4.6)	(4.6)
0.59	1.28	1.97	2.84	39.3	41.8	46.5
	(0.44)	(0.69)	(1.34)	(4.7)	(4.7)	(6.7)
2.97	1.49	2.04	2.57	41.4	42.8	45.4
	(0.37)	(0.57)	(0.88)	(4.2)	(3.5)	(5.6)
4.75	1.38	1.92	2.61	40.3	41.9	46.0
	(0.49)	(0.87)	(0.99)	(5.5)	(6.0)	(6.0)

TABLE 2.

Mean weight and length gain of juvenile red swamp crawfish, *P. clarkii*, exposed to different concentrations of nitrite for 30 days[a]

Nitrite-N (mg/L)	Weight Gain (%)		Length Gain (%)	
	15 day	30 day	15 day	30 day
0.00	46.7[b]	124.4[b]	8.1[b]	21.7[b]
0.59	51.8[b]	121.4[b]	6.0[b]	18.5[b,c]
2.97	37.2[b]	72.5[c]	3.6[b]	10.0[c]
4.75	37.1[b]	89.9[c]	3.8[b]	14.8[b,c]

[a] Data in the same column having different superscripts are significantly different ($p < 0.05$).
Note: length/weight gain (%) = (mean length/weight at time t − mean initial length/wt) × 100/(mean initial length/wt)

TABLE 3.

Mortality of juvenile red swamp crawfish, *P. clarkii*, exposed to different concentrations of nitrite for 30 days.

Nitrite-N (mg/L)	Mortality (%)		
	Cannibalism	Molting	Total
0.00	25.0	0.0	25.0
0.59	12.5	16.7	29.2
2.97	4.2	20.8	25.0
4.75	8.3	25.0	33.3

Chen et al. 1990a, Chen et al. 1990b, Chen and Lei 1990, Chen and Tu 1990). Hymel (1985) reported that high nitrite concentrations were unlikely in crawfish ponds because of the extensive nature of the cultivation practice in which no formulated feeds are used. However, further study should be conducted to identify the long-term effects of nitrite on the growth of crawfish. High nitrite concentrations may be a problem in soft-shell crawfish operations that use recirculating systems. Further research is needed to evaluate the toxicity of nitrite at different levels of chloride.

TABLE 4.

Measured nitrite-N concentrations in test containers with juvenile red swamp crawfish, *P. clarkii*, during 30-day exposure.

Nominal Nitrite (mg/L)	Measured Concentrations (mg/L)							
	Week 1		Week 2		Week 3		Week 4	
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.59	0.59	0.59	0.59	0.58	0.69	0.72	0.52	0.66
2.97	2.63	2.80	2.67	2.68	2.51	2.78	2.96	2.84
4.75	4.20	4.36	4.45	4.45	4.09	4.38	4.71	4.66

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NOTES ON THE BLUE CRAB FISHERY IN THE APALACHICOLA, FLORIDA ESTUARY

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ABSTRACT Harvested, cooked blue crabs were intermittently sampled and measured in a processing house over a period of 2 y. Information was gathered in the range of sizes, means, and modes of males, immature and mature females, and gravid females.

KEY WORDS: Blue crab, *Callinectes sapidus*, female maturity, population variation, sizes of sexes, estuarine dynamics

INTRODUCTION

Callinectes sapidus Rathbun 1896, a swimming crab, belongs to the family *Portunidae*. Its life history has been investigated by Barnes (1904), Hay (1905), Churchill (1921), Truitt (1939), Darnell (1959), and Tagatz (1968). An excellent summary is provided by the Gulf States Marine Fisheries Commission (1970).

Mating occurs in brackish water areas, where males remain throughout their life cycle. After fertilization, females migrate to more saline waters where spawning occurs (Tagatz 1968). Most spawning takes place in spring and summer. Larvae migrate to brackish water estuaries to mature. Growth occurs by ecdysis. This process is the basis of the soft crab fishery, an industry and its potential in Florida described by Otwell et al. (1980).

Many studies have shown inshore-offshore migrations of blue crabs from various areas: Van Engel (1958), Darnell (1959), Waterman (1961), Fischler and Walburg (1962), Tagatz (1968), Judy and Dudley (1970), Gulf States Marine Fisheries Commission (1970). Oesterling (1976), however, found indications of alongshore migrations on the western coast of Florida in the Gulf of Mexico. His tagging studies indicated that all nonlocal movement on the western coast was in a northerly direction along the peninsular portion of the state and westerly along the panhandle. Oesterling and Adams (1982) theorized that the Apalachicola estuarine system is the primary spawning ground for blue crabs on Florida's Gulf Coast. Oesterling's study was conducted for only a short time (less than a year). More tagging by others followed. The later tagging confirmed earlier findings of a long, northerly migration (Steele 1991, Lyons 1995).

This study was based on commercial production from Apalachicola Bay. This area is described in Ingle (1951), Ingle and Dawson (1953), and Livingston and Joyce (1977). It was conducted to determine the size and sex of hard blue crabs processed by commercial operations.

The average yearly volume of hard blue crabs landed in the Apalachicola Bay area (Franklin County) during the period 1974–1976 was more than 1,000,000 pounds, according to Landrum and Prochaska (1980). They describe a declining trend in pounds produced as the result of a decrease in effort that had prevailed since 1964, but increasing dockside prices compensated monetarily for the volume decline. The increase in dockside price and the consequent value of the fishery also offset the decrease in the number of fishermen, firms, and traps.

The decline in production continued after 1976. During a recent span of years (1990–1995), the average pounds landed was 258,000. During the last year of that period (1995), only 122,000 pounds were produced (Kennedy 1995).

Conversations with crab house owners indicated that the decrease in fishing effort is largely due to a bottleneck created by a decrease in labor. Funding by social programs competes for potential fishermen and employees who might seek work as crab pickers, according to industry representatives (personal communications 1981 and 1995).

PROCEDURE

Crabs caught in Apalachicola Bay and processed locally were sampled intermittently for a 2-y period (June 1979 to May 1981). Samples were measured after crabs had been cooked. Each sample consisted of a basket approximately 45 cm^3. Two samples were chosen randomly from the picking room of the crab house on each sampling date. Carapace widths between the extremities of the lateral spines of all crabs within the two baskets were then measured.

Data were recorded in size classes of a 4-mm range, from 80 to 200 mm, and divided into four categories. These groups were males, mature females, gravid females, and immature females. Crabs smaller than 80 mm were measured and recorded but were not included in any analyses because the numbers were insignificant.

Results from samples were totaled for each group and plotted on graphs by size frequencies. Range, mode, and mean of carapace width for each category were also plotted.

Several factors affected the size of crabs brought into the processing plant. The commonly used 3.25-cm-pore-size mesh chicken wire of which the traps were constructed prevented most crabs smaller than the mesh size from being captured. This is not critical because crabs of this size are not of commercial value. Peelers may have been removed for soft-shell production and not sold to the picking plant. The number of crabbers, traps per boat, and location of harvesting varied seasonally depending on the availability of crabs. The assumption is made that local fishermen place their traps in areas where prospects are high for catching desirable sizes of animals in commercial quantities.

RESULTS

The first sample was obtained in June 1979; the last was obtained in May 1981. During December 1980 and February and March 1981, very few crabs were harvested. No data are available for these months. The total number of crabs and the number of samples taken (N) are indicated for each month in Table 1, which also shows the mean size of all categories combined for each month.

The number of crabs per basket examined depended on the size of the animals and to some extent on the random alignment of the animals within the basket. No attempt was made to "pack" the crabs. The basket size containing the sample was always the same, but more crabs of smaller size were required to fill it than crabs of larger size. These data indicate that essentially the same size crab is processed year-round. Only 20 mm separated the low mean for June 1980 and the high mean for January 1981.

The percentage of each category caught each month is shown in

TABLE 1.

Number of crabs and samples taken and mean size of all categories combined for each month.

Month	n[a]	Total	Mean (mm)
June 1979	4	625	135
May 1980	8	1,309	139
June 1980	4	862	132
July 1980	6	1,122	132
August 1980	4	631	136
September 1980	6	964	137
October 1980	6	817	146
November 1980	4	629	148
January 1981	2	216	152
April 1981	8	1,234	141
May 1981	6	886	142

[a] n, number of baskets.

Figure 1. The largest percentage of male crabs by month was caught in June 1980. This percentage decreased during winter months and increased during the rest of the year. The percentage of mature females was highest in the winter and lowest during the summer. This seasonal change in relative abundance is compatible with previous observations in other blue crab studies. Female crabs move to deeper waters in warmer months for hatching (Cargo 1958, Fiedler 1930, Tagatz 1968).

From the data available, it appears that two peaks in the percentage of gravid females in the total population occur: one in August 1980 (27%) and one in April 1981 (25%). In general, gravid females are only a small percentage of the commercial catch, so the data above are worth noting. Gravid females were not seen in any samples in November 1980 or January 1981. No samples were taken in February or March 1981, so information is not available on the time that spawning began. However, by April, gravid females were fairly abundant in the commercial catch.

Immature females comprised only a small percentage of the crabs harvested each month. However, small numbers were present in every month sampled.

The range, mode, and mean of carapace width of all categories

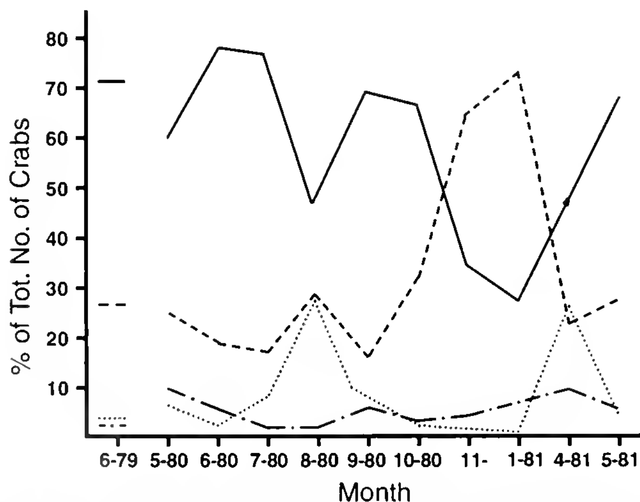

Figure 1. Percentage of total (Tot.) number (No.) of crabs by category for each month. Male (—), mature female (---), gravid female (· · · · · ·), immature female (· – · – ·).

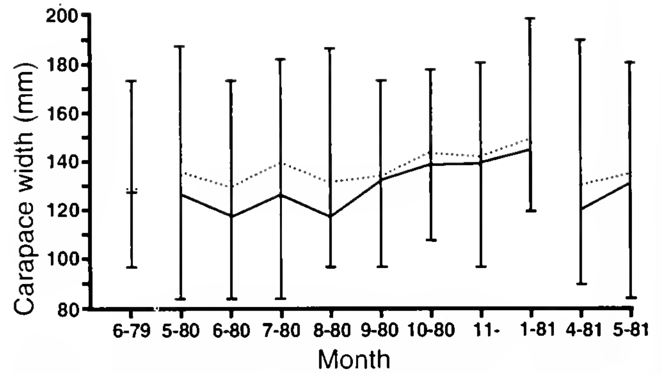

Figure 2. Range, mode, and mean of carapace width for males. Mode (—), mean (· · · · · ·).

