

THE EFFECT OF LOW SALINITY ON ESTABLISHED
INFECTIONS OF PERKINSUS MARINUS (APICOMPLEXA: PERKINSASIDA)
IN THE EASTERN OYSTER, CRASSOSTREA VIRGINICA

A Thesis

Presented to

The Faculty of the School of Marine Science
The College of William and Mary in Virginia

In Partial Fulfillment
of the Requirement for the Degree of
Master of Arts

by

Lisa Maria Ragone

1991

APPROVAL SHEET

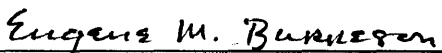
This thesis is submitted in partial fulfillment of
the requirements for the degree of

Master of Arts



Lisa M. Ragone

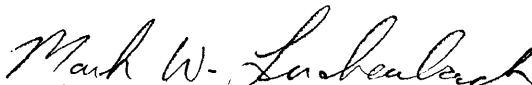
Approved, September 1991



Eugene M. Burreson, Ph.D.
Committee Chairman/Advisor



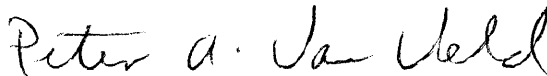
Fu-Lin E. Chu, Ph.D.



Mark W. Luckenbach, Ph.D.



Frank O. Perkins, Ph.D.



Peter A. Van Veld, Ph.D.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS	iv
LIST OF TABLES	v
LIST OF FIGURES .	vi
ABSTRACT	vii
INTRODUCTION	2
METHODS .	10
RESULTS . . .	21
DISCUSSION . . .	39
LITERATURE CITED	48
VITA	54

ACKNOWLEDGEMENTS

I am grateful to my major professor, Gene Burreson, for his guidance, advice, and review of this document. I thank my committee members Fu-Lin Chu, Mark Luckenbach, Frank Perkins, and Pete Van Veld for their helpful comments and review of my thesis. Thanks to Mark for his assistance with experimental design and statistical problems and to Dr. Perkins for support. Many other individuals assisted me along the way and I am very appreciative of their contributions. I extend thanks to Dan O'Connor for his interest, insights, advice, and encouragement; to Bruce Barber and Jerome La Peyre for their comments; to Al Curry for his strong back and encouragement; to Nita Walker and Pat Blake for sharing their histological expertise; and to Kenny Walker and Judy Meyers for assistance with the collection of oysters. Many thanks to Mike Castagna and his staff, especially Reed Boniwell, for sharing their laboratory with me and for providing assistance with setting up my experiment and keeping it going. Special thanks to Gustavo Calvo who assisted in almost every aspect of this project. His support and comments were especially helpful. Finally, I would like to thank my parents and family for their continuous support and encouragement.

LIST OF TABLES

Table	Page
1. Prevalence and intensity of <u>P. marinus</u> in oysters sampled after 14, 28, 42, and 56 days of exposure to treatment	22
2. Results of two factor analysis of variance for the effect of salinity and time on mean <u>P. marinus</u> prevalence	24
3. One-way analysis of variance of <u>P. marinus</u> prevalence between treatment salinities on each sample date	25
4. Mean percent cumulative oyster mortality following 14, 28, 42, and 56 days of exposure to treatment salinities	29
5. Results of two factor analysis of variance for the effect of salinity and time on mean cumulative mortality	31
6. One-way analysis of variance and multiple comparisons test (SNK) of mean cumulative percent mortality between treatments	32
7. Mean percent oyster mortality occurring durring each sampling interval	34
8. Prevalence of <u>H. nelsoni</u> , <u>B. cuculus</u> , <u>N. ostrearum</u> , and <u>P. marinus</u> in oysters sampled at the initiation of the experiment and on days 14, 28, 42, and 56	37

LIST OF FIGURES

Figure	Page
1. Oyster collection and transplantation sites	11
2. Prevalence and intensity of <u>P. marinus</u> at the initiation of the experiment	13
3. Site locations of oyster disease monitoring grounds and salinity stations	20
4. Mean prevalence of <u>P. marinus</u> at each treatment salinity after 14, 28, 42 and 56 days of exposure	23
5. Relative <u>P. marinus</u> infection levels at 6, 9, 12, and 20 ppt	26
6. Mean percent cumulative mortality of oysters exposed to 6, 9, 12, and 20 ppt following 14, 28, 42, and 56 days exposure ...	28
7. Mean percent mortality at each salinity treatment occurring during each sampling interval	33
8. Percent mortality of oysters transferred from low to high salinity in comparison to nontransferred groups ...	36
9. A. James River monitoring station salinities, 1989 and 1990	
B. <u>Perkinsus marinus</u> prevalence in oysters sampled from James River monitoring stations, 1989 and 1990	44

ABSTRACT

Environmental parameters are particularly important regulators of host parasite interactions. Understanding the influence of environmental factors can aid resource managers in preventing or terminating disease epizootics of commercially important species. The effect of salinity on Perkinsus marinus, a protozoan pathogen of the eastern oyster, Crassostrea virginica, was investigated. Oysters parasitized by P. marinus were exposed under laboratory conditions to 6, 9, 12 and 20 ppt salinity regimes at temperatures exceeding 20 degrees celsius, for a period of 8 weeks. Infection prevalence and intensity was assessed in samples (n=25) drawn from each treatment group following 2, 4, 6 and 8 weeks of exposure; oyster mortality was determined daily. The pathogen persisted throughout the course of the experiment at all salinities tested; however, development of P. marinus infections to lethal levels was delayed in oysters maintained at 12, 9, and 6 ppt. Cumulative mortalities at the termination of the experiment were 31.1, 32.1, 14.1, and 13.9 percent at 20, 12, 9, and 6 ppt, respectively. Oyster survival at 6 ppt was significantly higher than at 20 ppt. A critical range for parasite pathogenicity apparently exists between 9 and 12 ppt. Although P. marinus is able to tolerate salinities as low as 6 ppt it is less virulent at salinities less than 9 ppt.

THE EFFECT OF LOW SALINITY ON ESTABLISHED
INFECTIONS OF PERKINSUS MARINUS (APICOMPLEXA: PERKINSASIDA)
IN THE EASTERN OYSTER, CRASSOSTREA VIRGINICA

INTRODUCTION

Host parasite relationships in the marine environment are greatly dependent on environmental factors (Thorson, 1969; Rohde, 1982). For osmoconformers, such as marine and estuarine bivalves, salinity plays a particularly important role in modulating the complex interactions between hosts and parasites (Hepper, 1955; Bayne et al., 1978; Gauthier et al., 1990). The eastern oyster, Crassostrea virginica inhabits estuarine waters within both the polyhaline (30-18 ppt) and mesohaline (18-5 ppt) zones (Galtsolf, 1964). Generally, oyster parasites have a narrower salinity tolerance than their host and are more common in high saline areas (Hopkins, 1956; Wells, 1961; Andrews, 1964; Farley, 1975; Ford and Haskin, 1982; Andrews, 1983; Gauthier et al., 1990). For instance, it has been well documented (Farley, 1975; Ford and Haskin, 1982; Andrews, 1983; Ford, 1985) that the protozoan parasite Haplosporidium nelsoni (Acetospora) is intolerant of salinities below 10 ppt and exhibits full pathogenicity at salinities above 20 ppt. Experimental approaches involving field transplantations of infected oysters to various salinity regimes have provided evidence that the parasite can be eliminated from the host by exposure to salinities below 10 ppt (Andrews, 1983; Ford, 1985; Ford and Haskin, 1988).

Studies showing elimination of MSX infections by exposure to low salinities suggest that low salinity may also be a viable means for suppressing or preventing disease epizootics caused by Perkinsus marinus. This protozoan parasite has caused severe oyster mortalities

during the warmer months of the year in high salinity areas of the Chesapeake Bay at least since the 1950s (Andrews, 1988). Mortality caused by P. marinus has contributed to the dramatic decline in oyster landings which have decreased from an average of 3.5 million bushels per year prior to 1960 to a record low of 135,704 bushels in 1990 (Virginia Marine Resource Commission, unpublished data). Recent years (1985-1988) of drought conditions have caused record high salinities in upper estuarine areas of Virginia and allowed the pathogen to spread to previously disease free areas (Burreson, 1989). The current widespread distribution of P. marinus poses a serious threat to the oyster industry and increases the need to gain a more thorough understanding of the effect of salinity on P. marinus. Information concerning the role of salinity in controlling P. marinus is of practical importance to successful management of the oyster fishery.

Perkinsus marinus is a protozoan presently classified in the phylum Apicomplexa, class Perkinsasida (Levine 1988). Recurrent oyster mortalities occurring in the Gulf of Mexico during the late 1940s led to its discovery by Mackin et al. (1950). The authors at that time believed the parasite possessed fungal affinities and called it Dermocystidium marinum. Electron microscopic studies conducted by Perkins (1976) revealed that biflagellate zoospores produced by the parasite possessed an apical complex, an organelle typical of members of the phylum Apicomplexa. This discovery resulted in the reclassification of the parasite to its current status.

Numerous reviews have focused on the epizootiology and life history of P. marinus (Quick and Mackin, 1971; Lauckner, 1983; Andrews, 1988). Reproduction of the parasite is vegetative. Within living oysters the

cycle is known to be comprised of three cell stages; meronts (previously termed trophozoites), schizonts (previously termed sporangia), and zoosporangia (Perkins, 1991). Immature meronts, often found within phagosomes of hemocytes, are uninucleate, coccoid and range in size from 2-4 μm . As the cells mature they enlarge to 10-20 μm ; in the process they develop an eccentrically located vacuole which often contains a prominent vacuoplast. The cell nucleus has a central endosome and is located near the cell wall which gives the mature meront its characteristic signet ring configuration. The enlarged, mature meront undergoes successive bipartitioning, alternating karyokinesis and cytokinesis, and yields a multicellular (8-64 cell) schizont. The schizont ruptures liberating immature meronts and as the immature meronts enlarge the cycle is reinitiated (Perkins, 1991).

Meronts cultured in fluid thioglycollate medium (FTM), as well as those in moribund oyster tissue enlarge to form prezoosporangia. The prezoosporangia enlarge to 15-100 μm in FTM and are characterized by an extremely large vacuole which compresses the cytoplasm into a thin layer against the cell wall. Upon release into seawater the prezoosporangia initiate zoosporulation in which successive bipartitioning produces large numbers of biflagellate zoospores. Prior to first cleavage of the protoplast a discharge pore and tube develop from the cell wall of the zoosporangia. After being completely formed the zoospores are released through the pore and tube (Laukner, 1983; Perkins, 1988).

All stages of *P. marinus* with the possible exception of prezoosporangia are capable of invading new hosts (Laukner, 1983); however, the meronts are believed to be the normal agents of disease transmission (Perkins, 1988). Infections are initiated by ingestion of

infective cells with food and subsequent phagocytosis and transport by host cells in the stomach (Mackin, 1951) or by penetration and encystation of zoospores within or between cells of the gill, labial palp, or mantle epithelium (Perkins, 1988).

Once established in the host tissue the pathogen is dispersed by hemocytes to all parts of the body. Proliferation of the parasite causes lysis of host tissue and blockage of blood sinuses and organ functions (Ray et al., 1953; Lauckner, 1983; Andrews, 1988). At temperatures above 20 °C infections develop rapidly and death may occur within 4-5 weeks after the initial infection (Andrews, 1988).

