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EFFECT OF SALINITY ON INFECTION PROGRESSION AND PATHOGENICITY OF *PERKINSUS MARINUS* IN THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA* (GMELIN)

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ABSTRACT The effect of salinity on *Perkinsus marinus*, a protozoan pathogen of the eastern oyster, *Crassostrea virginica* (Gmelin 1791) was investigated. Oysters parasitized by *P. marinus* were exposed in the laboratory to 6, 9, 12, and 20 ppt at a temperature ranging from 20–25°C, for an eight week period. Infection prevalence and intensity were assessed in samples (n = 25) from each treatment following 2, 4, 6, and 8 weeks of exposure and oyster mortality was determined daily. The pathogen persisted, at high prevalences, throughout the course of the experiment at all treatment salinities; however, *P. marinus* infection development was retarded at 12 ppt and did not progress at 6 and 9 ppt. Cumulative oyster mortalities progressively increased with increasing salinity and at the termination of the experiment were 9.1, 11.6, 21.1, and 27.8 percent at 6, 9, 12, and 20 ppt, respectively. 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 below 9 ppt.

KEY WORDS: *Perkinsus*, oyster, salinity

INTRODUCTION

During the past three decades commercial oyster landings in Virginia have declined from an average of 3.5 million bushels per year prior to 1960, to a record low 0.1 million bushels in 1990–1991 (Virginia Marine Resource Commission landings data). This decline has been attributed to over fishing, declining water quality and disease (Hargis and Haven 1988). Factors that lead to disease epizootics in marine organisms are extremely complex and include biotic and abiotic parameters (Thorson 1969, Rohde 1982). For osmoconformers, such as the eastern oyster, *Crassostrea virginica*, salinity plays a major role in modulating its association with disease organisms (Hepper 1955, Bayne et al. 1978, Gauthier et al. 1990). Generally, oyster parasites have a narrower salinity tolerance than their host and are more common in high salinity areas (Hopkins 1956, Wells 1961, Andrews 1964, Farley 1975, Ford and Haskin 1982, Andrews 1983, Gauthier et al. 1990). Low salinity exposure (<10–15 ppt) often reduces the occurrence and the virulence of disease organisms. In the last decade salinity increases in Virginia's upper estuaries, resulting from four consecutive drought years (1985–1988), have caused an intensification of *Perkinsus marinus* (commonly known as Dermo), one of the Chesapeake Bay's most problematic oyster pathogens (Burreson and Andrews 1988, Burreson 1989). In response to increasing salinities in upper bay waters the parasite has spread to previously disease free seed areas and has had a severe impact on the oyster resource and industry. A more thorough understanding of the influence of salinity on the relationship between *P. marinus* and the eastern oyster will help elucidate the annual variability in the distribution and pathogenicity of this parasite and allow resource managers and oyster growers to forecast and perhaps avoid disease epizootics.

The influence of salinity on the activity of *P. marinus* has been the focus of numerous studies. Several investigators have documented a positive correlation between salinity and *P. marinus* infection intensity through field surveys (Mackin 1951, Mackin 1956, Andrews and Hewatt 1957, Soniat 1985, Craig et al. 1989, Gauthier et al. 1990, Crosby and Roberts 1990, Powell et al. 1992). Oysters grown in high salinity areas (15–30 ppt) experienced higher disease prevalence than those grown at lower salinities (<15 ppt). It has been 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 infective elements by freshwater inflow into the estuary (Mackin 1956, Ray 1954, Andrews and Hewatt 1957); however, disease development was retarded and oyster mortality was suppressed in infected oysters that were transplanted to a low salinity site (1–13 ppt) in the James River, Virginia (Andrews and Hewatt 1957) suggesting that salinity may have some physiological effect on the parasite.

Few laboratory studies investigating the effect of salinity on *P. marinus* have been conducted. Ray (1954) investigated the comparative development time of *P. marinus* in artificially infected oysters maintained at high (26–28 ppt) and low (10–13.5 ppt) salinity in closed aquaria. The parasite tolerated the low salinity treatment; however, development of infection and subsequent mortalities of oysters were delayed relative to the high salinity group. Similarly, Scott et al. (1985) found lower mortality in oysters held at 8–10 ppt than in oysters held at 21–25 ppt. Inhibition of *P. marinus* zoosporulation by low salinity (5–10 ppt) has been documented in vitro studies conducted by Perkins (1966) and Chu and Greene (1989).