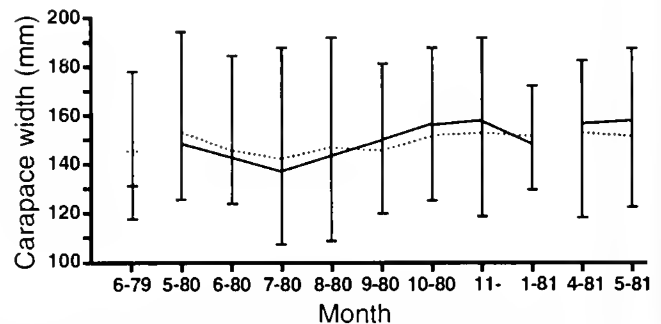

Figure 3. Range, mode, and mean of carapace width for mature females. Mode (—), mean (· · · · · ·).

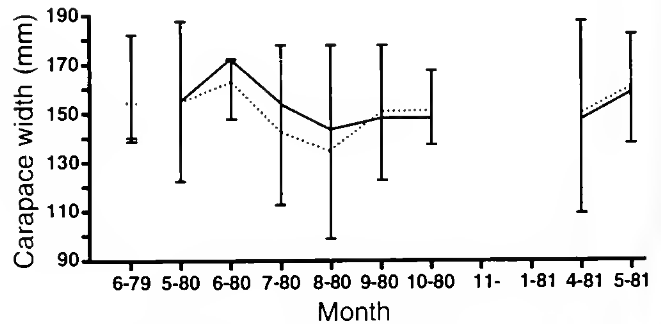

Figure 4. Range, mode, and mean of carapace width for gravid females. Mode (—), mean (· · · · · ·).

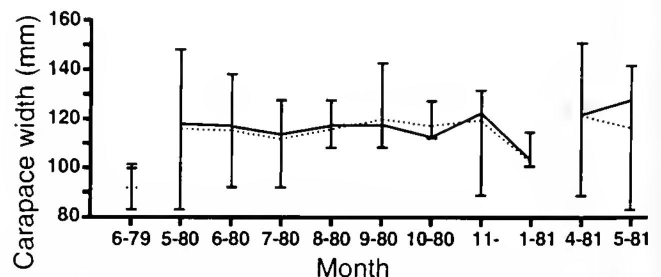

Figure 5. Range, mode, and mean of carapace width for immature females. Mode (—), mean (· · · · · ·).

TABLE 2.

Summary: carapace width (range, mode, and mean) of all categories by month.

Sex	Total No. of Individuals	Carapace width (mm) Range	Mode	Mean
June 1979				
Male	446	98–173	128	128
Mature female	167	118–178	133	144
Gravid female	10	138–183	138	154
Immature female	2	83–103	103	93
Total	625		n[a] = 4	
May 1980				
Male	786	83–188	128	136
Mature female	311	123–193	148	152
Gravid female	86	123–188	153	153
Immature female	126	83–148	118	117
Total	1,309		n = 8	
June 1980				
Male	661	83–173	118	129
Mature female	164	123–183	143	145
Gravid female	4	148–173	173	162
Immature female	33	93–138	118	116
Total	862		n = 4	
July 1980				
Male	842	83–183	128	128
Mature female	192	108–188	138	142
Gravid female	81	113–178	153	144
Immature female	7	93–128	113	112
Total	1,122		n = 6	
August 1980				
Male	285	98–188	118	131
Mature female	175	108–193	143	146
Gravid female	169	98–178	143	136
Immature female	9	108–128	118	116
Total	631		n = 4	
September 1980				
Male	659	98–173	133	134
Mature female	147	118–183	148	146
Gravid female	111	123–178	148	150
Immature female	47	108–143	118	121
Total	964		n = 6	
October 1980				
Male	535	108–178	138	143
Mature female	261	123–188	155	152
Gravid female	7	138–168	148	151
Immature female	14	113–128	113	118
Total	817		n = 6	
November 1980				
Male	212	98–183	138	141
Mature female	401	118–193	158	153
Gravid female	0			
Immature female	16	88–133	123	121
Total	629		n = 4	
January 1981				
Male	58	118–198	148	152
Mature female	157	128–173	148	152
Gravid female	0			
Immature female	1	111–115	113	113
Total	216		n = 2	
April 1981				
Male	568	88–188	118	133
Mature female	255	118–183	158	154
Gravid female	303	113–188	148	149
Immature female	108	88–153	123	123
Total	1,234		n = 8	
May 1981				
Male	595	83–183	133	139
Mature female	228	123–188	158	153
Gravid female	27	138–183	158	160
Immature female	36	83–143	128	119
Total	886		n = 6	

[a] n, number of baskets.

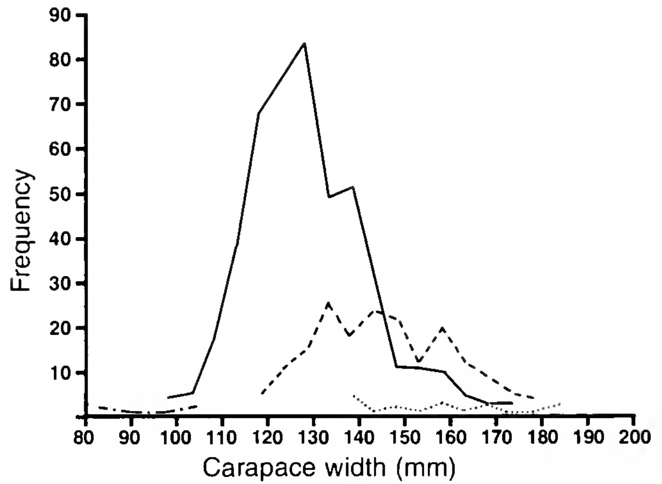

Figure 6. Size frequencies for all categories during June 1979. n = 8. Male (—), mature female (– – –), gravid female (· · · · · ·), immature female (· – · – ·).

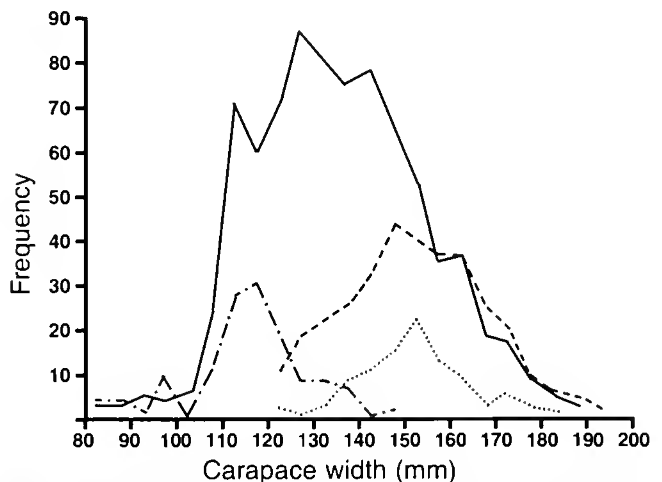

Figure 7. Size frequencies for all categories during May 1980. n = 8. Male (—), mature female (– – –), gravid female (· · · · · ·), immature female (· – · – ·).

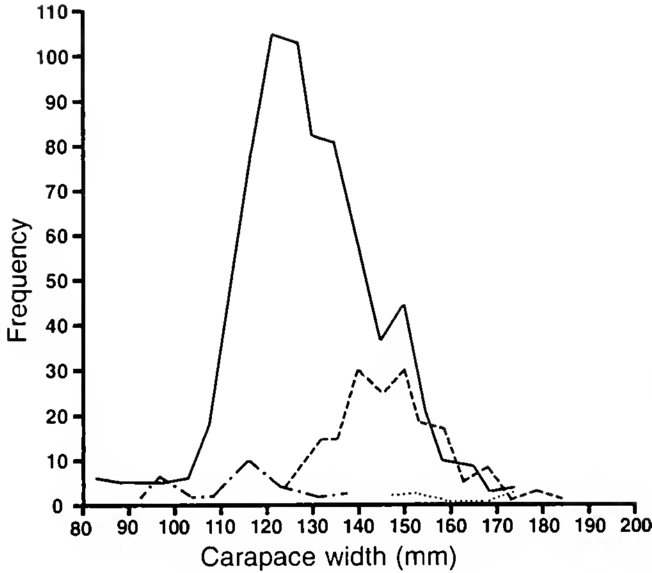

Figure 8. Size frequencies for all categories during June 1980. n = 4. Male (—), mature female (– – –), gravid female (· · · · · ·), immature female (· – · – ·).

are shown in Figures 2–5 and Table 2. This table also shows the total numbers of crabs measured. In general, males were smaller than mature and gravid females. Immature females had the smallest carapace width of all categories.

The largest range in carapace width occurred in males (83–198 mm); the smallest range occurred in immature females (83–153

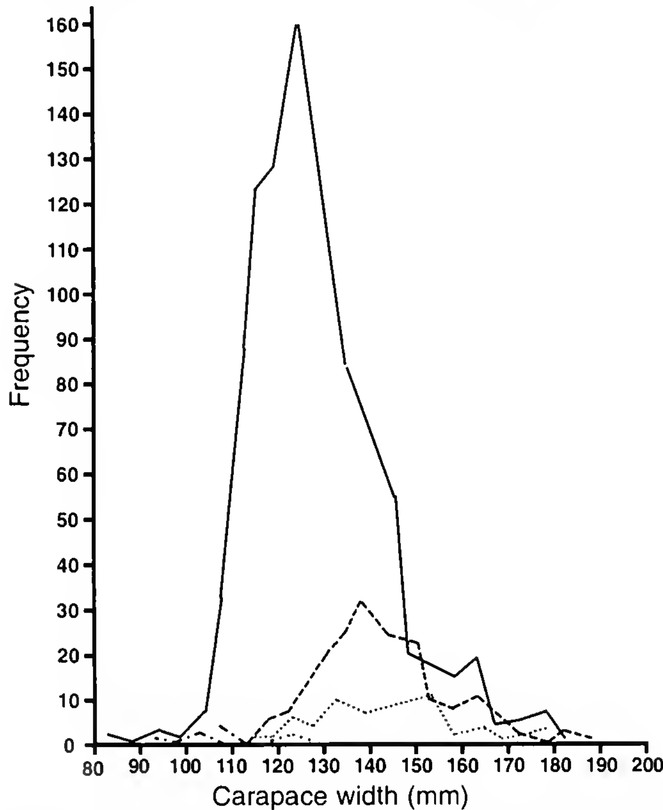

Figure 9. Size frequencies for all categories during July 1980. n = 6. Male (—), mature female (– – –), gravid female (· · · · · ·), immature female (· – · – ·).

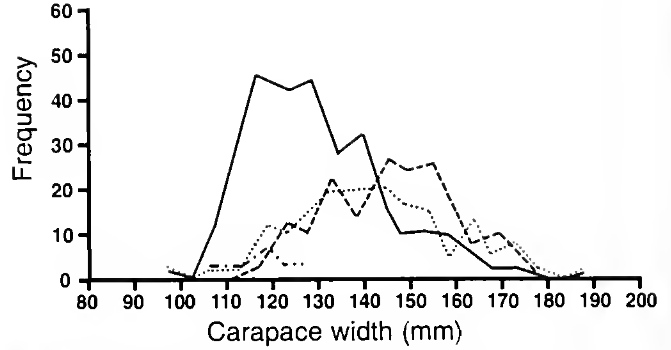

Figure 10. Size frequencies for all categories during August 1980. n = 4. Male (—), mature female (– – –), gravid female (· · · · · ·), immature female (· – · – ·).