Perkinsus marinus is distributed along the southeast coast of the United States from the Delaware Bay to Florida and along the coast and estuaries of the Gulf of Mexico (Andrews, 1988). This subtropical distribution implies the importance of temperature as a regulating factor and it appears that temperature is the most important environmental factor affecting its geographic distribution (Ray, 1954; Andrews and Hewatt, 1957; Quick and Mackin, 1971). The northern limit of P. marinus is believed to be controlled by minimum winter temperature. In the Chesapeake Bay epizootics of the parasite display a seasonal periodicity. Infection levels begin to increase in early spring as temperatures consistently exceed 20 °C and the parasite becomes active (Andrews and Hewatt, 1957; Andrews, 1988). The highest disease prevalence and associated mortalities occur during mid to late summer at temperatures above 25 °C (Ray, 1954; Andrews and Hewatt, 1957; Andrews, 1988). Prevalence of the pathogen declines in late fall and winter as temperature decreases; however, on occasion it has been

observed to persist at temperatures as low as 0-5 °C (Andrews, 1988). Laboratory studies suggest that development of established P. marinus infections is retarded and new infections do not appear at 15 °C (Andrews and Hewatt, 1957). It has been suggested that lower water temperatures reduce the metabolic activity of the parasite as well as enhance the oysters ability to combat infection (Ray, 1954).

The influence of salinity on the activity of P. marinus has been the focus of numerous studies in both the Gulf of Mexico and the Chesapeake Bay. Several authors have documented a direct correlation between salinity values and infection intensity (Mackin, 1951; Mackin, 1956; Andrews and Hewatt, 1957; Soniat, 1985; Craig et al. 1989; Gauthier et al. 1990; Crosby and Roberts, 1990). Mackin (1951) reported that oysters grown in Louisiana in high salinity areas (20-30 ppt) experienced greater disease pressure and mortality than those grown in low salinity areas (8-12 ppt). Later investigations by Mackin (1956) showed that the salinity tolerance of the parasite in Louisiana estuaries was almost as great as that of oysters. Andrews and Hewatt (1957) observed the parasite to be restricted in the Chesapeake Bay to waters with salinities greater than 15 ppt. In recent years P. marinus has also been observed in areas with lower salinities (Burreson, 1989; 1990; 1991). Soniat (1985) sampled oysters from the Galveston Bay for a period of 26 months (during which time the salinity ranged from 3.0 ppt to 28.5 ppt and temperature ranged from 9.3 to 30.5 °C) and found a significant relationship between weighted incidence (a measure of disease intensity) and salinity. Soniat also noted prevalence and intensity of P. marinus to decline in late summer, an apparent result of

freshets. Both P. marinus prevalence and intensity were positively correlated with salinity in a recent investigation in the Gulf of Mexico (Craig et al., 1989); however, salinity data were only recorded at the time of collection and did not adequately represent the values to which the oysters were exposed. Gauthier et al. (1990) surveyed oysters along a salinity gradient (lower estuary site 15-20 ppt; middle estuary site 10-15 ppt and upper estuary site 5-10 ppt) in coastal areas of Louisiana and found a positive correlation between P. marinus occurrence and salinity. Perkinsus marinus infection intensity in oysters from a high salinity (29.3-34.9 ppt) habitat in South Carolina positively correlated with both temperature and salinity (Crosby and Roberts, 1990).

Mackin (1956) suggested that the correlation between disease level and salinity is not a result of a limiting physiological effect on host or parasite but rather is due to the dilution of waterborne infective elements by freshwater inflow into the estuary. The author supported this hypothesis with a laboratory study in which the parasite persisted in infected oysters placed in low salinity (8.6-14.6 ppt). In agreement with Mackin, Andrews and Hewatt (1957) suggested that the circulation patterns and flushing rates of a particular area are of primary importance in regulating the disease. The authors also suggested that the low prevalence of the pathogen in Chesapeake waters with salinities less than 15 ppt and its presence in Redfish Bay, Louisiana in 7-8 ppt may be related to flushing rates of the two areas.

In contrast, transplantation and laboratory experiments have demonstrated that low salinity exposure has a direct effect on development of P. marinus infections within the oyster. Transplantation experiments in which infected oysters were moved to low salinity areas

(1-13 ppt) demonstrated that the parasite can persist in waters with low salinities; however, it appeared that the development of the disease was delayed, oyster mortalities were suppressed, and no infections were acquired by adjacent uninfected oysters (Andrews and Hewatt, 1957). Ray (1954) investigated the comparative development time of P. marinus in oysters held in closed aquaria with high salinity (26-28 ppt) and low salinity (10-13.5 ppt) water. Oysters in both groups were infected by injection of minced tissue from diseased oysters. Once again the parasite tolerated low salinity; however, development of infection and subsequent mortalities of oysters were delayed relative to the high salinity group. Scott et al. (1985) suggested that differences in survival between high and low salinity oyster populations are related to physiological differences in oysters exposed to different salinities rather than to infective density. The authors compared mortalities of infected oysters held in low salinity (8-10 ppt) to those held in high salinity (21-25 ppt). During the first 33 days of exposure no significant difference between mortality in the two groups was observed. After 60 days oyster mortality in the low salinity groups averaged 25% less than in the high salinity groups.

In vitro studies on the relationship of temperature, salinity and P. marinus infection have been conducted by Perkins (1966) and Chu and Greene (1989). Perkins (1966) found salinities from 10-35 ppt to be relatively unimportant as a limiting factor. Salinities from 5-10 ppt inhibited zoosporulation. Similar findings were reported by Chu and Greene (1989). Salinities below 6 ppt were found to have an inhibitory effect on development of zoosporulation. Some degree of zoosporulation was observed at higher salinities (6-34 ppt).

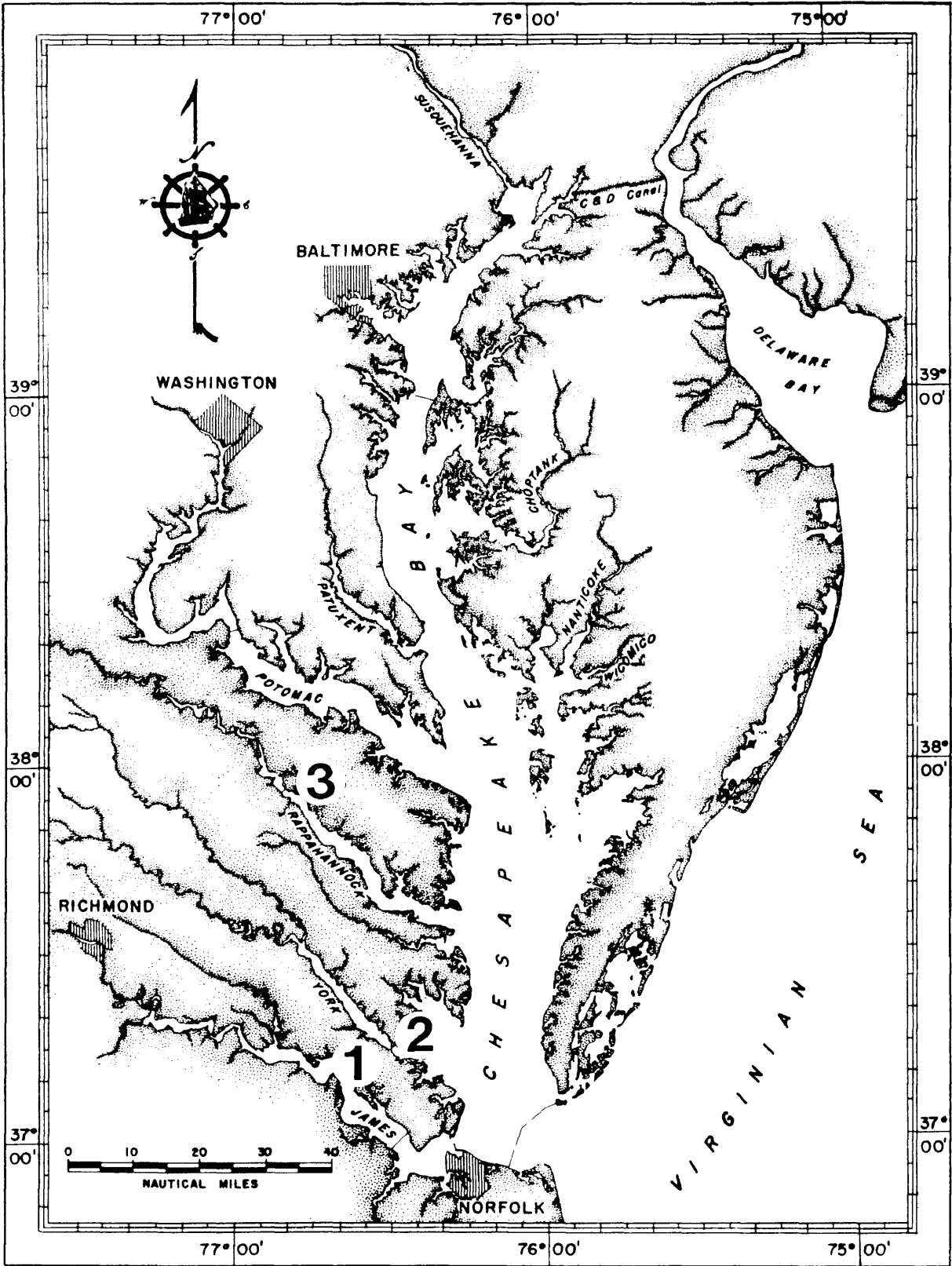
In an effort to further define the influence of low salinity on the pathogenicity and survival of P. marinus the following investigation was undertaken. The laboratory experiment reported here investigated the effect of low salinity exposure on established infections of P. marinus in the eastern oyster, by examining the hypothesis that exposure of P. marinus-infected oysters to low salinity will decrease the prevalence and intensity of infections and thus reduce oyster mortality. Three objectives were addressed. The first objective was to identify salinity conditions in which the pathogenic effect of the P. marinus is reduced by comparing mortality of infected oysters exposed to various salinity regimes. The second objective was to determine if exposure of infected oysters to low salinity results in expulsion of the parasite; and if expulsion is observed, to determine the salinity condition and the time period of exposure required to induce the response. The third objective was to determine if P. marinus infection development differs at various salinity exposures.

MATERIALS AND METHODS

Collection of oysters

Approximately 900 adult oysters (60-110 mm) were collected in May 1989 from Deep Water Shoal, a natural oyster reef located in the upper, low saline region of Virginia's James River (Figure 1). Immediately following collection the oysters were divided into two groups, placed in two trays (120 x 60 x 14 cm) and transplanted to the lower York River, where they were suspended from a pier at the Virginia Institute of Marine Science, Gloucester Point, Virginia (Figure 1). Disease monitoring by the Virginia Institute of Marine Science has verified that P. marinus is endemic at the transplantation site and that oysters maintained in this area during the summer months acquire the disease at relatively high prevalences (Burreson, 1989, 1990). The Deep Water Shoal native oysters collected in May were maintained at this location until mid-September 1989. During this time the oysters were naturally exposed to and infected by P. marinus. The mean daily salinity at the site during the exposure period ranged from 14-22 ppt and the mean daily water temperature ranged from 19-27 degrees celsius. Periodically, oysters were sampled and analyzed for disease prevalence so that the acquisition and progression of the disease could be monitored. In September the oysters were transferred to the laboratory and cleaned of fouling organisms. Three replicate samples (n=25) were analyzed for P. marinus intensity and prevalence. It was inferred from the samples that

Figure 1. Oyster collection and transplantation sites; (1) Deepwater Shoal, James River, (2) Virginia Institute of Marine Science, York River, (3) Ross' Rock, Rappahannock River.