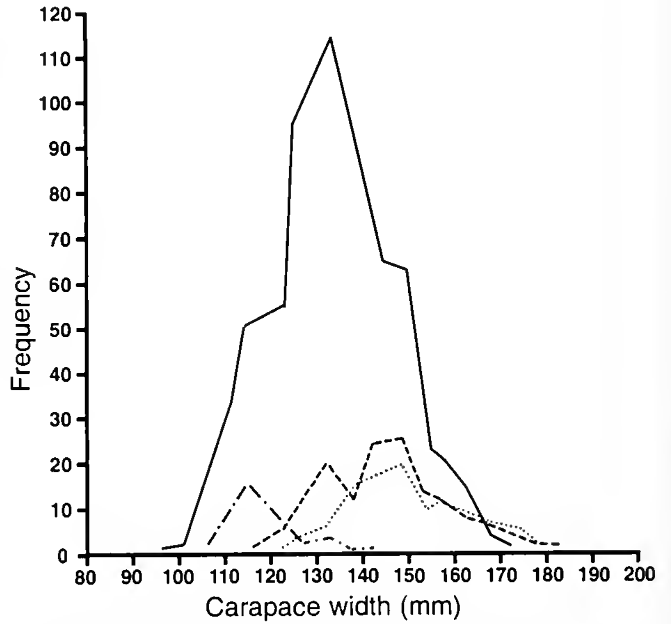

Figure 11. Size frequencies for all categories during September 1980. n = 6. Male (—), mature female (– – –), gravid female (· · · · · ·), immature female (· – · – ·).

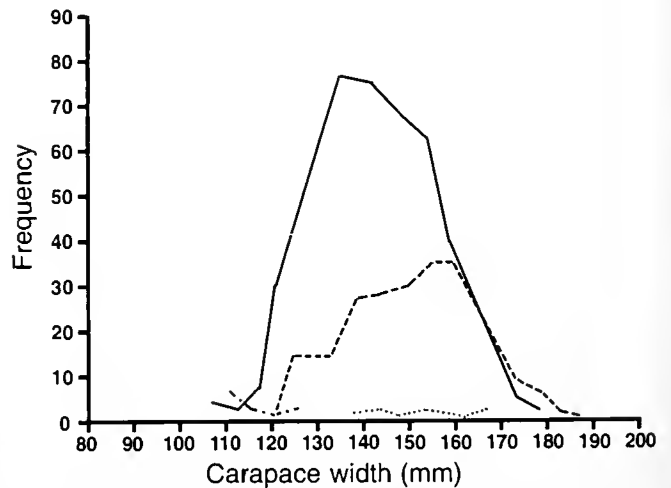

Figure 12. Size frequencies for all categories during October 1980. n = 6. Male (—), mature female (– – –), gravid female (· · · · · ·), immature female (· – · – ·).

mm). Mature females ranged from 108 to 193 mm. Figures 6–16 show the same information as Figures 2–5, plotted by month rather than categorized by sex for all four groups.

DISCUSSION

Laughlin (1979) found that larger crabs were most abundant in spring and summer in the Apalachicola Estuary. High temperature and salinity appeared conducive to the occurrence of larger crabs. Our data indicate a slight tendency toward more small crabs during summer than winter months. However, the size differences are small (Table 2). It appears that essentially the same mean size crab is caught year-round.

Male crabs, with a smaller mean size than mature females, are relatively less abundant in winter (November 1980 and January 1981). Cooler temperatures apparently depress the development of eggs; therefore, during lower temperatures, many individuals that would be in the gravid category (under warmer conditions) are lumped with mature individuals when eggs were not visible. Females (mature) with a larger mean size exerted a greater influence on the overall mean size of crabs in winter samples because of the relative decline in the numbers of males of lower mean size.

Because spawning occurs during the warmest months of the year, the number of gravid females would be expected to increase during these months, with a resultant decrease in the relative number of mature females. Our data reflect this: mature females were proportionately greater in winter than in summer months.

Putatively, it is illegal to harvest gravid females in Florida. However, egg-bearing crabs are processed. Less than 10% of the catch is composed of these females. Because females spawn offshore and most traps are placed inside the protection of St. George Island, there is a built-in bias in our data. Because gravid females migrate offshore to spawn, their relative abundance in the estuary declines.

Even if the Apalachicola region is not the main spawning ground for the blue crab on the upper western coast of peninsular Florida, as proposed by Oesterling et al. (1982), the area (Apalachee Bay) does maintain a large population of blue crabs. The population is of such a large size that Laughlin (1979) felt that cannibalism, by larger crabs, might play a role in the observed population distribution.

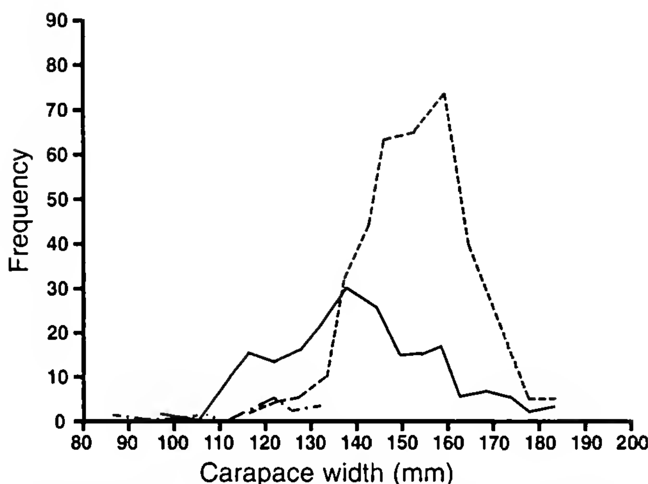

Figure 13. Size frequencies for all categories during November 1980 n = 4. Male (—), mature female (---), gravid female (· · · · · · ·), immature female (· – · – ·).

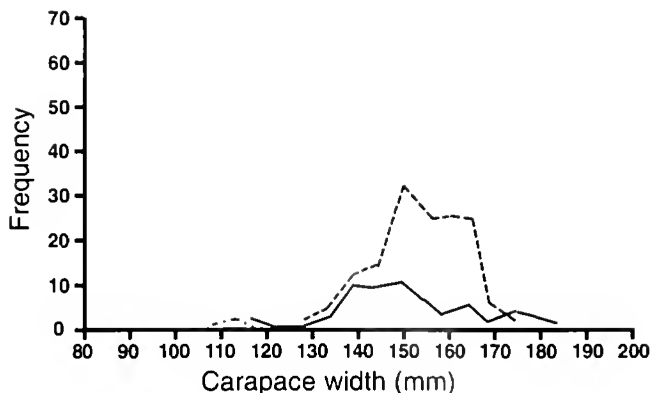

Figure 14. Size frequencies for all categories during January 1981. n = 2. Male (—), mature female (---), gravid female (· · · · · ·) [none]), immature female (· – · – ·).

It was putatively unlawful at the time of this study for any person to possess for sale blue crabs measuring less than 5 inches (126.5 mm) between the points of lateral spines in an amount greater than 10% of the total number of blue crabs in such person's possession. However, if the fisherman possessed a special permit for peelers or the bait trade, the law did not hold. A special permit was easily obtained from the Department of Natural Resources. Crabs less than 126.5 mm could be sold as peeler crabs, which bring a higher price. It should be noted that at the time of this study, regulations were basically ignored when they concerned crab harvests in the area.

The few crabs smaller than 126.5 mm that entered the processing house were predominantly immature females, which comprised 10% or less of the total catch each month. Mature and gravid females were seldom less than the legal size. On three occasions (June 1980, August 1980, and April 1981), the male carapace width mode was less than the prescribed length. The mean of these crabs on those dates, however, was larger than 126.5 mm.

All of the data are presumed to be affected by the proclivity of males and females to favor different habitats during certain periods of their life cycles. Males tend to occupy shallows, whereas females are known to move into deeper water, especially at spawning time. The data presented here must be judged against a background of highly variable physiologic functions, some of which result from unstable environmental conditions. Tagatz (1968) reported on studies made in the St. Johns River, its estuary, and offshore ocean waters. He found that after reaching maturity, females were impregnated. Truitt (1939) found that multiple spawnings can be expected from one impregnation. Although the transfer of sperm from male to female usually occurs in less saline waters, after spawning, the females migrate to saltier, often offshore, waters when hatching occurs. This movement has also been noted by Fiedler (1930) and Cargo (1958).

In the St. Johns River area, the female migration offshore was most pronounced in the spring and fall (Tagatz 1968). Tagatz concluded that larval development took place mainly in the ocean and that the final metamorphosis from magalops to first crab stage takes place most frequently there. As in the St. Johns' area, Van Engel (1958) reported that when females migrated to saltier waters (in Chesapeake Bay), males generally tended to remain in areas further upstream.

Postlarval instars were estimated to be 20 for males and 18 for

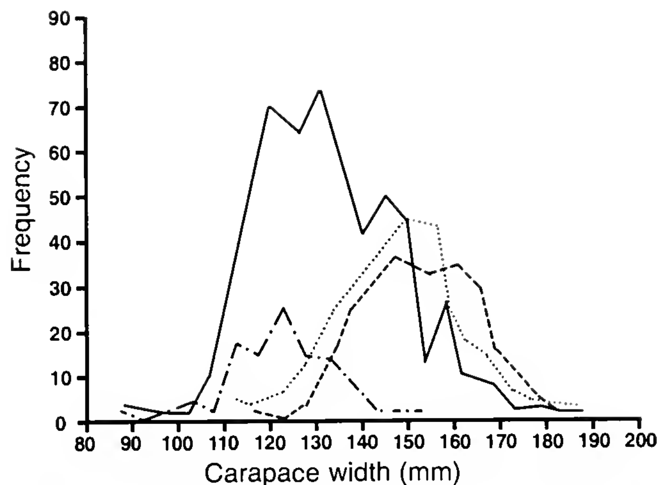

Figure 15. Size frequencies for all categories during April 1981. n = 8. Male (—), mature female (---), gravid female (· · · · · ·), immature female (· – · – ·).

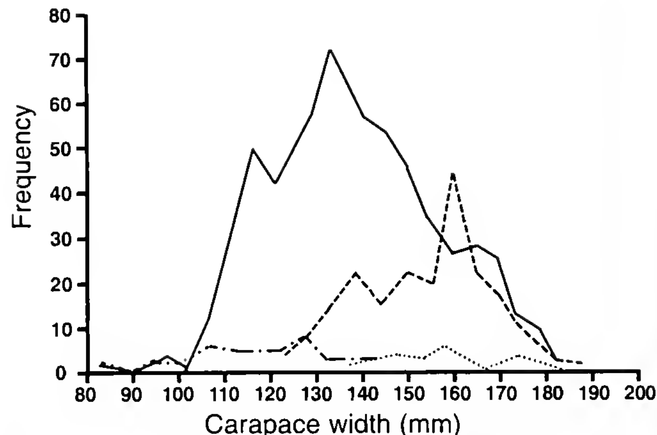

Figure 16. Size frequencies for all categories during May 1981. n = 6. Male (—), mature female (---), gravid female (· · · · · ·), immature female (· – · – ·).

females (Newcombe et al. 1949). They also concluded that environmental conditions, especially salinity, influence the percent increase in size per molt.

Most of the crabs of commercial size caught during most of the year in the lower St. Johns River were females (Tagatz 1968). Nearly all crabs caught in the ocean were females, except in the fall. Tagatz also found that the population of males and females that matured at a small size was larger in salt water than in fresh water. The smallest mature females measured in the St. Johns River were 90 mm. The largest immature female was 177 mm.

Because crab production varied, primarily because of weather, the number of samples per month in our study was not uniform. For instance, only two samples were checked in January 1981, whereas in two months, May 1980 and April 1981, data were derived from eight samples each month.

ACKNOWLEDGMENT

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A METHOD FOR QUANTITATIVELY SAMPLING NEKTON ON INTERTIDAL OYSTER REEFS

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ABSTRACT We developed a sampling methodology using a 24-m^2 lift net to quantitatively sample intertidal oyster reefs as a part of a long-term study of their functional ecology. The method involved surrounding an area of oyster reef with a buried net at low tide, allowing the water level to rise, raising the net at high tide to trap motile organisms, allowing the water to recede, and collecting the entrapped nekton. Natural and artificially constructed reefs were sampled, and efficiency (mark-recapture) studies were performed to evaluate the method. The advantages of this method are: (1) the habitat in the area to be sampled receives minimal damage; (2) the size and shape of the net system are flexible and can be adapted to fit a variety of habitats; (3) no permanent structures, other than a shallow perimeter trench, are present to act as attractants; and (4) it is relatively inexpensive to purchase and maintain gear. One disadvantage to the method is that it is very labor intensive, typically using three to five people. This method proved more efficient on natural reefs than artificial reefs, and the return rate was slightly better for *Fundulus heteroclitus* than for *Palaemonetes* spp. Seventeen decapod and 24 fish taxa were collected from initial spring, summer, and fall 1995 sampling.

KEY WORDS: Lift net, sampling techniques, intertidal oyster reefs, fishes, decapod crustaceans, habitat complexity

INTRODUCTION

Oyster reefs are a conspicuous feature of the intertidal zone in most estuaries in the southeastern United States (portions of North Carolina, South Carolina, Georgia and portions of northeast Florida). By forming intensive biogenic intertidal reefs, often adjacent to emergent marsh vegetation, *Crassostrea virginica* provides the only three-dimensional structural relief in an otherwise unvegetated, soft-bottom, benthic habitat. In areas otherwise devoid of naturally occurring hard substrate, the many crevices and expansive surface area found within an oyster reef provide a refuge and attachment for numerous small invertebrates (e.g., Dame 1979, Bahr 1974, Klemanowicz 1985, Powell 1994).

Although intertidal oyster reefs are prominent in the region, information is generally lacking on the importance of these reefs as habitat for juvenile and adult fishes, crabs, and shrimp, which move on and off the reefs with the tide. Anecdotal information for South Carolina suggests that fishes such as bay anchovy *Anchoa mitchilli*, silversides *Menidia* spp., and killifishes *Fundulus* spp. are attracted to oyster reefs because of their complex three-dimensional structure, which provides them with a refuge from fish predators (e.g., sciaenids and paralichthid flounders). Large predators including spotted seatrout *Cynoscion nebulosus*, red drum *Sciaenops ocellatus*, summer flounder *Paralichthys dentatus*, and sheepshead *Archosargus probatocephalus* migrate onto oyster reefs on flood tides to consume small crabs, shrimps, and fishes that reside in and around the reef structure (Coen et al. 1996). Oyster reefs in high-salinity waters are also an important habitat for juveniles of several important fish species such as sheepshead *A. probatocephalus*, gag grouper *Mycteroperca microlepis*, and snapper *Lutjanus* spp., as well as stone crab *Menippe mercenaria* and blue crab *Callinectes sapidus* (Cain and Dean 1976, Grant and McDonald 1979, Reiss and Dean 1981, Crabtree and Dean 1982, Wilson et al. 1982, Kleypas and Dean 1983, Wenner and Stokes 1984).

Quantifying the use of oyster reefs by various life history stages of motile fauna has been limited by sample gear. To our knowledge, only Bahr (1974), Crabtree and Dean (1982), and Powell (1994) have previously attempted to quantify transient nekton associated with intertidal or shallow subtidal oyster reefs. Powell (1994) conducted a visual census by diving on reefs, where he observed numerous pinfish *Lagodon rhomboides* and several sheepshead *A. probatocephalus* at high tide. Poor visibility (<10–15 cm) precludes comprehensive visual censuses on most southeastern oyster reefs, and disturbance by divers in these shallow areas likely biases results. Open-topped traps (Crabtree and Dean 1982) are biased for the collection of small nekton and do not provide data for the quantification of densities within a portion of the reef habitat. Conventional methods for sampling soft-bottom habitats, such as trawls and seines, cannot be used on intertidal oyster reefs because of hangs and tears from oyster clusters. Other devices such as drop samplers (Wenner and Beatty 1992) can be deployed in hard-bottom habitats, but they sample a relatively small area (<10 m^2) and may not seal properly along the bottom when placed over dense clusters of living oysters. Poisons, primarily rotenone, have been used to sample areas with oyster habitat (Weinstein 1979), but the broad spatial effect of the treatment precludes the determination of specific habitat use.

This article describes a modification of the lift net (Rozas 1992) and flume weir (Kneib 1991) used to quantitatively sample nekton in emergent vegetation such as intertidal marshes. Our net system is designed to completely surround a defined (e.g., 24-m^2) area of intertidal oyster reef, with minimal disturbance during deployment. It is being used to quantitatively sample nektonic species as part of a multiyear interdisciplinary study that determines the functional role of intertidal oyster reefs in southeastern estuaries (Coen et al. 1996). Although the purpose of this article is to describe the lift net system and its efficiency, we provide data on species composition and the abundance of fishes and decapod crustaceans collected by the gear to emphasize its effectiveness and versatility.

MATERIALS AND METHODS

Study Sites and Reef Fabrication

Two study areas with similar salinity regimes, bed grades, base sediments, wave disturbance, adjacent oyster communities, and elevation above mean high water were selected near Charleston Harbor, SC (Fig. 1). One area was located near Toler's Cove Marina (henceforth referred to as the Toler's site), a moderate-sized marina with approximately 138 slips, located within a small tidal creek (depth <3 m). Oyster reefs in this area are bordered by an extensive *Spartina alterniflora* salt marsh. A previous study by

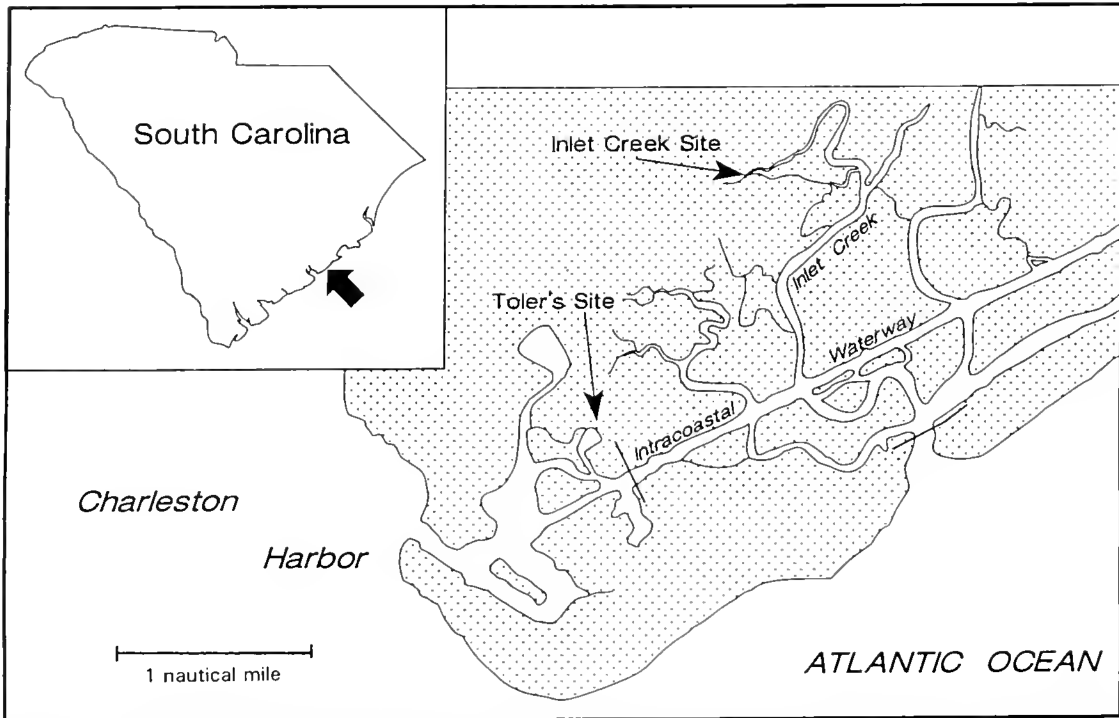

Figure 1. Study area located east of Charleston Harbor, SC. The Toler's Cove site (located around 32°46.27′ N latitude and 79°51.16′ W longitude) is a disturbed site because of marina activity, whereas the Inlet Creek site (located around 32°48.02′ N Latitude and 79°49.50′ W longitude) has no adjacent development.

Van Dolah et al. (1992) measured the levels of contaminants, oyster growth and general health and density of spat settlement at this site, which is closed to shellfish harvesting. The second area selected for study was located in the upper reaches of Inlet Creek (henceforth referred to as the Inlet site), a relatively pristine site, with broad expanses of reefs under private lease, a large buffer zone (>300 m) of *S. alterniflora*, and relatively little adjacent development. Both sites are dominated by fine sediments, often >75% silt/clay with little or no sand. Water quality variables of temperature, salinity, and dissolved oxygen, as determined from deployed Hydrolab Datasonde 3S™, were similar between sites (Coen et al. 1996). Both sites are subject to semi-diurnal tides, with a mean range of 1.5–2 m.

Three natural oyster reefs and three artificially constructed reefs (henceforth referred to as experimental) were sampled at each site (Fig. 2). Experimental reefs were constructed of 0.46- × 0.31- × 0.11-m perforated plastic trays lined with a 1.3-mm fiberglass mesh screen and filled with oyster shell. Trays were filled with shell (~8 kg each) to a standard height of ~0.11 m. All oysters and shell were removed from an area equivalent to reef size (roughly, 8.2 × 2.9 m) before fabrication of the experimental reefs in October 1994. Each experimental reef measured 2.92 × 8.17 m (23.86 m^2) and consisted of 26 rows of six trays placed end to end. Experimental reefs were designed to approximate the size of a natural oyster reef and to be sufficiently large so as to avoid repeated sampling and disturbance of areas sampled for another component of the overall study dealing with resident reef species (Coen et al. 1996). In close proximity to each experimental reef, an adjacent area of natural oyster reef, 24 m^2 (3 × 8 m) with approximately the same elevation and configuration and with live oysters and shell, was marked for sampling and is henceforth referred to as a natural reef. The paired natural and experimental reefs combined to make up more than half of the total reef area or "mound" on which they were located.