80% of the oysters were infected. Infection intensities ranged from negative to heavy (Figure 2).

Uninfected control oysters were collected just prior to the initiation of the laboratory experiment from Ross' Rock located in the upper Rappahannock River, Virginia (Figure 1). Analysis of oysters sampled (n=25) from the group revealed no detectable P. marinus infections. Deep Water Shoal oysters could not be used as uninfected controls because P. marinus became established in the area during the summer months.

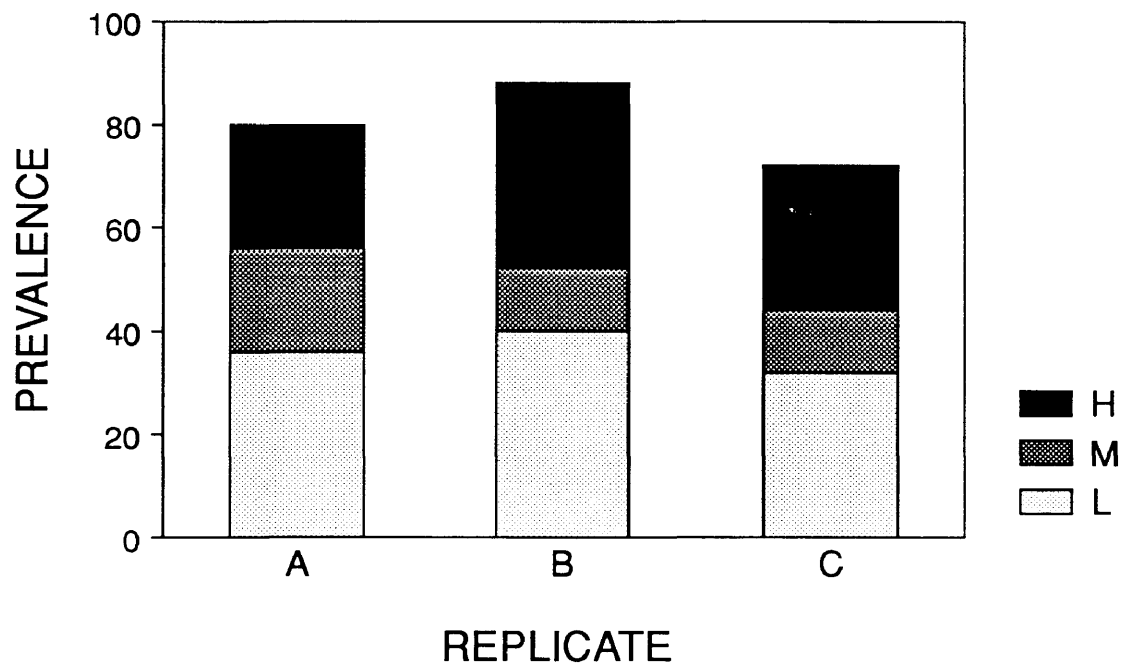
Experimental design

The laboratory portion of the experiment was conducted at the Virginia Institute of Marine Science Eastern Shore Laboratory in Wachapreague, Virginia. Oysters were randomly assigned to one of four salinity treatments; one high salinity treatment, 20 ppt, and three low salinity treatments, 12, 9, and 6 ppt. Five replicate, 50-liter, polypropylene tanks ("Tuf Box", Melmat Inc., Harbor City, CA.), each containing 30 oysters, were established at each salinity treatment. One tank containing 30 control oysters was also established at each salinity. The tanks were placed on a three tier wooden rack structure and all oyster groups were randomly assigned to a tank location.

All oysters were conditioned to salinity change so that no greater than a 5 ppt change in a 24 hour period was experienced. Water of the desired salinity was prepared daily by diluting filtered seawater (input from Finney Creek; ranging from 20-33 ppt) with fresh well water. The seawater was filtered through a series of filters: a 25 micron bag filter; two sand filters (TM40, Helder, Morristown, N.J.), the first containing sand and the second containing sand and activated carbon; and

Figure 2. Prevalence and intensity of P. marinus in oysters sampled at the initiation of the experiment. A, B, and C represent three replicate samples (n=25). Total height of the bars indicates prevalence (percent of sample infected). The patterned divisions within each bar represent the percent of individuals having light (L), moderate (M), and heavy (H) infections.

PREVALENCE AND INFECTION INTENSITY INITIAL SAMPLE



finally a one micron bag filter. Filtration removed natural food from the seawater, ensuring that food availability did not vary between treatments, and reduced the possibility of exposure to P. marinus and other parasites which may have been present in influent water.

Following filtration the water was mixed with well water in 44 gallon plastic containers, aerated and maintained at room temperature for 24 hours. The water was transferred to oyster tanks using a submersible water pump. The water in each aquarium was aerated and changed daily. Oysters were fed a commercial algal diet ("Diet A", Coast Oyster Co., WA.) once daily. An aliquot of the algal mix (2.5 ml) was diluted with 250 mls of filtered seawater and added to each aquarium. The food source was apparently adequate as evidenced by production of feces and pseudofeces by most individuals; and the overall condition of sampled oysters (ie. dark digestive glands, firm and opaquely colored tissue, and well developed gonads).

Salinity was closely monitored using a temperature compensated refractometer (Reichert Model 10419) and was maintained within 0.5 ppt of the desired value. Water temperature was controlled by an air conditioner or electric space heater and averaged $23.3^{\circ}\text{C} \pm 1.9$ ($\bar{x} \pm \text{sd}$).

The experiment was conducted for a period of eight weeks. Oyster mortality was recorded daily. All gapers (dead oysters) were removed from the aquaria within 24 hours of death and examined for parasite presence. Random samples of live oysters, 5 from each replicate were taken from each salinity group on day 14, 28, 42 and 56 (uninfected control oysters excluded). The oysters were shucked and oyster tissue was analyzed for parasite prevalence and intensity using both

thioglycollate and histological methods (as described below). Parasite levels in the uninfected control groups were only evaluated at the termination of the experiment, day 56.

To determine if infections which may have become subpatent or inhibited by low salinity exposure would reappear upon reexposure to high salinity a portion of low salinity exposed oysters were transferred to 20 ppt on day 28. Oysters (n=25) which had been exposed to 6, 9 and 12 ppt were transferred to three newly established 20 ppt aquariums. Mortality was followed daily for the remainder of the experiment and all remaining live oysters at the termination of the experiment were analysed for parasites.

Determination of parasite prevalence and intensity

Each oyster sampled and all gapers were examined for P. marinus prevalence and intensity using a modification of the fluid thioglycollate medium assay described by Ray (1952, 1966). Oysters were shucked and a small piece of rectum, gill, and mantle tissue was excised with a scapel and placed in a culture tube containing 10 ml of thioglycollate medium. Antibiotic, 0.5 ml of potassium penicillin G-streptomycin sulfate solution (2500 units of each/ml), was added to each culture tube to inhibit bacterial growth and the tubes were incubated at room temperature for 5 days. Following the incubation period the tissue was removed from the culture tubes, placed on microscope slides, macerated with a scapel and stained with Lugol's iodine solution. Coverslips were pressed onto the slides and the slides were examined at 40x and 100x using a light microscope. Perkinsus marinus cells appeared against the light orange stained oyster tissue as iodine stained (blue-black) spheres ranging in size from 10-200 um. Infection level was

categorized on a scale of 0-6 according to the method described by Ray (1954) and Quick (1971) which is based on the number of cells per unit volume of tissue. Rating of infection was as follows:

1. Negative - 0 cells in the entire preparation.
2. Very light infections - 1 to 10 cells in the entire preparation.
3. Light infections - 10 to 100 cells in the entire preparation.
4. Light-moderate infections - some areas free of parasites and other localized areas with 25-50 cells, 101-1000 cells in the entire preparation.
3. Moderate infections - some cells in every field at 100x with heavy concentrations in some areas and relatively few in others, 31-300 cells per field at 40x.
6. Moderate-heavy infections - large numbers of parasites with less than half of the tissue macroscopically appearing green-blue to blue-black, 301-1000 cells per field at 40x.
7. Heavy infections - large numbers of parasites with a major portion of the tissue macroscopically appearing dull green-blue to blue-black after staining with Lugol's solution, greater than 1000 cells per field at 40x.

Oyster death is usually caused by infections of moderate intensity or above (Quick and Mackin, 1971).

In order to detect the presence of other oyster pathogens and to assess tissue condition each oyster sampled and all gapers were also examined using standard paraffin histological techniques. After excising tissue for the thioglycollate assay a 4-5 mm cross section of oyster visceral mass was cut and preserved in Davidson's AFA (10% glycerin, 20% formalin, 30% 95% ETOH, 30% distilled water and 10%

glacial acetic acid). Preserved oyster tissue remained in Davidson's AFA for a minimum of 24 hours after which time the tissue was trimmed, placed in embedding capsules and transferred to 70% ethyl alcohol. The tissue was dehydrated using a sequence of chemical changes including ascending ethyl alcohols and S-29 Tissue Dry (Fisher Scientific, NJ). The tissue was then cleared with UC-670 Tissue Clear (Fisher Scientific, NJ) and infiltrated with Tissue Prep Paraffin (Fisher Scientific, NJ) using an automatic tissue processor (Autotechnicon, Technicon Corp., NJ). Paraffin blocks were sectioned at 6 μ m using a rotary microtome. Ribbons were floated in a warm water bath and affixed to slides coated with albumin and 2% formalin solution. The slides were dried at 45 degrees celsius for a minimum of 24 hours and stained with Harris' hematoxylin and eosin following routine methods (Luna, 1968).

The slides were examined at 400x using a light microscope and the presence of P. marinus and other oyster parasites (Haplosporidium nelsoni, Bucephalus cuculus and Nematopsis ostrearum) was recorded.

Assessment of oyster mortality

Percent mortality occurring during 14 day intervals was determined for each replicate tank. Percent mortality was calculated by dividing the number of oysters dying during an interval by the number of oysters which were alive at the beginning of the interval and multiplying the product by 100. Cumulative mortality was then determined by adding successive interval mortalities.

Statistical analysis

A total of 395 live oysters were sampled and analyzed for P. marinus prevalence and intensity. A hierarchical log-linear test (log-likelihood ratio test; G-test) was utilized to detect differences between salinity

treatments and through time in the distribution of oysters within P. marinus intensity categories (Sokal and Rohlf, 1981). The number of intensity categories was reduced from 7 to 4 so that the number of categories having low frequencies could be reduced thereby enhancing the power of the test. For this purpose moderate-heavy and heavy infections were combined and designated as heavy; moderate and light-moderate infections were combined and designated as moderate; light and very light infections were combined and designated as light; and the fourth category, negative, remained unchanged.