Net Design

Our sampling procedure was adapted from methods used previously to sample intertidal vegetated habitat (McIvor and Odum 1986, Kneib 1991, Rozas 1992, and Wenner and Beatty 1992) that involved isolating a discrete area of habitat with a net and extracting the animals trapped within the area. Samples were collected by surrounding an experimental or natural reef with a lift net at low tide when the reef was exposed (Fig. 3), allowing time for the tide to rise, raising the lift net at high slack water during daylight hours, allowing the water to recede, and collecting specimens trapped in the net.

Six months after reef construction and a few weeks before sampling, site preparation was completed. This involved driving 12 1.5-m sections of 12.7-mm concrete reinforcing rods (rebar) into the substrate surrounding each rectangular reef so that one was in each corner, three were spaced evenly along each long side, and one was placed in the center of each end. These were to mark placement and to support the net poles. A shallow trench was then excavated outside of the rebar, and a stainless steel cable, 4.8 mm in diameter, was secured in the bottom of the trench with a J-shaped 9.5-mm rebar. The purpose of the cable was to prevent the lifting of the bottom of the net during the net-raising procedure. The site perimeter was protected by placing 0.75- × 2.2-m removable plywood walkways around it during construction.

Sampling apparatus was set up on the low tide preceding the high tide during which sampling would take place. Before the nets were rigged, the site perimeter was again protected by the placement of the plywood walkways around it. Walkways were re-

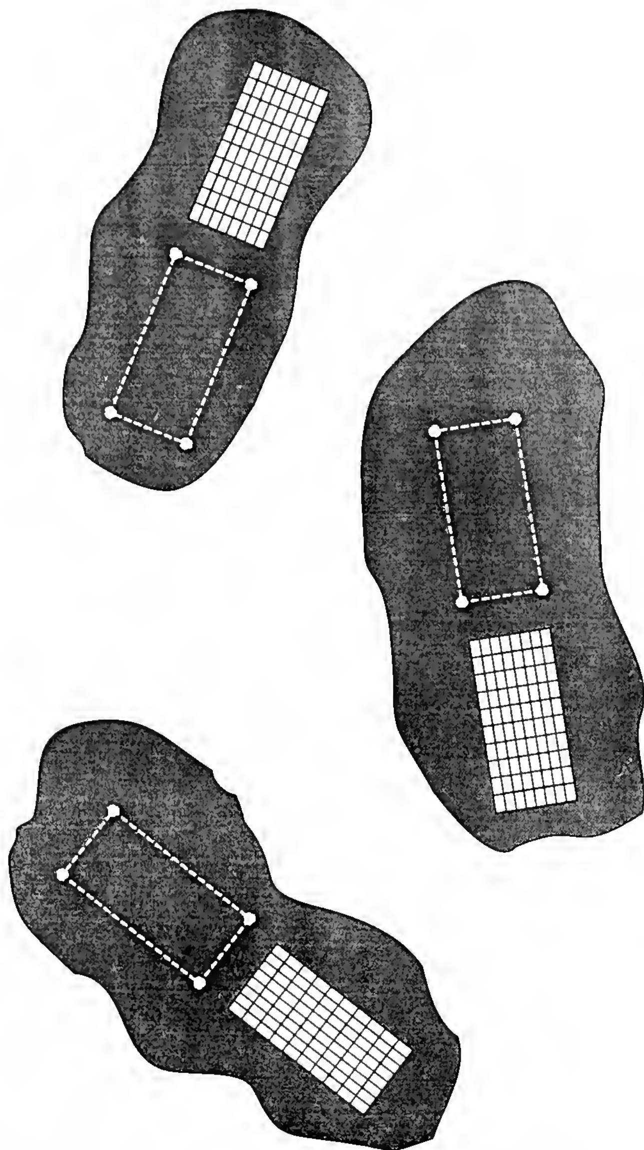

Figure 2. Conceptual layout of a reef site with three pairs of reefs, one natural reef and one experimental reef in each pair.

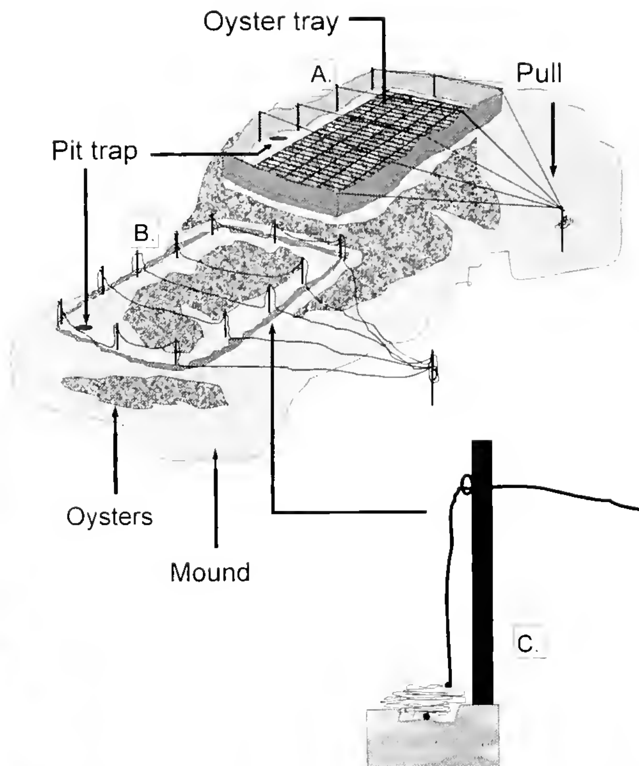

Figure 3. Reef mound containing a pair of reefs: an artificial reef (A) (with the lift net up) and a natural reef (B) (with the lift net down). Before sampling, the net is folded over the lead line and cable in a shallow trench (C) and covered with sediment. The pull line is attached to the top of the net and threaded through an eye bolt in the top of the pole.

moved once the site was ready, so as to not interfere with ambient flow around the reef. An aluminum pole ~2.2 m long was placed over each of the rebar supports and driven into the substrate. The length of the poles was determined with data on tide height, so that the tops of the poles, when in place, were ~20–30 cm above high water.

The lift net was constructed of 3.2-mm-pore-size mesh netting that was 2.44 m wide. The net was long enough to enclose the site and overlap ~2 m so that a secure seal could be achieved (Fig. 3A). A sleeve was sewn into the top and bottom of the net, with a line placed in the top sleeve for support and a 6.4-mm chain fed through the bottom sleeve to help weight the bottom and achieve a tight seal. After the net encircled the site, the net was secured to the cable with cable ties ~10 cm above the chain to avoid the lifting of the bottom of the net when the net was raised. The lift net was then folded into the shallow trench (Fig. 3B and C) so that the top of the net was approximately even with the top of the trench; the net was then covered with sediment (pluff mud) to help conceal it and hold it down over a tidal cycle.

Five 8.0-mm polypropylene lines were attached to the net in order to raise it from a remote station ~15 m away, thereby lessening the degree of disturbance to the sample site before the net was raised (Fig. 3). These lines were attached to rings on the head rope and were threaded through the eye bolt in the top of each pole. The lines at each end were threaded through a ring and eyebolt at each end pole.

A pit trap was positioned inside the area sampled by the net at the lowest elevation into which organisms could accumulate as the tide receded. The pit traps were constructed by excavating a hole ~30 cm in diameter and ~45 cm deep and inserting a polyvinyl chloride bucket with a removable 3.2-mm-pore-size mesh basket. Each pit was covered with a weighted top as the tide was rising to prevent it from biasing the sample in any way. The pit-trap covers were removed after the net was raised.

The time of high slack tide was estimated from NOAA tide tables, and the actual time was determined by visually monitoring water current speed at the site. Although each area contained three pairs of reefs (a natural reef and an experimental reef in each pair), only two pairs could be sampled simultaneously because of manpower limitations. Lift nets were raised simultaneously by two teams of four people, who pulled on the lifting lines from the remote stations. Lifting the nets took less than 6 sec. After the nets were raised, the lift lines were tied to the anchor post and the field crew proceeded to secure the top line of the net to the net poles so that the net would not be pulled down as the tide receded.

After the water level dropped and the reefs were exposed, the

plywood walkways were again put in place. The lift net was lowered to permit two teams of two individuals to move along the walkways in opposite directions and collect all animals trapped at the base of the net. Animals were also retrieved from the pit trap by removing the insert. All specimens were preserved in 10% buffered formalin. In order to minimize disturbance to the reefs, only those animals observed from the perimeter were collected from interior sections of each reef. To date, we have successfully used this technique to sample 12 reefs: 3 paired reefs at the Toler's site and 3 paired reefs at the Inlet Creek site, during daylight hours in May, July, and October 1995 (n = 6 reefs/site per date).

All specimens collected were returned to the laboratory, identified to species, and enumerated, with the occasional exception of grass shrimp *Palaemonetes* spp.; *P. pugio* and *P. vulgaris* were sometimes encountered in numbers large enough (>1,000) to necessitate subsampling. Our subsampling procedure consisted of randomly selecting a subsample that was 10% by weight of the total sample of all *Palaemonetes* spp. and then identifying and sorting the sample by species. Each species was then enumerated and weighed, and the ratios of number/weight in the subsample were used to estimate total number and weight for each species from the total weight of the group.

Capture Efficiency

Efficiency tests were conducted at two of the paired experimental and natural reef sites in Inlet Creek on three consecutive days in March 1995. After nets were raised at high slack tide as described above, 50 specimens of mummichog *Fundulus heteroclitus*, with clipped anal fins, and 50 grass shrimp *Palaemonetes* spp. stained with alcian blue (Coen et al. 1982) were released into the sampling area. These species were chosen because they were readily available at the sampling sites and are not demersal residents of oyster reefs. Five paired replicate tests were conducted, and the mean return rates were used to represent efficiency.

A two-sample *t*-test for independent samples was used to determine whether the mean number of recaptured individuals differed between natural and experimental reefs. Variances were determined to be equal by use of Levene's test (SPSS, Inc. 1993). Chi-square analysis of pooled frequencies was used to test the null hypothesis that the sample of recaptured individuals fit an expected 1:1 ratio between natural and experimental reefs (Sokal and Rohlf 1981). The significance level in all statistical tests was α = 0.05.

RESULTS

Of the 17 decapod and 24 fish taxa collected on oyster reefs in May, July, and October 1995, the grass shrimps *P. vulgaris* and *P. pugio* were the most abundant (Table 1). Overall, these species constituted 70 and 12%, respectively, of the total 27,850 individuals captured during the three sample periods. The overall density of *P. vulgaris* for both sites during the sampling period was 22 individuals/m^2, whereas *P. pugio* occurred at a much lower density of 4 individuals/m^2. Other numerically important species collected were the bay anchovy *A. mitchilli* (5%), the naked goby *Gobiosoma bosci* (3%), brown shrimp *Penaeus aztecus* (3%), and white shrimp *Penaeus setiferus* (2.5%). Grass shrimps, bay anchovy, and naked goby abundances were consistent during each collection date, whereas brown and white shrimp abundances were high in May and June, respectively.