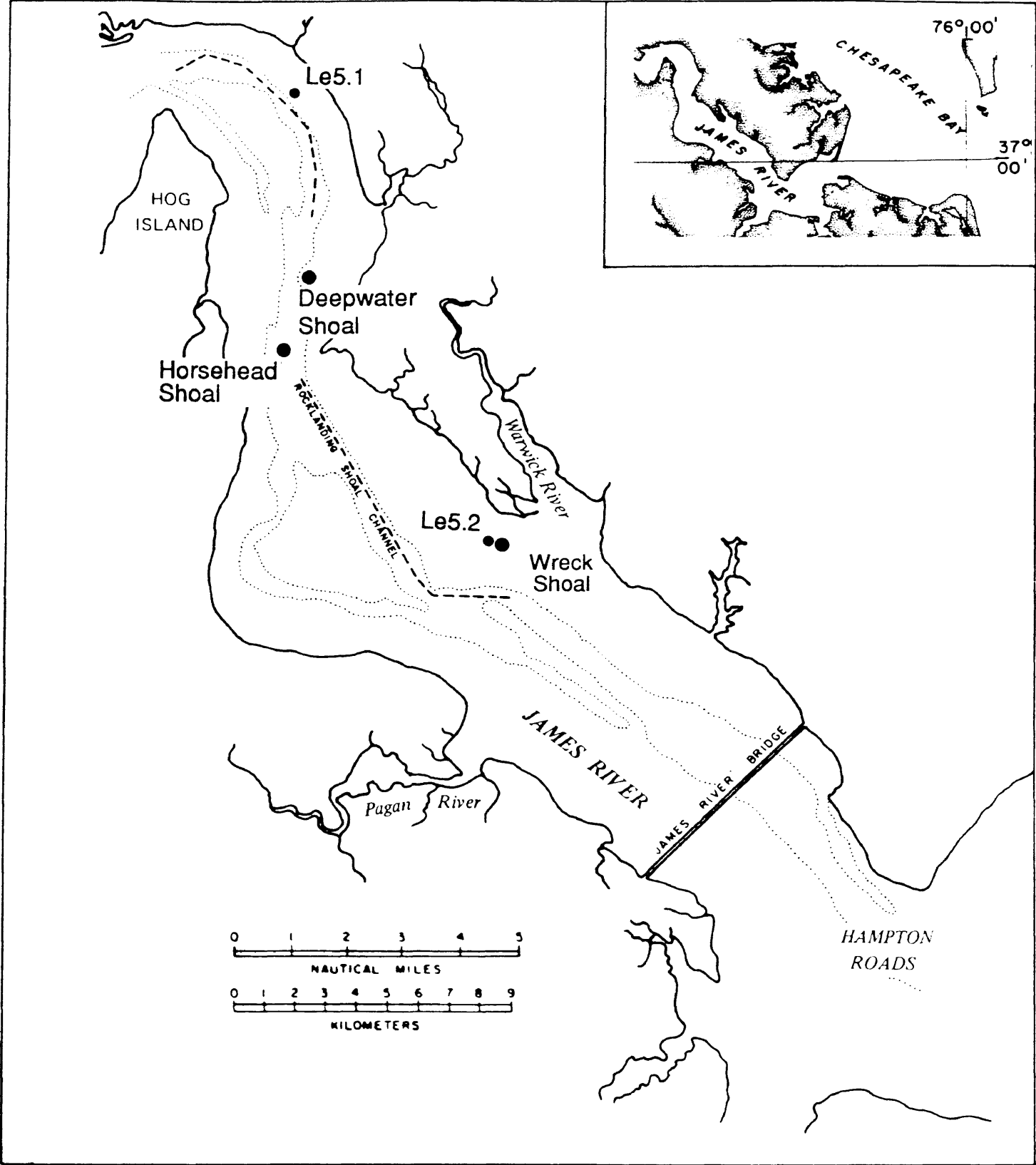
Differences in mean prevalence and mean cumulative percent mortality between salinity treatments and through time were determined using a two factor model I analysis of variance and when appropriate a Student-Newman-Keuls multiple comparison test (Zar, 1984). Prior to analysis the dependent variable was arcsine transformed and evaluated for compliance to the assumptions of the tests. Normality was examined using a normal plot and a Komogorov-Smirnov goodness of fit test and homoscedasticity was evaluated with a Cochrans C test (Sokal and Rohlf, 1981; Zar, 1984). Test results indicated that these assumptions were not violated. All tests were judged significant at an alpha value of 0.05. Computations were made on a Prime computer at the Virginia Institute of Marine Science using a SPSS-X statistical package.

Field observations

For discussion purposes information regarding P. marinus prevalence and intensity in the James River, Virginia is cited from Burreson (1990 and 1991). Corresponding James River salinity values were compiled from data collected by the Virginia Water Control Board. This salinity data was collected bimonthly, during slack water from stations LE5.1, which

is located near Deep Water Shoal, and LE5.2, which is located near Wreck Shoal (Figure 3).

Figure 3. Site locations of oyster disease monitoring grounds and salinity stations.



RESULTS

Perkinsus marinus prevalence and intensity

Perkinsus marinus prevalence and intensity was assessed, using thioglycollate assays, in oysters sampled from each of the four salinity treatments on days 14, 28, 42 and 56. Prevalence ranged from 76 to 100 percent and was generally lower in the 6 and 9 ppt treatments (Table 1; Figure 4). A two-way analysis of variance indicated that the effect of salinity on prevalence was marginally significant, while the effect of time and the interaction of time and salinity were insignificant (Table 2). Analysis of the data, collapsed across time, using a subsequent one-way analysis of variance followed by a Student-Newman-Keuls test showed that differences in prevalence were significant only between the 9 and 12 ppt groups (DF=3, F Ratio=3.344, P=0.0235). On any given day, prevalence did not significantly differ between treatment groups (Table 3). Prevalence of P. marinus in control oysters sampled at the termination of the experiment were 0% at 20 ppt, 4% at 12 ppt, 12% at 9 ppt and 0% at 6 ppt (Table 1).

A log-linear test of frequencies indicated that the distribution of the four intensity categories differed significantly (G test: DF=9, P=0.0338, $\chi^2=18.2$) between treatments. The 6 and 9 ppt groups had a higher total number of negative and light infections and a lower total number of moderate and heavy infections than the 12 ppt and 20 ppt groups (Table 1; Figure 5). The 12 ppt group had considerably fewer

Table 1 Prevalence (PREV) and intensity of P. marinus in oysters sampled after 14, 28, 42 and 56 days exposure to treatment salinities. Infection intensity categories are designated as negative (N), light (L), moderate (M), and heavy (H). Sample size shown as N is the total from five replicate samples (n=5).

	SALINITY TREATMENT	INFECTION INTENSITY				N	PREV
		N	L	M	H		
DAY 14	20 PPT	2	8	6	9	25	92
	12 PPT	2	11	2	10	25	92
	9 PPT	6	14	3	2	25	76
	6 PPT	5	9	5	6	25	80
DAY 28	20 PPT	2	11	3	9	25	92
	12 PPT	1	7	5	12	25	96
	9 PPT	2	10	4	9	25	92
	6 PPT	3	11	2	9	25	88
DAY 42	20 PPT	2	12	3	8	25	92
	12 PPT	0	13	4	8	25	100
	9 PPT	4	13	2	6	25	84
	6 PPT	1	16	3	5	25	96
DAY 56	20 PPT	2	10	6	7	25	92
	12 PPT	0	6	6	8	20	100
	9 PPT	4	8	6	7	25	84
	6 PPT	4	7	4	10	25	84
TOTAL	20 PPT	8	41	18	33	100	92
	12 PPT	3	37	17	38	95	97
	9 PPT	16	45	15	24	100	84
	6 PPT	13	43	14	30	100	87

Figure 4 Mean prevalence (± 1 standard deviation) of P. marinus in oysters sampled from 6, 9, 12 and 20 ppt treatment groups after 14, 28, 42 and 56 days of exposure to the respective salinities (mean is based on five replicate samples, n=5).

MEAN PREVALENCE

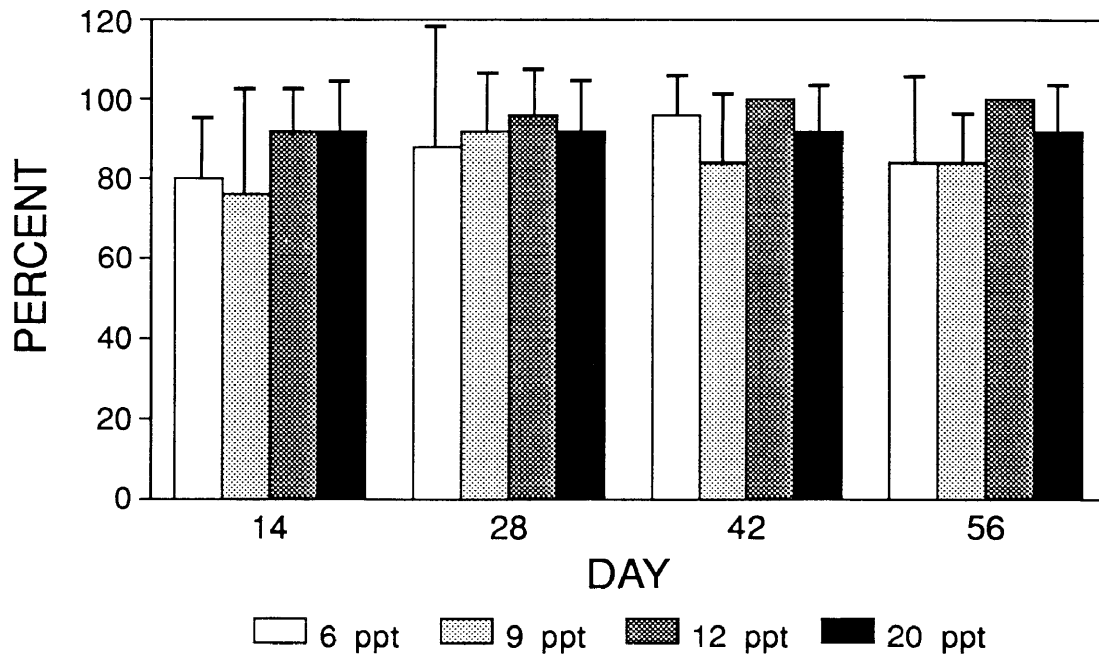


Table 2 Results of two factor analysis of variance for the effect of salinity and time on mean P. marinus prevalence.

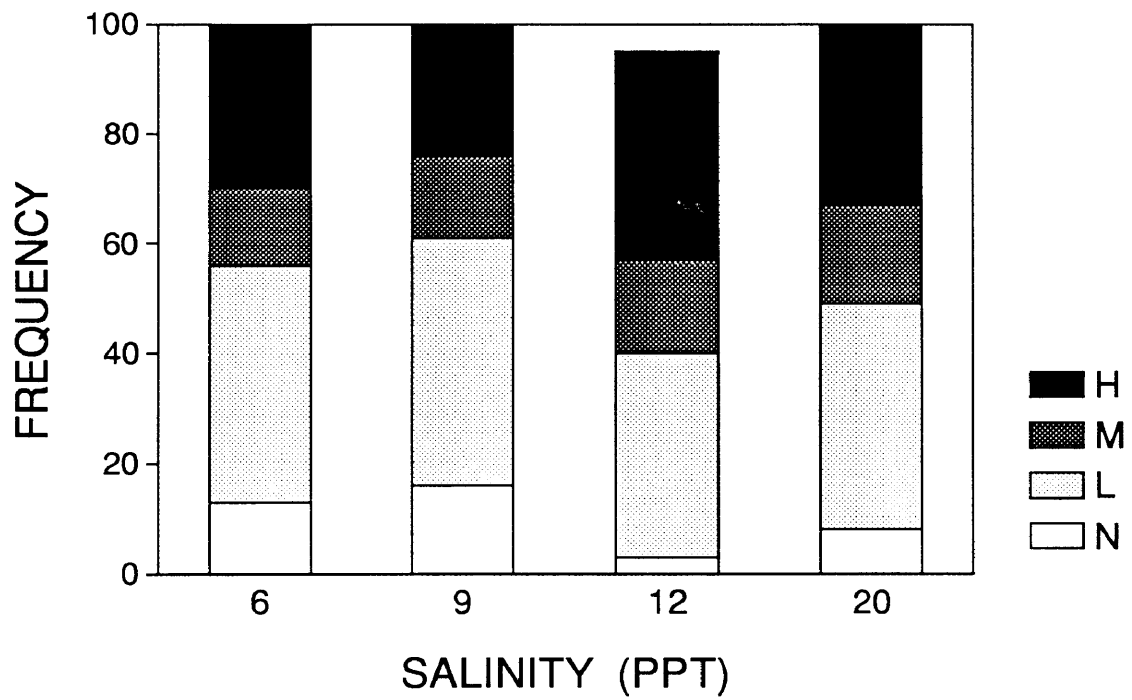
<u>Source of variation</u>	<u>DF</u>	<u>F Ratio</u>	<u>F Probability</u>
Salinity	3	3.15	0.031
Time	3	1.29	0.285
Salinity x Time	9	0.43	0.915

Table 3 Results of one-way analysis of variance of P. marinus prevalence by treatment salinity on each sample date.

<u>Day</u>	<u>DF</u>	<u>F Ratio</u>	<u>F Probability</u>
14	3	0.963	0.3910
28	3	0.124	0.9446
42	3	1.871	0.1752
56	3	2.006	0.1538

Figure 5 Relative P. marinus infection levels between 6, 9, 12 and 20 ppt oyster groups. Frequencies are based on the total number of live oysters sampled during the experimental period. Infection levels are negative (N), light (L), moderate (M), and heavy (H).

RELATIVE INFECTION INTENSITY
ALL SAMPLES POOLED



negatives than all other treatment groups and this alone may account for the significant difference indicated above. On day 14 there were relatively large differences between groups in the number of light and heavy infections. The pattern of fewer advanced infections and more numerous negative and light infections in oysters exposed to 6 and 9 ppt, indicates infection development was delayed relative to the higher salinity groups. The distribution of the four intensity categories did not significantly differ (G test: DF=9, P=0.0624, $\chi^2=16.2$) through time. A significant increase in the frequency of negative and light infections was not observed as the experiment progressed, hence, no evidence was obtained to indicate the occurrence of parasite expulsion. No significant interactive effect due to time and salinity was observed (G test: DF=27, P=0.7087, $\chi^2=22.6$).

Mortality

Despite the high prevalence of disease at all four salinity treatments a marked difference in disease-associated mortality between treatments was observed. Mean cumulative percent mortality increased in all four groups as the experiment progressed, but increased at a more rapid rate in the higher salinity treatments (Figure 6; Table 4). Cumulative oyster mortality during the first 14 days of the experiment was higher at 20 ppt than at 12, 9, and 6 ppt (7.3% vs 2.0%, 2.0%, and 0.7% respectively). After 28 days of exposure the 20 ppt exposed oysters continued to exhibit the highest rate of mortality, 18.4%, as compared to 10.3% at 12 ppt, 6.9% at 9 ppt, and 1.5% at 6 ppt. By day 42 the rate of oyster mortality at 20 and 12 ppt was virtually the same (22.5% vs. 21.2%); while oyster mortality at 9 and 6 ppt remained

Figure 6 Mean percent cumulative mortality of oysters exposed to 6, 9, 12 and 20 ppt following 14, 28, 42 and 56 days of treatment (mean is based on five replicate groups).

MEAN CUMULATIVE MORTALITY

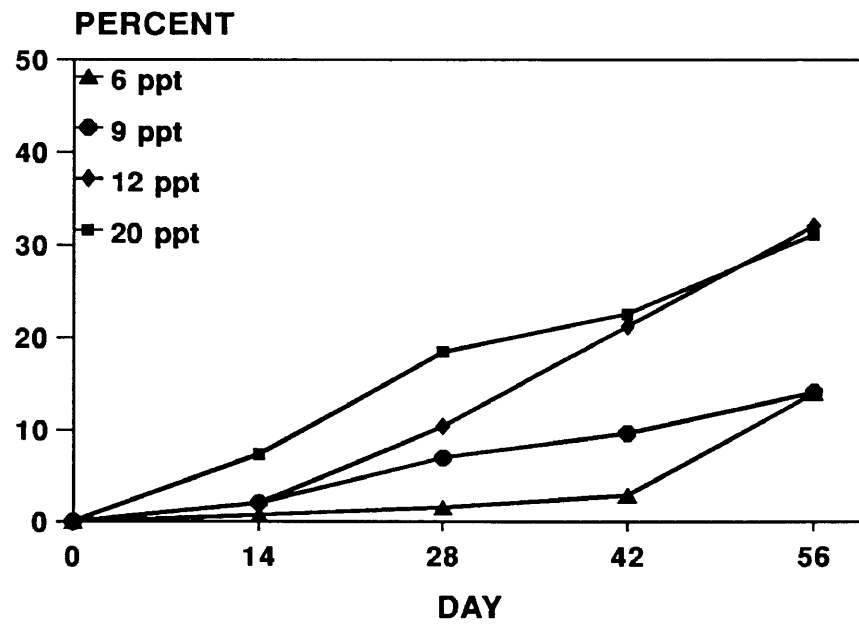


Table 4 Mean percent cumulative oyster mortality (\pm 1 standard deviation) at each treatment salinity following 14, 28, 42, and 56 days of exposure. Mean is based on five replicate groups.

<u>DAY</u>	<u>REP #</u>	<u>TREATMENT</u>			
		<u>20 PPT</u>	<u>12 PPT</u>	<u>9 PPT</u>	<u>6 PPT</u>
14	\bar{x} =	7.33	2.00	2.00	0.67
	sd =	5.48	4.47	2.98	1.49
28	\bar{x} =	18.41	10.33	6.94	1.47
	sd =	3.93	11.06	8.10	2.02
42	\bar{x} =	22.50	21.20	9.61	2.80
	sd =	8.99	20.80	5.72	2.85
56	\bar{x} =	31.14	32.06	14.05	13.91
	sd =	13.57	25.96	4.30	13.81

considerably reduced at 9.6% and 2.8%. During the final 14 days of the experiment oyster mortality at 6 ppt increased to 13.9% becoming nearly equivalent to mortality at 9 ppt, and mortality at 12 and 20 ppt increased to 32.1% and 31.1%, respectively. After 56 days of exposure oyster mortalities at 6 ppt and 9 ppt were reduced relative to the higher salinity groups, by an average of 64%. Mean cumulative mortality differed significantly between both sample dates and treatments (Table 5). The results of the SNK test indicate that significant differences were primarily between the 6 ppt and 20 ppt treatments (Table 6).

Disparities between relative oyster mortalities at each treatment for each time interval are emphasized in Figure 7 and Table 7. Mortality occurring during the first time interval, day 0 - day 14, was considerably lower in the 6, 9, and 12 ppt treatments than in the 20 ppt group. During the second interval the rate of mortality at 6 ppt was virtually equivalent to that observed during interval 1, and greatly reduced relative to the high salinity, 20 ppt, treatment. An increase in the rate of mortality was observed at both 9 ppt and 12 ppt; however, percent mortality was still less than that observed at 20 ppt. From day 29 through 42 mortality was highest at 12 ppt. The rate of mortality at 20 ppt declined during the third interval; however, it was still greater than mortality at 6 ppt and 9 ppt. While the rate of mortality at 9 ppt remained relatively stable from day 15-56 a sharp rise in mortality was observed at 6 ppt during the last interval. Slight increases in mortality were also observed at 12 and 20 ppt.

Oysters transferred to high salinity (20 ppt) following a 28 day exposure period at low salinity (6, 9, and 12 ppt) experienced a much higher rate of mortality than those remaining continuously at the

Table 5 Results of two factor analysis of variance for the effect of salinity and time on mean cumulative mortality.

<u>Source of variation</u>	<u>DF</u>	<u>F Ratio</u>	<u>F Probability</u>
Salinity	3	11.21	0.000
Time	3	14.91	0.000
Salinity x Time	9	0.62	0.780

Table 6 One-way analysis of variance and multiple comparisons (SNK) of mean cumulative percent mortality between treatment salinities. Lines connect treatment means that do not significantly differ ($P > 0.05$).

Day	\bar{x}	Salinity Treatment				F Ratio	F Prob.
		20 ppt	12 ppt	9 ppt	6 ppt		
14	\bar{x}	<u>7.33</u>	<u>2.00</u>	<u>2.00</u>	<u>0.67</u>	2.505	.0961
28	\bar{x}	<u>18.41</u>	<u>10.33</u>	<u>6.94</u>	<u>1.47</u>	4.004	.0265
42	\bar{x}	<u>22.50</u>	<u>21.20</u>	9.61	2.80	5.556	.0083
56	\bar{x}	<u>31.14</u>	<u>32.06</u>	<u>14.05</u>	13.91	3.560	.0381

\bar{x} = mean cumulative mortality

Figure 7 Mean percent interval mortality (+ 1 standard error) of oysters exposed to 6, 9, 12 and 20 ppt occurring during days 0-14, 15-28, 29-42 and 43-56.