The percentage of individuals recaptured during the capture efficiency study was greatest on the natural reefs, with 68.5% (standard error, SE = 10.1) of the mummichogs recovered and 58% (SE = 11.4) of the grass shrimp recovered. On the experimental reefs, 54% (SE = 10.9) of the mummichogs and 43% (SE = 8.5) of the *Palaemonetes* spp. were recovered. Although no significant difference (two-sample *t*-test) was detected between the mean number of individual *Fundulus* or *Palaemonetes* recaptured on natural and experimental reefs (Fig. 4), total numbers recovered for each species differed significantly from 1:1 for reef treatments (pooled χ^2 = 4.52, $p < 0.05$ for *Fundulus;* pooled χ^2 = 5.14, $p < 0.05$ for *Palaemonetes*), with more individuals of each species captured on the natural reefs.

DISCUSSION

The recapture efficiency of the net system we used to sample oyster reefs compared favorably with similar techniques used to sample salt marshes. Rozas (1992) estimated the efficiency of a bottomless lift net to range from 32 to 93%, depending on the species, with the method being less efficient in capturing *Palaemonetes* (32%) and more efficient (81%) in capturing Gulf killifish (*Fundulus grandis*). Similarly, Kneib (1991) found that a flume weir recovered *F. heteroclitus* and *P. pugio* less efficiently from the marsh surface than it did other species. He (Kneib 1991) conducted several efficiency tests where test animals were released into an area that was then sampled three consecutive times. An average of 62% of the released *Fundulus* and an average of 42–72% (depending on size) of the released grass shrimp were recovered on the first retrieval efforts of the tests.

Various explanations have been given for the efficiency levels of drop nets and flume weirs. Rozas (1992) attributed his lowered efficiencies to the escape of organisms through holes in the netting made by blue crabs. We have also found holes (generally <2 cm) around the base of our nets and have frequently seen blue crabs tearing at the net in an attempt to grab organisms caught in the folds of the mesh. Although Rozas (1992) discounted a possible loss of efficiency due to the avoidance of collecting pans by organisms remaining on the marsh, such avoidance may be a significant factor in our study. In our study, the additional structure created by the experimental trays and complete coverage by oyster shell on the experimental reefs undoubtedly provided more refuges and natural depressions than the patchy shell density of natural reefs, thereby reducing capture efficiency. Also, our reefs were sufficiently large that thorough inspection was not possible without walking on the reef, a situation deemed to be an undesirable disturbance. However, small dip nets were used to strain water-filled depressions that formed along the base of the net and trays to recover organisms using them as refuges.

Our modification of the lift net system used previously to sample emergent vegetation has many of the same advantages noted by Rozas (1992) for his sampling of intertidal marshes. Because we deployed the nets immediately before each sampling event and took them down at completion, there were no permanent posts or walkways and little habitat modification. A shallow trench to hold the net was the only necessary construction. It was fitted with netting covered by mud so that nekton could move onto the reef without being impeded or scared away by the net.

Another major advantage of this technique is its flexibility in size for sampling a variety of intertidal habitats. Rozas (1992) described the use of the bottomless lift net for sampling small (6-m^2), discrete salt marsh areas. Our adaptation is designed to

TABLE 1.

Rank abundance for species collected on intertidal oyster reefs in May, July, and October 1995 (IC = Inlet Creek; TC = Tolers Cove).[a]

Species Name	IC Control (No. of Individuals)	IC Experimental (No. of Individuals)	TC Control (No. of Individuals)	TC Experimental (No. of Individuals)	Grand Total	Abundance (%)	Rank (% Abundance)
Alpheus heterochaelis	1	0	0	1	2	0.01	40
Anchoa mitchilli	619	311	321	129	1,380	4.96	3
Archosargus probatocephalus	1	0	2	3	6	0.02	31
Bairdiella chrysoura	10	6	1	1	18	0.06	24
Callinectes sapidus	26	21	14	11	72	0.26	11
Callinectes similis	3	3	0	0	6	0.02	31
Chasmodes bosquianus	10	16	62	14	102	0.37	10
Clibanarius vittatus	0	0	9	0	9	0.03	30
Eucinostomus argenteus	60	42	44	88	234	0.84	7
Eucinostomus sp.	0	0	14	12	26	0.09	20
Eurypanopeus depressus	3	0	1	1	5	0.02	35
Eurytium limosum	6	10	23	16	55	0.2	14
Evorthodus lyricus	0	0	1	0	1	0	41
Fundulus heteroclitus	4	6	5	1	16	0.06	26
Gobionellus boleosoma	21	18	25	42	106	0.38	9
Gobiosoma bosci	84	217	290	304	895	3.21	4
Lagodon rhomboides	7	9	31	10	57	0.2	12
Leiostomus xanthurus	4	8	23	9	44	0.16	16
Lutjanus griseus	4	9	6	15	34	0.12	18
Menidia menidia	12	97	17	10	136	0.49	8
Mugil cephalus	5	7	2	2	16	0.06	26
Mugil curema	0	3	13	25	41	0.15	17
Myrophis punctatus	0	0	1	0	1	0	41
Opsanus tau	0	0	4	10	14	0.05	28
Orthopristis chrysopterus	0	0	4	13	17	0.06	25
Palaemonetes pugio	146	237	872	2,142	3,397	12.2	2
Palaemonetes sp.	0	0	0	5	5	0.02	35
Palaemonetes vulgaris	1,699	1,910	8,673	7,101	19,383	69.6	1
Panopeus herbstii	10	6	10	6	32	0.11	19
Panopeus obesus	11	14	15	16	56	0.2	13
Panopeus sp.	9	7	3	3	22	0.08	23
Paralichthys lethostigma	1	0	0	0	1	0	41
Paralicthys dentatus	4	3	9	7	23	0.08	22
Penaeus aztecus	238	177	332	88	835	3	5
Penaeus duorarum	3	0	1	2	6	0.02	31
Penaeus setiferus	213	77	322	82	694	2.49	6
Sciaenops ocellatus	2	3	2	3	10	0.04	29
Sesarma sp.	0	1	0	0	1	0	41
Syngnathus louisianae	1	1	0	2	4	0.01	38
Uca minax	1	3	0	1	5	0.02	35
Uca pugilator	6	1	5	13	25	0.09	21
Uca pugnax	1	1	4	0	6	0.02	31
Uca sp.	15	9	8	15	47	0.17	15
Total	3,242	3,235	11,170	10,203	27,850	100	

[a] Number of individuals is total number of specimens from three experimental or three control reefs at each site.

sample a larger (24-m^2) intertidal area, regardless of reef shape. We caution that the expansion of the area sampled might complicate raising the net sides by requiring more liftlines and greater deployment time. We cannot attest to the use of this net in marsh habitats but suggest that it would be feasible because of its similarity to the net described by Rozas (1992).

Although Kneib (1991) noted that the use of a flume weir in habitats with little or no emergent structure could bias results, our technique likely avoided such bias for several reasons. We did not install a permanent boardwalk around the netting, thereby avoiding shadows, which may attract fish. Removable plywood walkways around each reef minimized disturbance during sampling. Oyster reefs have a vertical structure (15–25 cm) that far exceeds that of the buried net within its trench, so the avoidance or attraction of nekton to the net should be minimal. Escape was minimized as the result of the ability to pull the nets upward from a submerged position rather than lowering panels (Kneib 1991) or a lead line (McIvor and Odum 1986, Wenner and Beatty 1992). The use of lift lines from a location away from the net also reduced site disturbance. The height of the net was sufficient to be above the high-water level in estuarine systems with a mean tidal amplitude of ~1.5–2.0 m.

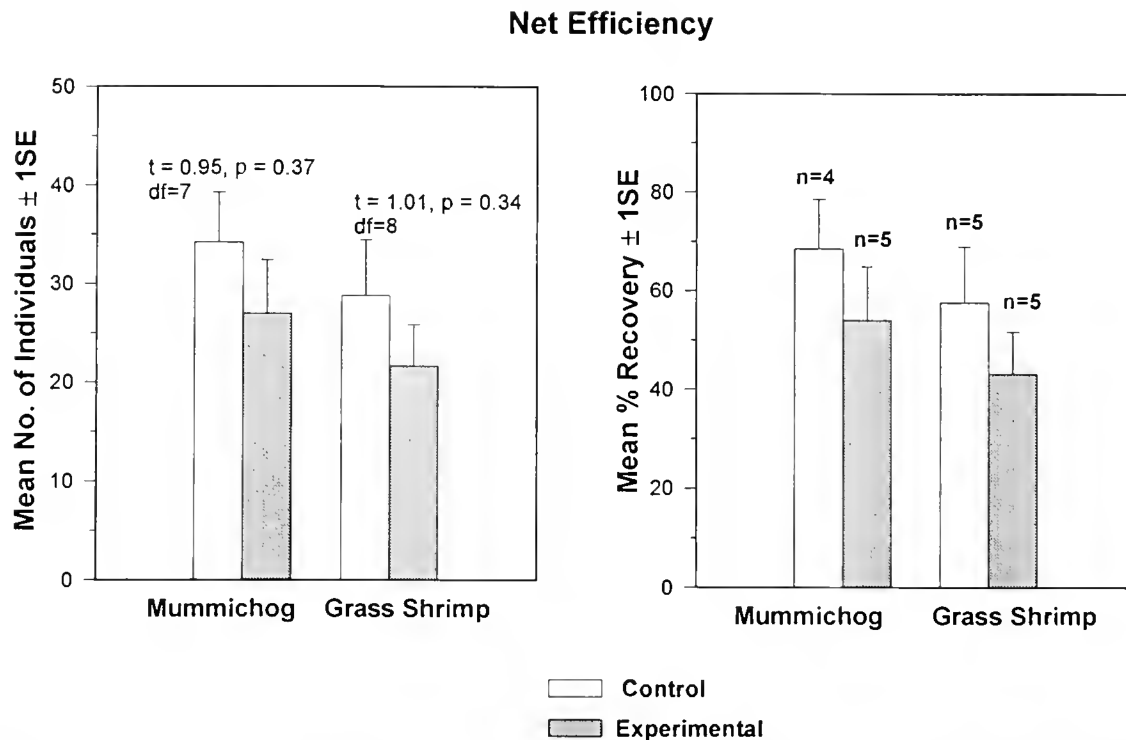

Figure 4. Efficiency of recapture for *F. heteroclitus* and *P. pugio* released in an area enclosed by a lift net. The mean number of individuals and the mean % recovery from replicate releases on control and experimental reefs are presented. Results of a *t*-test to determine significant differences among mean numbers of recaptured individuals between control and experimental reefs are shown.

The cost of materials was ~$1,000 per sampling unit (one reef), including net, chain, line, poles, rebar, and cable. Because nets were deployed and removed in a day and not left in the field for an extended period, they experience little deterioration. Nets were rinsed and dried immediately after sampling to prolong their use. Typically, six paired reefs could be sampled per week.

A major disadvantage to this sampling technique is the number of people necessary to set up and deploy each net. We used teams of four individuals per site (two per net) during our study, all of whom could work comfortably out of a single vessel. A pair of nets could be fully prepared in 1–2 h. The logistics of sampling depends largely on the number of replicates in the study.