MEAN INTERVAL MORTALITY

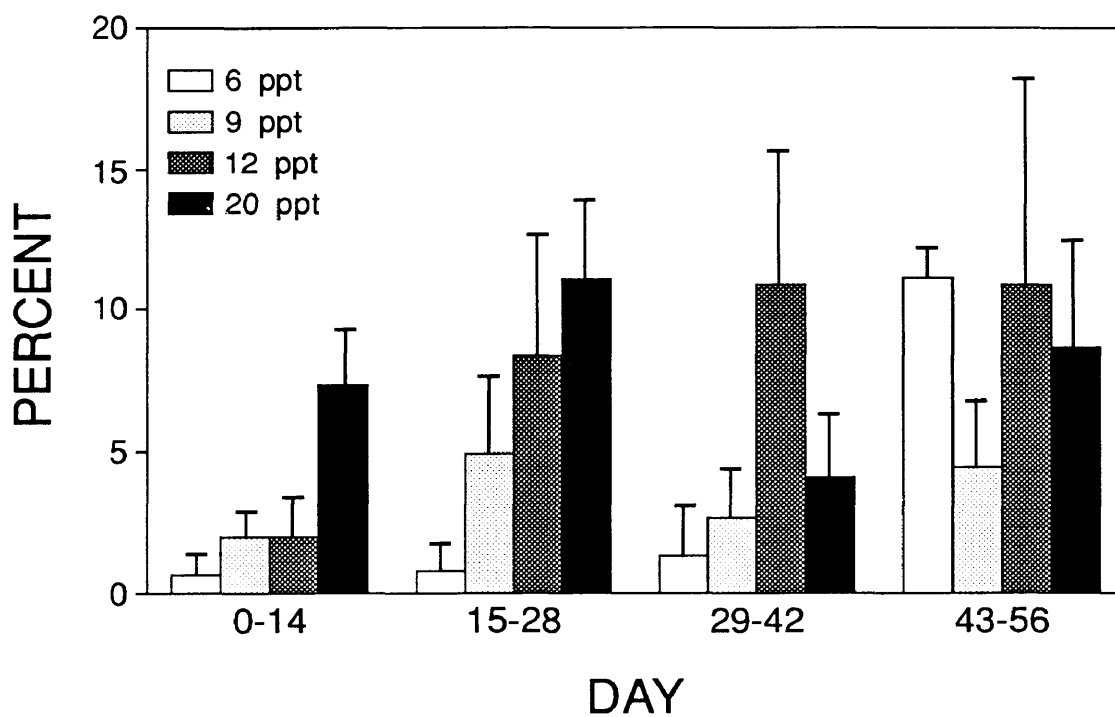


Table 7 Mean percent oyster mortality occurring at each treatment salinity during each sampling interval; day 0-14, day 15-28, day 29-42 and day 43-56 (sd = standard deviation).

<u>INTERVAL</u>	<u>REP #</u>	<u>TREATMENT</u>			
		<u>20 PPT</u>	<u>12 PPT</u>	<u>9 PPT</u>	<u>6 PPT</u>
0-14	\bar{x} =	7.33	2.00	2.00	0.67
	sd =	5.48	4.47	2.98	1.49
15-28	\bar{x} =	11.08	8.33	4.94	0.80
	sd =	6.87	8.71	7.24	1.79
29-42	\bar{x} =	4.10	10.87	2.67	1.33
	sd =	6.02	12.92	3.65	2.98
43-56	\bar{x} =	8.64	10.86	4.44	11.11
	sd =	8.22	17.43	6.09	11.11

original treatment salinity. The mortality occurred soon after the transfer and continued until the termination of the experiment (Figure 8). Given the small number of oysters transferred statistical comparisons between groups were not made.

Mortality of the uninfected control oysters was as follows: 4% at 20 ppt; 12% at 12 ppt; 4% at 9 ppt and 0% at 6 ppt. Perkinsus marinus was determined to be present in three gapers at very low intensities, the others were uninfected. Perkinsus marinus was also detected in four of the live control oysters, one from 12 ppt and three from 9 ppt, examined at the termination of the experiment. Although an initial sample indicated that the control oysters were free of P. marinus it is possible that the parasite was present at undetectable levels. Samples taken in October 1989 at the original collection site were diagnosed positive for P. marinus. Since P. marinus is also endemic in the Wachapreague area it is possible that infective particles were received in incoming water during the course of the experiment. It is believed that such low infection levels, if also experienced by the treatment groups, would not significantly confound the results presented above.

Histology

Histological analysis revealed the presence of Haplosporidium nelsoni, Bucephalus cuculus and Nematopsis ostrearum in a relatively small percent of the oysters (Table 8). Haplosporidium nelsoni prevalence, in any given sample, ranged from 0 to 12%. Of the total number of live oysters analyzed only 3% were diagnosed as being infected by H. nelsoni. Bucephalus cuculus prevalence ranged from 0 to 8% and less than 4% of the total number of oysters sampled were observed to

Figure 8 Percent mortality of oysters transferred to 20 ppt after 28 days exposure to 6, 9, and 12 ppt (6 to 20, 9 to 20 and 12 to 20) in comparison to those maintained at their original salinity treatment. Percent mortalities are calculated for days 29-56. Values shown for 6, 9, 12 and 20 ppt represent means of five replicates. Transferred groups were not replicated.

PERCENT MORTALITY DAY 29-56
OYSTERS TRANSFERRED TO HIGH SALINITY
AND NONTRANSFERRED OYSTERS

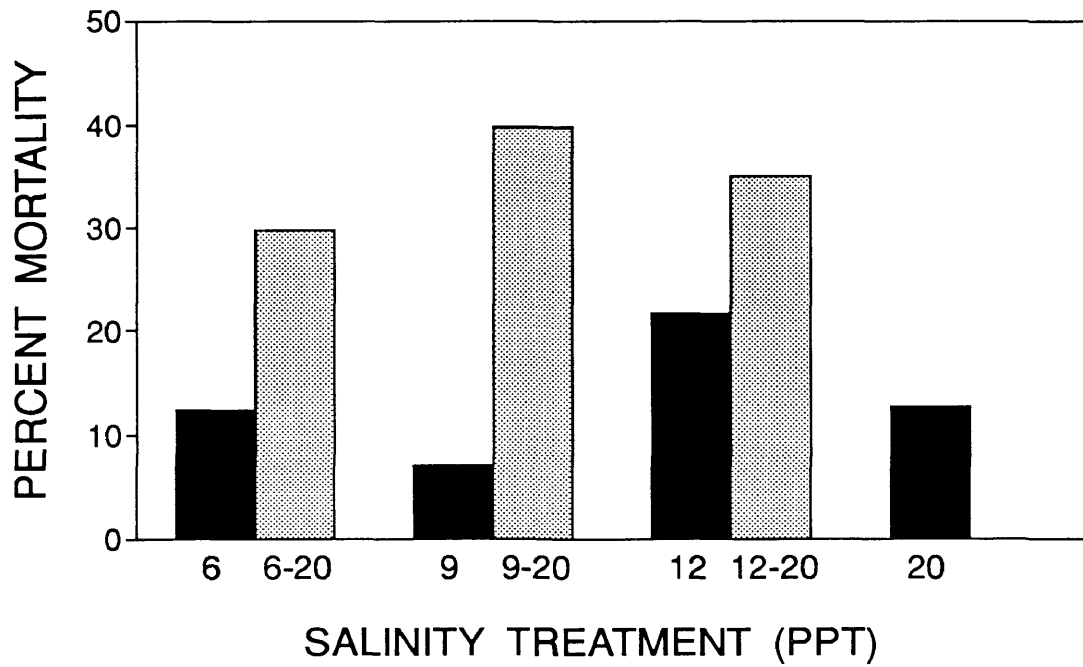


Table 8 Prevalence of H. nelsoni, B. cuculus, N. ostrearum and P. marinus in oysters sampled at the initiation of the experiment and on days 14, 28, 42 and 56. All diagnoses were made on histological preparations. Sample size is designated as n.

Day	Group	Prevalence				n
		<u>H. nelsoni</u>	<u>B. cuculus</u>	<u>N. ostrearum</u>	<u>P. marinus</u>	
0	A	16	24	20	24	25
	B	16	8	24	20	25
	C	4	8	20	8	25
14	20 ppt	8	8	28	44	25
	12 ppt	0	4	8	24	25
	9 ppt	0	8	20	16	25
	6 ppt	0	0	8	16	25
28	20 ppt	12	8	12	32	25
	12 ppt	8	0	16	48	25
	9 ppt	4	8	0	16	25
	6 ppt	0	0	8	8	25
42	20 ppt	0	0	16	48	25
	12 ppt	8	8	4	40	25
	9 ppt	0	0	8	56	25
	6 ppt	0	4	16	44	25
56	20 ppt	8	4	0	56	25
	12 ppt	0	8	4	44	20
	9 ppt	0	4	4	48	25
	6 ppt	0	0	4	48	25

have B. cuculus infections. Nematopsis ostrearum prevalence ranged from 0 to 28% and had an overall prevalence of 20%.

Most P. marinus infections diagnosed by thioglycollate as being advanced were also detected by histological analysis. No differences in the nature of infection or the morphology of the parasite associated with salinity exposure were observed.

A total of 73 gapers from the treatment groups were collected and analyzed histologically. In agreement with the thioglycollate results all were determined to be positive for P. marinus. Three of the gapers were also diagnosed positive for H. nelsoni. Diagnosis by thioglycollate indicated that 75% of the gapers had heavy infections, 8% had moderate-heavy infections, 7% had moderate infections, 6% had light infections and 4% had very light infections.

DISCUSSION

Previous investigations have shown that low salinity suppresses oyster mortality caused by P. marinus (Andrews and Hewatt, 1957; Ray, 1954; Scott et al., 1985). Andrews and Hewatt (1957) followed oysters transplanted in the field to an area having a salinity ranging from 1-13 ppt. Investigations by Ray (1954) and Scott et al. (1985) were conducted in the laboratory. Ray (1954) compared mortality of oysters exposed to 26-28 ppt to those exposed to 10-13.5 ppt; and Scott et al. (1985) contrasted mortalities of oysters exposed to 21-25 ppt with those exposed to 8-10 ppt. All three studies indicated that disease-induced oyster mortality was delayed and reduced by low salinity conditions. This investigation substantiates their results and further extends our understanding of this relationship by suggesting that 9-12 ppt is a critical range for the activity of P. marinus; while mortality was reduced at 9 ppt the parasite exhibited full pathogenicity at 12 ppt. At the termination of the experiment oyster survival at 6 and 9 ppt was more than 60% higher than at 12 and 20 ppt. Additionally, oyster mortality was delayed at 6, 9 and 12 ppt, relative to 20 ppt. Oysters exposed to 20 ppt began dying soon after the initiation of the experiment and continued to die through the duration of the experiment as infection development progressed. The pattern at 12 ppt was similar although initial mortality was slightly delayed relative to the 20 ppt group. Mortality of oysters exposed to 6 and 9 ppt primarily occurred during the final two weeks of the experiment, presumably as a result of

advanced infections which were present at the start of the experiment. Reduction in mortality would likely have occurred following the death of oysters which had advanced infections at the beginning of the experiment.

Enhanced survival was not a permanent attribute of low salinity exposed oysters. When transferred to high salinity conditions the oysters died at a relatively high rate. The sharp rise in mortality may reflect increased multiplication of the parasite in response to more favorable conditions for the pathogen. It is also possible that the change in salinity may have created additional stress thereby increasing mortality of oysters which had already been weakened by disease.

Although exposure of infected oysters to low salinity reduced oyster mortality, a concomitant decrease in P. marinus prevalence was not observed. Perkinsus marinus once established in the eastern oyster can tolerate salinities as low as 6 ppt for a period of at least 56 days. During the course of the experiment prevalence of the pathogen did not decrease significantly at any of the four salinity treatments; hence no evidence was obtained to suggest that low salinity exposure causes parasite expulsion.