The sampling method described here provides a means for the quantitative assessment of nektonic species associated with intertidal oyster reefs. Previous methods attempting to quantify organisms associated with subtidal reefs were not suitable for the calculation of density estimates of transient reef species. This method quantitatively samples small reefs (tens of square meters), requires little modification to the reef, is deployed with a minimum disturbance of organisms in situ, and is relatively inexpensive to construct and maintain. Information obtained from the sampling of intertidal oyster reefs aids in determining the relative value of various critical habitats in southeastern U.S. estuaries and helps to elucidate how this extensive oyster reef habitat contributes to the broader functioning of estuarine ecosystems.

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aerated, running seawater (5 L/min) at ambient water temperature (14–16°C). Each size category was divided into three feeding treatments: 30 urchins were fed *N. luetkeana*, 30 were fed *Z. marina*, and 30 were starved. Within each feeding treatment, urchins were randomly assigned to three replicate tanks (10 urchins per tank). Treatments requiring *Z. marina* and *N. luetkeana* were provided with enough material so that individual urchins were in close contact with the available food source at all times. Each tank was closely monitored daily for urchin mortality and cleaned as required. After 90 days, the experiment was terminated. The TD and JL of each urchin were measured as described above.

Percent change in TD (d) was calculated as $d = 100 (T_t - T_0)/T_0$, where T_t = the mean final TD (in millimeters) for one replicate tank of urchins after 90 days, and T_0 = the mean initial TD (in millimeters) for one replicate tank of urchins at 0 days. Percent change in JL (j) was calculated as $j = 100 (J_t - J_0)/J_0$, where J_t = the mean final JL (in millimeters) for one replicate tank of urchins after 90 days, and J_0 = the mean JL (in millimeters) of urchins from that particular size group sacrificed before the experiment. The mean and standard error of d and j for the three replicate tanks from each size group per food treatment were calculated. The final JL/TD ratio was calculated for each of the wild and treated urchins; means and standard errors for JL/TD were calculated for all urchins in each treatment. Percent mortality (x) was calculated on a weekly basis for urchins within each tank as $x = 100 (M_t/N_0)$, where M_t = the total number of mortalities at a given time, and N_0 = the initial number of urchins at 0 days. A two-way analysis of variance (ANOVA) was used to compare each growth parameter and mortality estimate of urchins between different feeding treatments and initial size groups after the data were arcsine transformed. There were significant differences ($p < 0.05$) in the interaction between size and treatments in the growth parameters so the Tukey test was used for post hoc multiple pairwise mean comparisons (Wilkinson et al. 1992). The power curve of the linear regression form $\ln JL = \ln a + b \ln TD$ was used to approximate (with the least-squares method) the relationship between the final JL and TD of individual urchins collected from each of the wild and the three experimental treatments after the data were natural log (ln) transformed. Analysis of covariance (ANCOVA) was used to test between the treatment homogeneity of the slope and the elevation coefficients of the regressions (Zar 1984).

RESULTS

The percent growth of TD and JL declined with the increase in size categories for fed red sea urchins (Fig. 1A and B). Growth was greater for urchins fed *Nereocystis* than *Zostera*, and growth was nearly zero for urchins that were starved (Fig. 1A and B). There was no significant percent change (Tukey test, $p > 0.05$) in growth for all sizes of urchins that were starved (Fig. 1). There were significant differences (Tukey test, $p < 0.05$) in percent change in TD, JL, and the JL/TD ratio between all urchin feed treatments except for the starved and wild sample urchins (Fig. 1C). The average mortality per tank was 15% ($n = 10$), with 95% of these deaths occurring within the first 2 wk of the experiment, probably due to handling effects. There were no significant differences in mortality (ANOVA, $p > 0.05$) between feeding treatments or size groups. The JL/TD ratio was higher for starved and wild urchins at each size category than for urchins fed *Zostera*, and it was lowest for those fed *Nereocystis* (Fig. 1C). There was no

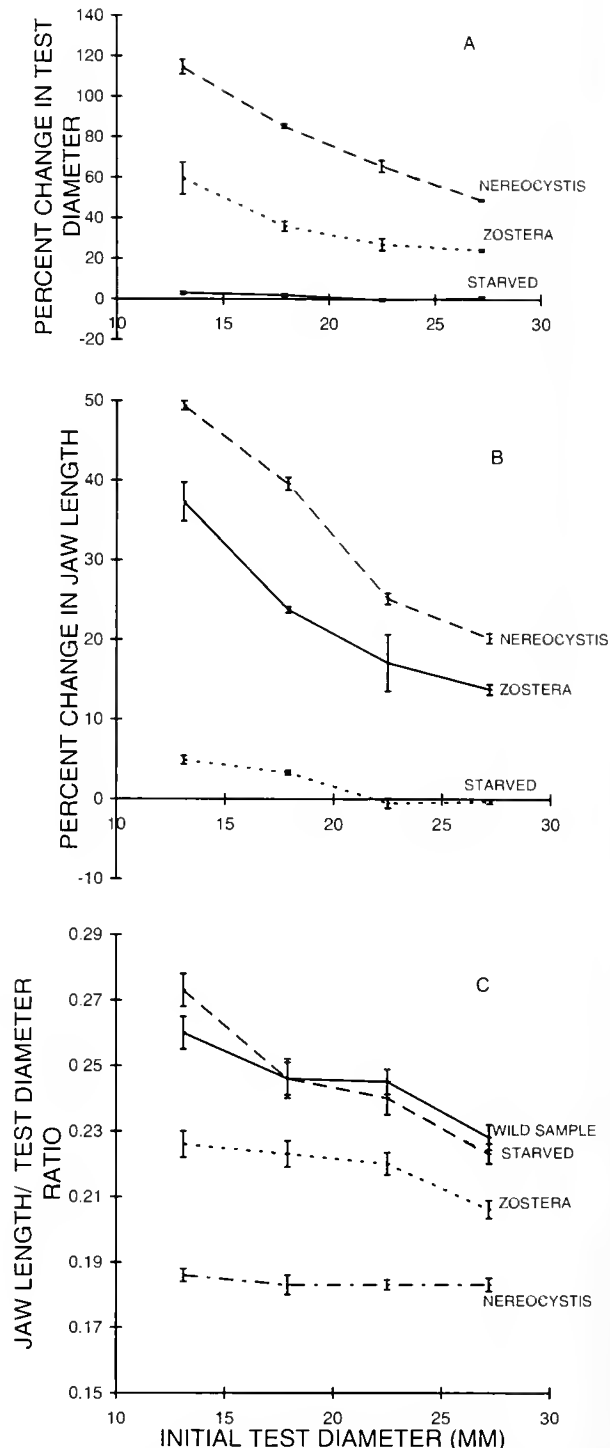

Figure 1. (A) TD growth, (B) JL growth, and (C) final JL/TD ratio in relation to the initial TD of four groups of red sea urchin juveniles fed *Nereocystis* or *Zostera* or not fed (starved) for 90 days in aquaria. The JL/TD ratios for the "wild sample" are based on red sea urchins at the start of the experiment. Vertical bars are standard errors about the means (dots).

significant difference (Tukey test, $p > 0.05$) in JL/TD ratios between the different size groups of urchins fed *Nereocystis* (Fig. 1C). The ANCOVA confirmed, for JLs adjusted for the covariate TD (slopes were homogeneous), that although there was no dif-

TABLE 1.

Relationship between the final JL (in mm millimeters) and TD by use of the linear equation ln JL = ln a + b ln TD for juvenile red sea urchins from the wild and three laboratory treatments.[a]

	Regression Coefficients				Test Diameter (mm)	
Treatment	ln a	b	r^2	N	Min	Max
Wild sample	−0.805 a	0.796	0.892	80	10	30
Starved	−0.613 a	0.735	0.899	98	11	30
Zostera	−0.723 b	0.760	0.802	102	16	37
Nereocystis	−1.214 c	0.866	0.819	105	23	45

[a] N is the number of urchins in the sample; r^2 is the coefficient of determination. There was no overall difference ($p > 0.05$) between four slopes (b) by the use of ANCOVA for the homogeneity of slopes. Elevations (ln a) followed by the same letter were not significantly different ($p > 0.05$), whereas elevations followed by different letters were significantly different ($p < 0.05$) by the use of pairwise comparisons of treatments with ANCOVA, adjusting the ln JL values for the covariate ln TD.

ference ($p > 0.05$) between wild caught urchins and starved urchins, there were significant differences ($p < 0.05$) in all other combinations between the starved urchins and those fed *Zostera* and *Nereocystis* (Table 1).

DISCUSSION

Nereocystis is clearly a better food for the growth of *S. franciscanus* juveniles than is *Zostera*. Although growth in urchin juveniles fed *Zostera* was almost 50% less than those fed on *Nereocystis*, there are no previous studies showing the capacity of *S. franciscanus* to digest *Zostera*. Echinoids are generally incapable of producing the pectinase enzyme required to break down the pectin polysaccharide found in *Z. marina* (Lawrence 1975, Lowe and Lawrence 1976, Whyte and Englar 1977, J. N. C. Whyte pers. comm.). The fact that urchins grew after eating *Zostera* indicated their ability to digest and absorb some nutrients from the plant. The pectin polysaccharide constitutes a large proportion of the cellulose fiber found in *Zostera;* this would inhibit the digestion of the more readily absorbed components. However, chewing by urchins could break down the fibrous component of *Zostera* to some degree, allowing enzymatic interaction with the digestible components of the plant. Several studies have shown that polysaccharide-degrading bacteria were present in the guts of the sea urchins and have suggested that bacteria may play a role in digestion (Guerinot and Patriquin 1981, Lasker and Giese 1954, Yano et al. 1993). Red sea urchins may also have bacteria capable of assisting digestion.

Percent change in the TD and the JL of *S. franciscanus* fed *Zostera* or *Nereocystis* declined with an increase in TD size, which according to Lawrence (1975), is due to decreased absorption and the assimilation of urchins with increasing age. Also, the increased allocation of nutrients for sexual maturation and gonad development would reduce the nutrients available for somatic growth (Fuji 1967, Gonzalez et al. 1993). Small, immature gonad development was visible in all *S. franciscanus* fed *Nereocystis* and in many of the urchins fed with *Zostera*. In contrast to the growth of fed urchins, the ability of *S. franciscanus* to maintain the same TD over 90 days of starvation supports the theory that urchins can live in a feast-and-famine environment (Andrew 1989), maintaining their size over periods of starvation and capitalizing on an abundance of nutritious food when available by growing rapidly (Vadas 1977, Larson et al. 1980, Andrew 1986, Lawrence and Lane 1982).

The JL/TD ratios of the urchins starved for 90 days in the laboratory were similar to those of the original wild urchins for the same size categories. The large JL/TD ratio suggests that wild urchins were also starved, probably because of the observed general absence of marine flora attached to the substrate, accompanied by high densities of red urchins (20–30 urchins per m^2) at the collection site (A. Campbell unpub. data). High-quality food such as *Nereocystis* allowed juvenile red sea urchins to rapidly grow their test and jaws at similar relative rates throughout the TD size range observed. Our results on juvenile *S. franciscanus* generally agree with the interpretation that sea urchins have an adaptive morphological plasticity in which individual urchins may allocate more resources to the food-gathering Aristotle's lantern during food scarcity and allocate more resources to test growth during high-quality food abundance (Ebert 1980, Edwards and Ebert 1991). Although the perennial *Zostera* is a lower quality food source for *S. franciscanus* compared with the annual *Nereocystis*, the presence of *Zostera* as a drift plant material may be important in maintaining high levels of red sea urchin density when sufficient *Nereocystis* is unavailable in the coastal waters of Clayoquot Sound.

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omy, increased sample size, and the ability to survey several species simultaneously.

WDFW INTERTIDAL BIVALVE POPULATION ASSESSMENT IN PUGET SOUND AND HOOD CANAL, WASHINGTON. William W. Campbell and **Jennifer A. Cahalan,** Washington Department of Fish and Wildlife, Point Whitney Shellfish Laboratory, 1000 Point Whitney Road, Brinnon, WA 98320.

The intertidal shellfish project is responsible for determining clam and oyster populations on selected beaches in Hood Canal and Puget Sound. Clam and oyster population assessments have been conducted on Puget Sound beaches since the mid-1970s. Current survey methods are modified from methods developed in 1986. Approximately 30 to 50 clam and 20 to 30 oyster population assessments are conducted each season.

Surveys are conducted on a beach specific basis using a systematic random sampling design. Clam and oyster surveys are similar in nature, differing only in sample collection methods. Surveys are conducted on days with minus tides on 1.0 ft MLLW or lower, for two hours on either side of low tide. The density of the resource and area of the population are estimated from these data, and population totals are calculated. Length-frequency information is also collected during the surveys. Total allowable catch estimates are derived from the size-frequency distribution and population totals. The allowable catch is then applied to the following harvest season.

New survey methods are currently being developed, incorporating global positioning system (GPS) technology to determine individual sample locations. These sample locations will be used in conjunction with geographic information system (GIS, MapInfo) to produce maps of beaches and the surveyed resources. In addition, significant landmarks, enhancement plots, and resource density gradients will also be portrayed.

KEY WORDS: Resource assessment, intertidal bivalves, GPS/GIS

POSITIONING YOUR SHELLFISH IN CHINA. Jerry Chang* and **Kenneth K. Chew,** School of Fisheries, Box 357980, University of Washington, Seattle, WA 98195–7980.

The fast economic growth in mainland China demands more and more shellfish products from USA, particularly the Northwest. Oysters, clams, mussels, lobsters and geoducks are the primary products exported in alive, frozen, smoke and canned forms. Presently there is no well-established channel for shellfish growers to market their products in China. There are also some constraints challenging shellfish exporters, such as under-developed markets, poor infrastructure, inefficient brokers and agents, inadequate distribution system as well as tariff and culture barriers to do business with China. Comments will reflect upon the present status of the seafood industry in China and what can be expected in the near and distant future.

INTERTIDAL SHELLFISH MANAGEMENT ON PUBLIC TIDELANDS IN PUGET SOUND, WASHINGTON—THE MANAGEMENT PROCESS. Anita E. Cook, Washington Department of Fish and Wildlife, Point Whitney Shellfish Laboratory, 1000 Point Whitney Road, Brinnon, WA 98320.

Intertidal clam and oyster harvest comprises one of the largest recreational shellfish fisheries in Washington and provides harvesting opportunities to thousands of harvesters each year. This paper describes current management techniques and challenges in the wake of the federal court decision regarding tribal shellfish rights.

The intertidal shellfish management unit is primarily responsible for the development of regional management plans and establishing and implementing shellfish harvest regulations. The focus of this presentation is to elaborate on the process involved in developing fishery regulations and to discuss how recreational harvest estimates and population assessment information are integrated into management decisions. Consideration will be given to: 1) development of regional management plans, 2) how estimates of recreational allowable catch are made within a cooperative state/tribal management planning process, 3) how pre-season projections are developed, 4) inseason management, including season adjustments and harvest reporting, and 5) special challenges, including mixed species management, mixed fisheries (commercial and recreational), and information dissemination.

KEY WORDS: Resource management, regulations, tribal, clams, oysters

PATTERNS IN RAZOR CLAM ABUNDANCE ESTIMATES IN COASTAL WASHINGTON. Annette Hoffmann,* Jack V. Tagart, and **Dan Ayres,** Washington Department of Fish and Wildlife, Washington, 98501.

Precise estimates of razor clam (*Siliqua patula*) abundance are necessary for responsible harvest management and for monitoring of long term population trends. Understanding "patchiness," or how the patterns of densities are distributed can greatly help one improve the precision of an abundance estimator by stratifying the beach into areas of like densities. Stratification will improve the precision of an estimator if the within strata variance is smaller, on average, than the among strata variance. However, stratification will degrade the precision if the within variance is the same or greater than the among variance, or if the process of stratifying intensifies existing biases. If stratification variables can be identified that are consistent across beaches and persistent across years, then we will use them in our abundance monitoring and harvest management plans.

We investigated the use of stratifying razor clam densities along elevational and latitudinal (transects perpendicular to the surf) gradients on three beaches using pump survey data collected from 1994–1996. Analysis results showed that elevation was a good stratification variable, but that latitude was not. However, a secondary study showed that the "patchiness" of razor clam dis-

tribution was on a smaller scale than the latitudinal transects and therefore, would be unlikely to show up as a latitudinal effect. Stratification by elevation resulted in improved precision in 5 out of 6 beach-years. The case where elevation was not significant occurred in a year of relatively low abundance. In this case we also encountered both kinds of problems one can experience with stratification: intensified bias and greater within than among strata variances. With these data, we cannot discern the cause of the non-significance.

These results suggest that elevation may be a consistent stratification variable, but may not be persistent. However, these results are preliminary. We will further investigate both variables on more beaches and over more years before concluding that they should or should not be used in a monitoring plan.

TASMANIAN PACIFIC OYSTERS, *CRASSOSTREA GIGAS*, IN WASHINGTON STATE: CHARACTERIZATION OF A TRANSPLANTED POPULATION. **Manfred T. Kittel*** and **Kenneth K. Chew**, School of Fisheries, Box 357980, University of Washington, Seattle, WA 98195.

In 1995, 32 Pacific oysters from Tasmania, Australia, were artificially spawned in quarantine in the state of Washington and the resulting F_1 generation was outplanted at three different locations of Puget Sound. We have begun studies to characterize these oysters at several levels. Survival, growth rates and shell morphology of oysters grown under different environmental regimes are determined and compared to similar data from *C. gigas* of local origin. Patterns of gametogenesis and glycogen storage will be examined from histological sections of oysters taken over the period of one growing season. The ability of the imported oysters to form viable hybrids with *C. gigas* of local origin and closely related species such as *C. sikamea* will be determined from reciprocal crosses between selected oysters. Molecular analysis of the transplanted oysters will focus on establishing their species identity and genetic relatedness to local *C. gigas* populations. Preliminary findings of some of the ongoing studies will be presented.

UPDATE ON THE MOLLUSCAN BROODSTOCK PROGRAM—PRODUCTION OF FAMILIES. **Chris J. Langdon,*** Hatfield Marine Science Center, Oregon State University, Newport, OR 97365.

The focus of the Molluscan Broodstock Program (MBP) during the first year of funding has been rearing two groups of about 50 families of Pacific oyster spat for planting and evaluation at commercial test sites on the West Coast, U.S.

Pairs of oysters from either Willapa or Dabob Bay "wild" populations were crossed to produce full-sib families. Larvae were reared in 100 l tanks at a concentration of 4 larvae/ml. Competent larvae were exposed for 2 h to 2×10^{-4} M epinephrine to induce metamorphosis. Throughout the culture period, larvae were fed on a mixed algal diet of *Chaetoceros* sp. and a flagellate (*Pseudoisochrysis* or *Isochrysis* sp.).

Spat were initially cultured in a small (MINI) upweller system at 25°C and fed on the mixed algal diet. When all the spat from a family had been collected, the number of spat per family was reduced to 20,000 by random partitioning. When spat could be retained on a 1.5 mm mesh, they were transferred to a larger (MAXI) upweller system and cultured at 20–25°C on a diet consisting of mainly *Chaetoceros* sp. When spat in the MAXI system could be retained on a ¼ inch mesh, they were transferred to ⅛ inch mesh bags suspended in Yaquina Bay, Oregon, and held for planting at commercial test sites in late summer/fall 1996.

Survival, growth and meat yields of planted families will be compared when they reach market size and top performing families will be used to produce the next MBP generation.

IMPACT OF CRYOPROTECTANTS DIMETHYL SULFOXIDE, ETHYLENE GLYCOL, METHANOL, GLYCEROL, SUCROSE AND POLYVINYLPYRROLIDONE ON OYSTER (*CRASSOSTREA GIGAS*) EGGS BEFORE FREEZING. **Xin Liu*** and **A. M. Robinson,** Department of Fisheries and Wildlife, Hatfield Marine Science Center, Oregon State University, Newport, OR 97365.

As a basis for Pacific oyster cryopreservation study, information on impact of cryoprotectants on oyster eggs before freezing is an important factor. Oyster eggs were exposed to various concentrations of six cryoprotective compounds, dimethyl sulfoxide (DMSO), ethylene glycol (EG), methanol, glycerol, sucrose and polyvinylpyrrolidone (PVP) at room temperature (21–24°C) for 30 minutes. The results showed that glycerol at tested concentrations and methanol at greater than 1.2 M concentrations were highly toxic to oyster eggs, whereas sucrose at tested concentrations and PVP at less than 10% concentrations did not have toxic effects. The results of time exposure experiment conducted with 1.4 M DMSO, 1.8 M EG, 2.4 M methanol, 1.4 M glycerol, 0.292 M sucrose and 10% PVP concentrations for 25 minutes indicated that the exposure time should be less than 20 minutes to minimize the injury of the eggs caused by cryoprotectants. The combination of 0.7 M DMSO + 0.141 M sucrose concentration mixture (15 minute exposure) improved oyster egg survival rate. It could be attributed to the replacement of DMSO fractions with sucrose rather than any specific toxicity blocking mechanism. Exposure to the cryoprotectants before freezing can cause major biochemical or/and osmotic injury on oyster eggs.

GROWTH OF THE PACIFIC OYSTER, *CRASSOSTREA GIGAS* (THUNBERG) AT 18 SITES IN PUGET SOUND AND HOOD CANAL. **Terrie A. Manning** and **Jennifer A. Cahalan,** Department of Fish and Wildlife, Pt. Whitney Shellfish Laboratory, 1000 Pt. Whitney Rd., Brinnon, WA 98320.

A mark and recapture study was conducted at 18 beaches in Hood Canal and Puget Sound, Washington to obtain accurate estimates of Pacific oyster growth rates. These population parameter

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Cameron P. Goater and Amy W. Weber
Factors affecting the distribution and abundance of *Mytilicola orientalis* (Copepoda) in the mussel, *Mytilus trossulus*, in Barkley Sound, B.C. 681

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COVER PHOTO: Pearl oyster collectors in the waters in front of Nusa Tupe, ICLARM's Fieldstation in the Western Province of Solomon Islands. (Photo by Mike McKoy)

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