This result strongly contrasts with reports documenting the effect which salinity exerts on H. nelsoni. It has been well documented that H. nelsoni is restricted to salinities above 10 ppt and is readily eliminated by the host at salinities below 10 ppt (Ford, 1985 and Andrews, 1983). Ford (1985) showed that at temperatures above 20 degrees celsius patent MSX infections disappeared after only 2 weeks exposure to a mean salinity of 10 ppt or less. Contrary to expectation, P. marinus appears to be much more tolerant than H. nelsoni of low

salinity exposure. This investigation has demonstrated that at temperatures above 20 degrees celsius salinity exerts a minimal influence on parasite prevalence once oysters are infected.

Infection intensities were also indicative of a lack of parasite expulsion. Had low salinity induced expulsion, a coincident decline in parasite intensity would have been observed in oysters sampled during the course of the study. A striking decrease of parasite intensity was not observed at any salinity treatment. However, a comparison of infection levels of live oysters sampled during the course of the experiment revealed that low salinity exposure delayed development of the parasite to pathogenic levels. Advanced infections were more numerous in oysters maintained at 12 and 20 ppt than in oysters maintained at 6 and 9 ppt. In addition, the progression from light to advanced infections occurred at a slower rate in the low salinity exposed oysters. On day 14 oysters sampled from 6 and 9 ppt exhibited infection intensities similar to the initial levels while infections at 12 and 20 ppt had already begun to progress and cause mortality. The proportion of oysters having advanced infections at the two lower salinities, however, increased as the experiment progressed, finally reaching at the termination of the experiment a frequency which appeared to be equivalent to that at 20 ppt. It is important to note that as the study progressed the number of oysters sampled from the higher salinity groups having advanced infections is obscured by the high mortality experienced by these groups. Many oysters from the 12 and 20 ppt groups perished early in the study; hence, these were not included in subsequent samples and there was a bias toward sampling more resistant

oysters or those which had negative or light infections at the initiation of the experiment.

Previous investigations have also shown that low salinity restricts disease progression. Ray (1954) showed that the development of infections and subsequent mortality of oysters held at 10-13.5 ppt was delayed relative to oysters at 26-28 ppt and Andrews and Hewatt (1957) demonstrated that infection development was retarded in oysters transplanted in the field to a low salinity area (1-13 ppt). By expanding the range of salinity conditions evaluated, and reducing external variables this investigation further elucidated our understanding of the relationship between salinity and P. marinus infection progression.

Additionally, this study has provided information that is essential for making appropriate management decisions. As a consequence of several drought years P. marinus has become established on all public oyster grounds in Virginia (Burreson, 1990), including normally low salinity areas which had previously been parasite "free". It is evident from this study that a return to normal low salinities, even as low as 6 ppt, will not eradicate the parasite from these areas in a relatively short time frame (< 56 days). Nor will a temporary translocation of infected oysters to low salinity followed by a return to high salinity abate the pathogen. However, the results also indicate that severe oyster mortalities are unlikely to result in areas which maintain a salinity below 9 ppt. Oyster growers may be able to avoid disease-induced mortality by maintaining oysters in low salinity areas (< 9 ppt), while oysters maintained at 12 ppt are likely to experience mortality comparable to oysters maintained at 20 ppt. High mortalities

experienced by oysters which were transferred to high salinity following a period of low salinity exposure, suggest that increases in salinity which occur in areas normally having low salinities will undoubtedly allow infections to rapidly develop and subsequently cause mortality. To reduce or avoid oyster mortality it is essential that disease levels and salinities of oyster grounds be closely monitored, and be taken into consideration when making decisions regarding fishing and management strategies.

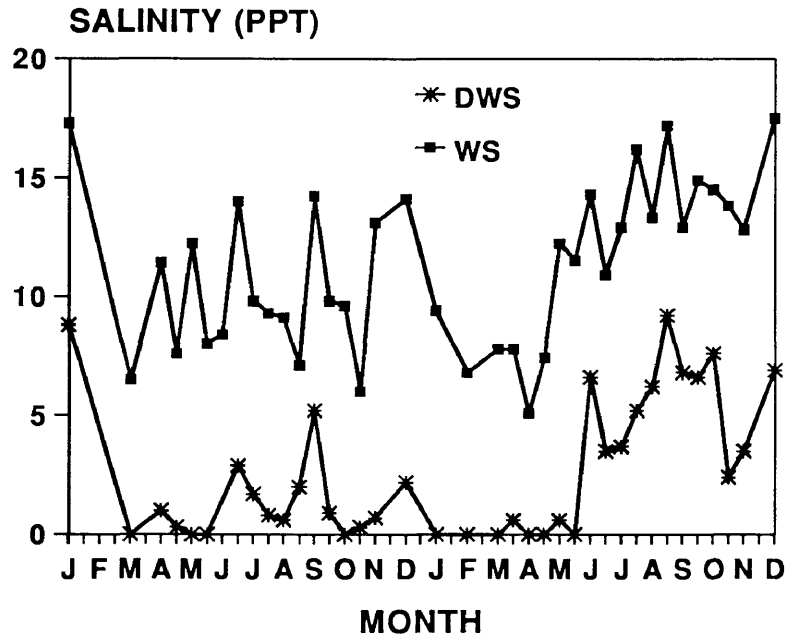
In general the results of this study are consistent with recent observations of the occurrence and activity of P. marinus in Virginian waters. Despite the occurrence of low salinities (0.0-9.2 ppt) in 1989 and 1990 at both Deep Water Shoal and Horse Head Shoal P. marinus continued to be prevalent during the summer and fall months (Figure 9; Figure 3). However, parasite intensities at both of these upper river locations were much lower than at Wreck Shoal (6.0-17.2 ppt). Perkinsus marinus intensities were particularly low at Deep Water Shoal- during the two year period only 2 heavy infections were recorded. This low intensity may indicate that P. marinus infection is inhibited by low salinity, as suggested by this investigation. Since few advanced infections were detected in Deep Water Shoal oysters it is likely that oyster mortality was minimal.

It is generally agreed that development of P. marinus is delayed by low salinity exposure and as a consequence disease associated oyster mortalities are reduced; however, the mode of action which produces this effect is somewhat controversial. Mackin (1961) and Andrews and Hewatt (1957) proposed that disease mortality of oysters inhabiting low salinity waters is reduced because infective agents which are waterborne

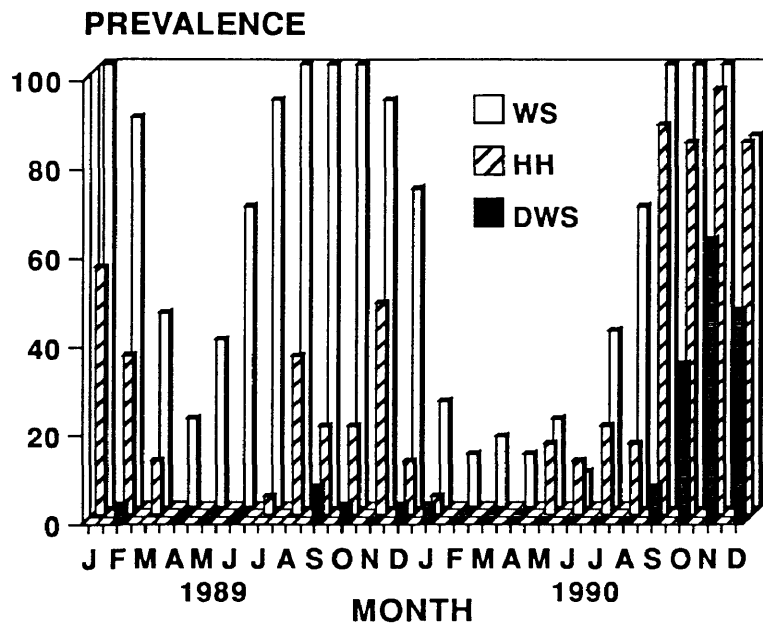
Figure 9 A. Salinities (ppt) of bimonthly water samples collected from stations in the proximity of Deep Water Shoal (DWS) and Wreck Shoal (WS) of the James River, Virginia during 1989 and 1990.

B. P. marinus prevalence in oysters sampled from James River monitoring stations (Deep Water Shoal = DWS, Horse Head Shoal = HH, and Wreck Shoal = WS). Bars represent prevalence in samples (n=25) collected once monthly during 1989 and 1990.

A. SALINITY AT JAMES RIVER STATIONS



B. PREVALENCE AT JAMES RIVER STATIONS



are diluted by increased flow rates characteristic of many low salinity areas. Alternatively, Scott et al. (1985) suggested that differences in survival between oysters maintained at high and low salinities are not related to differences in infective cell density in sea water, but rather reflect physiological differences in oysters exposed to different salinities. Consistent with the later hypothesis this investigation has indicated that differences in survival between oysters exposed to high and low salinity may not be related to the density of infective agents in sea water. As in the experiment presented by Scott et al. (1985) the oysters used in this study were infected at the initiation of the experiment and the experiment was conducted in a closed system; hence, dilution of infective cells was not a factor and could not have been responsible for the reduced mortality observed under low salinity conditions. While dilution of infective particles by inflowing water and flushing of oyster beds may have some influence on disease prevalence in the natural environment, interpretation of the results of this study suggest that a physiological response of host and/ or parasite may also be operative.

The effect of salinity on physiological aspects of P. marinus and C. virginica has been the focus of only a few studies. Perkins (1966) and Chu and Greene (1989) have shown that salinities from 5-10 ppt inhibit zoosporulation of P. marinus indicating that salinity may have a direct effect on the parasite. Other studies have indicated that the oysters defense mechanism may be altered by environmental conditions such as temperature and salinity. Fisher and Newell (1986) and Fisher et al. (1987) have shown that hemocyte activities such as locomotion rate, ability to spread, and adherence capacity are reduced by elevations in

salinity in C. virginica and Ostrea edulis under both acute and acclimated conditions. In addition the presence of oyster lysosomal enzymes, which are believed to function in defense of invading microorganisms, has also been correlated with salinity. Analysing oysters sampled from this experiment La Payre et al. (1990) found higher lysozyme concentrations in low salinity exposed oysters than in oysters exposed to higher salinities. While the roles of hemocytes and lysosomal enzymes in the oysters defense of P. marinus has not been clarified, it seems apparent that salinity may influence their activity. Future investigations are required to elucidate the modulation of host and parasite physiologies by salinity.

Whether a physiological response of host, parasite or a combination of both, the results of this investigation indicate that salinity influences P. marinus pathogenicity. This experiment was conducted at temperatures exceeding 20°C because it has been well documented that this range in temperature is most favorable to the parasite; therefore inhibition of parasite activity by temperature would not confound the results. It is possible that at warm temperatures P. marinus metabolism is optimized and any influence which salinity might exert is minimized. Dissimilar results, particularly in respect to expulsion, may have occurred if this experiment were conducted at a lower temperature. Feng and Stauber (1968) discussed the delicate balance associated with host-parasite relationships. The authors suggested that activities of both hosts and parasites are enhanced by optimal temperatures. Host defense activities as well as parasite multiplication, metabolite production and nutritional requirements can be altered by thermal depressions or

elevations. A delicate balance between host and parasite exist and such alterations can effect the outcome of the interaction (Feng and Stauber, 1968). Perhaps for P. marinus temperature is the primary environmental influence, however it seems plausible that as the balance shifts other extrinsic environmental factors such as salinity are likely to become more important. It is possible that the combined effect of low salinity and low temperatures could be more important than either factor alone.

In conclusion, this investigation has demonstrated that P. marinus can tolerate salinities as low as 6 ppt, for a period of at least 56 days; however, infection development appeared to be delayed and oyster mortality was reduced by exposure to 6 and 9 ppt. Mean cumulative mortality of oysters maintained at 6 and 9 ppt for 56 days was 64% less than oysters maintained at 12 and 20 ppt. Mortality of oysters exposed to 12 ppt was similar to the mortality of 20 ppt exposed oysters. Contrary to the original hypothesis, expulsion of P. marinus was not detected at any salinity treatment. This suggests that unlike H. nelsoni, P. marinus is not strongly affected by low salinity. These results contribute to our understanding of the influence of salinity on P. marinus and can aide in predicting the response of the parasite to fluctuating environmental conditions. Future investigations focusing on the synergistic influence of temperature and salinity on host and parasite physiologies are required to further elucidate the influence of environmental conditions on interactions between C. virginica and P. marinus.

LITERATURE CITED

- Andrews, J.D. 1964. Oyster mortality studies in Virginia IV. MSX in James River public seed beds. Proceedings of the National Shellfisheries Association 53:65-84.
- Andrews, J.D. 1983. Minchinia nelsoni (MSX) infections in James River seed-Oyster areas and their expulsion in spring. Estuarine, Coastal and Shelf Science 16:255-209.
- Andrews, J.D. 1988. Epizootiology of of the disease caused by the oyster pathogen Perkinsus marinus and its effects on the oyster industry. American Fisheries Society Special Publication 18:47-63.
- Andrews, J.D. and W.G. Hewatt. 1957. Oyster mortality studies in Virginia. II. The fungus disease caused by Dermocystidium marinum in oysters of the Chesapeake Bay. Ecological Monographs 27(1):1-25.
- Andrews, J.D. and S.M. Ray. 1988. Management strategies to control the disease caused by Perkinsus marinus. American Fisheries Society Special Publication 18:257-264.
- Bayne, B.L., J.M. Gee, J.T. Davey and C. Scullaid. 1978. Physiological responses of Mytilus edulis to parasitic infestation by Mytilicola intestinalis. Journal de Conseil International Pour L'exploration de la Mer 38:12-17.
- Burreson, E.M. 1989. Prevalence of the major oyster diseases of Virginia waters - 1988. A summary of the annual monitoring program. Virginia Institute of Marine Science, Gloucester Point, Virginia.
- Burreson, E.M. 1990. Status of the major oyster diseases in Virginia - 1989. A summary of the annual monitoring program. Marine Resource

- Report 90-1 Virginia Institute of Marine Science, Gloucester Point, Virginia.
- Burreson, E.M. 1991. Status of the major oyster diseases in Virginia - 1990. A summary of the annual monitoring program. Marine Resource Report 91-1 Virginia Institute of Marine Science, Gloucester Point, Virginia.
- Chu, F.E. and K.H. Greene. 1989. Effect of temperature and salinity on in vitro culture of the oyster pathogen, Perkinsus marinus (Apicomplexa: Perkinsea). Journal of Invertebrate Pathology 53:260-268.
- Craig, A., E.N. Powell, R.R. Fay and J.M. Brooks. 1989. Distribution of Perkinsus marinus in Gulf Coast oyster populations. Estuaries 12(2):82-91.
- Crosby, M.P. and C.F. Roberts. 1990. Seasonal infection intensity cycle of the parasite Perkinsus marinus (and an absence of Haplosporidium spp.) in oysters from a South Carolina salt marsh. Diseases of Aquatic Organisms 9:149-155.
- Farley, C.A. 1975. Epizootic aspects of Minchinia nelsoni (Haplosporidia) disease in Maryland oysters. Journal of Protozoology 22:418-427.
- Feng, S.Y. and L.A. Stauber. 1968. Experimental hexamitiasis in the oyster Crassostrea virginica. Journal of Invertebrate Pathology 10:94-110.
- Fisher, W.S., N. Auffret and G. Balouet. 1987. Response of European flat oyster (Ostrea edulis) hemocytes to acute salinity and temperature changes. Aquaculture 67:179-190.

- Fisher, W.S. and R.E.I. Newell. 1986. Salinity effects on the activity of granular hemocytes of American oysters, Crassostrea virginica. Biological Bulletin 170:122-134.
- Ford, S.E. 1985. Effects of salinity on survival of the MSX parasite Haplosporidium nelsoni (Haskin, Stauber, and Mackin) in oysters. Journal of Shellfish Research 5(2):85-90.
- Ford, S.E. and H.H. Haskin. 1982. History and epizootiology of Haplosporidium nelsoni (MSX), an oyster pathogen, in Delaware Bay, 1957-1980. Journal of Invertebrate Pathology 40:118-141.
- Ford, S.E. and H.H. Haskin. 1988. Management strategies for MSX (Haplosporidium nelsoni) disease in eastern oysters. American Fisheries Society Special Publications 18:249-256.
- Galtsoff, P.S. 1964. The American oyster, Crassostrea virginica Gmelin. U.S. Fish and Wildlife Service Fisheries Bulletin 64. 480 pp.
- Gauthier, J.D., T.M. Soniat and J.S. Rogers. 1990. A parasitological survey of oysters along salinity gradients in coastal Louisiana. Journal of the World Aquacultural Society 21(2):105-115.
- Hepper, B.T. 1955. Environmental factors governing the infection of mussels, Mytilus edulis by Myticola intestinalis. Fisheries Investigations Ministry of Agriculture, Fisheries and Food (Great Britain) Ser. II 20:1-21.
- Hopkins, S.H. 1956. Notes on the boring sponges in Gulf coast estuaries and their relation to salinity. Bulletin of Marine Science of the Gulf and Caribbean 6(1):44-56.
- La Peyre, J.F., F.E. Chu and L.M. Ragone. 1990. The effect of salinity and Perkinsus marinus infection on some immunological parameters of

- the oyster Crassostrea virginica. National Shellfisheries Association Annual Meeting April 1-5, 1990. Williamsburg, Virginia.
- Lauckner, G. 1983. Diseases of Mollusca: Bivalvia. In Diseases of Marine Animals, Volume 2, ed. O. Kinne, 477-962. Hamburg, West Germany: Biologische Anstalt Helgoland.
- Levine, N.D. 1988. The Protozoan Phylum Apicomplexa. Boca Raton: CRC Press, Inc.
- Luna, L.G. 1968. Manual of Histological Staining Methods of the Armed Forces Institute of Pathology, 3rd ed. New York: McGraw-Hill. 258 pp.
- Mackin, J.G. 1951. Histopathology of infection of Crassostrea virginica (Gmelin) by Dermocystidium marinum Mackin, Owen and Collier. Bulletin of Marine Science of the Gulf and Caribbean 1:72-87.
- Mackin, J.G. 1956. Dermocystidium marinum and other microorganisms in Louisiana. Proceedings National Shellfisheries Association 46:116-133.
- Mackin, J.G. 1961. Oyster disease caused by Dermocystidium marinum and other microorganisms in Louisiana. Texas Institute of Marine Science Publication 7:132-229.
- Mackin, J.G., H.M. Owen and A. Collier. 1950. Preliminary note on the occurrence of a new protistan parasite, Dermocystidium marinum n. sp., in Crassostrea virginica (Gmelin). Science 111(2883):328-329.
- Perkins, F.O. 1966. Life history studies of Dermocystidium marinum, an oyster pathogen. Dissertation, Florida State University. 273 pp.

- Perkins, F.O. 1976. Zoospores of the oyster pathogen, Dermocystidium. I. Fine structure of the conocoid and other sporozoan-like organelles. *Journal of Parasitology* 62:959-974.
- Perkins, F.O. 1988. Structure of protistan parasites found in bivalve molluscs. *American Fisheries Society Special Publication* 18:93-111.
- Perkins, F.O. 1991. Infectious diseases of molluscs. In *Pathobiology of Marine and Estuarine Organisms*, eds. J. Couch, J. Fournier, and R. Menzer, New York, Van Nostrand Reinhold, in press.
- Quick, J.A. Jr. 1971. Pathological parasitological effects of elevated temperatures on the oyster Crassostrea virginica with an emphasis on the pathogen Labyrinthomyxa marina. Florida Department of Natural Resources Marine Research Laboratory, St Petersburg, Florida Professional Papers Series 15:105-190.
- Quick, J.A. and J.G. Mackin. 1971. Oyster parasitism by Labyrinthomyxa marina in Florida. Florida Department Natural Resources Marine Laboratory, Professional paper series 13, 55pp.
- Ray, S.M. 1952. A culture technique for the diagnosis of infections with Dermocystidium marinum (Mackin, Owen, and Collier) in oysters. *Science* 116(3014):360-361.
- Ray, S.M. 1954. Biological studies of Dermocystidium marinum, a fungus parasite of oysters. Rice Institute Pamphlet, 114 pp. (Monograph in Biology Special Series Issue).
- Ray, S.M. 1966. A review of the culture methods for detecting Dermocystidium marinum with suggested modifications and precautions. *Proceedings National Shellfisheries Association* 54:55-59.

- Ray, S.M., J.G. Mackin and J.L. Boswell. 1953. Quantitative measurement of the effects of oysters on disease caused by Dermocystidium marinum. Bulletin of Marine Science of the Gulf and Caribbean 3:6-33.
- Rohde, K. 1982. Ecology of Parasites. New York: University of Queensland Press. 245 pp.
- Scott, G.I., D.P. Middaugh, and T.I. Sammons. 1985. Interactions of chlorine-produced oxidants (CPO) and salinity in affecting lethal and sub-lethal effects in the eastern or American oyster, Crassostrea virginica (Gmelin), infected with the protistan parasite, Perkinsus marinus. In Marine Pollution and Physiology: Recent Advances, eds. F.J. Vernberg, F.P. Thurberg, A. Calabrese, and W.B. Vernberg, 351-376. University of South Carolina Press.
- Sokal, R.R., and F.J. Rohlf. 1981. Biometry. 2nd ed., San Francisco: W.H. Freeman and Co.. 859 pp.
- Soniat, T.M. 1985. Changes in levels of infection of oysters infected by Perkinsus marinus, with special reference to the interaction of temperature and salinity upon parasitism. Northwest Gulf Science 7(2):171-174.
- Thorson, R.E. 1969. Environmental stimuli and response of parasitic helminths. Bioscience 19(2):126-130.
- Wells, H.W. 1961. The fauna of oyster beds, with special reference to the salinity factor. Ecological Monographs 31:239-266.
- Zar, J.H. 1984. Biostatistical Analysis. 2nd ed. Prentice Hall Inc., Englewood, New Jersey. 718 pp.

VITA

Lisa M. Ragone

Born in Vineland, New Jersey, 23 October 1961. Graduated from Delsea Regional High School, Franklinville, New Jersey, in 1980. Earned a B.A. in Biology and Geography from the University of Delaware, Newark, Delaware, in 1984. Employed as a laboratory technician at the Rutgers University Shellfish Research Laboratory, Port Norris, New Jersey, October 1984 through August 1988. Entered the Masters program of the College of William and Mary, School of Marine Science in 1988. Employed at the college as a graduate research assistant from September 1988 through December 1990. Employed as a marine scientist at the Virginia Institute of Marine Science from January 1991 through present.