Studies to date have greatly enhanced our understanding of the influence of salinity on the relationship between *C. virginica* and *P. marinus;* however, further investigations under controlled conditions are needed to substantiate and elaborate current knowledge. The experiment reported here investigated the effect of low salinity exposure (6, 9, and 12 ppt) on established infections of *P. marinus*.

MATERIALS AND METHODS

Approximately 900 oysters (60–110 mm) were collected 5 May 1989 from Deep Water Shoal, the uppermost natural oyster reef in the James River, Virginia. Immediately following collection the oysters were placed in two trays and suspended from a pier at the Virginia Institute of Marine Science in the lower York River, Virginia. The oysters remained at this location until mid Septem-

ber 1989, during which time they acquired *P. marinus* infections. 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°C. On 14 September 1989 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.

Oysters serving as uninfected controls were collected 8 September 1989 from Ross' Rock located in the upper Rappahannock River, Virginia. At the time of collection a sample of 25 oysters was examined for *P. marinus* prevalence and intensity.

The laboratory portion of the experiment was conducted at the Virginia Institute of Marine Science Eastern Shore Laboratory, Wachapreague, Virginia. Oysters were randomly assigned to one of four salinity treatments; a high salinity (20 ppt) control treatment and three low salinity treatments, 12, 9, and 6 ppt. Five replicate, 50 liter, polypropylene tanks, each containing 30 oysters were established at each salinity treatment. One tank containing 30 uninfected control oysters was also established at each salinity treatment.

All oysters were conditioned to salinity treatments so that no greater than a 5 ppt change in salinity was experienced in a 24 hour period. Water of the desired treatment salinity was prepared daily by diluting filtered sea water (pumped from Finnery Creek) with fresh well water in 44 gallon plastic containers. The sea water was filtered through a series of filters including a 25 micron bag filter, two sand filters containing sand and activated carbon, and a 1 micron bag filter. Filtration removed seston, ensuring that food availability did not vary between treatments, and reduced the possibility of exposure to *P. marinus* and other oyster parasites which may have been present in influent water. Aquaria water was aerated and changed daily. Mean daily water temperature was 23.3°C (±1.9 s.d.).

Oysters were fed a commercial algal diet (Diet A, Coast Oyster Co., WA) daily. An aliquot of the algal mix (2.5 ml) was diluted with 250 mls of filtered sea water and added to each aquaria. The food source was adequate on the basis of feces and pseudofeces production by most individuals and by the overall condition of uninfected oysters and oysters with low level infections (i.e. firm and opaquely colored tissue and well developed gonads).

The experiment was conducted for a period of eight weeks. Oyster mortality was recorded daily. All gaping oysters were removed from aquaria and examined for disease organisms. Random samples of live oysters, five from each replicate tank, were taken from each treatment group on day 14, 28, 42, and 56. The oysters were shucked and *P. marinus* prevalence and intensity were determined using thioglycollate culture of rectal, gill, and mantle tissue; infection intensities were rated as negative, light, moderate, and heavy (Ray 1952). Diagnosis of other oyster parasites [*Haplosporidium nelsoni* (Haskin, Stauber, and Mackin), *Bucephalus cuculus* McGrady and *Nematopsis ostrearum* Prytherch] was by routine paraffin histology of tissue fixed in Davidson's AFA. Parasite intensity and prevalence in the control groups were evaluated only at the termination of the experiment.

On day 28 of the experiment, 25 oysters from each low salinity (6, 9, and 12 ppt) treatment group were transferred to 20 ppt in order to determine if infections that may have become subpatent would reappear upon exposure to high salinity. Mortality of the transferred oysters was followed daily for the remainder of the experiment and at the termination of the experiment all live oysters were analyzed for *P. marinus* and other parasites. Control oysters were not treated similarly; hence, we did not have an appropriate control to assess the solitary effect of the salinity change on the survival of oysters transferred from the low to high salinity conditions.

Cumulative mortality was determined for each treatment replicate on day 14, 28, 42, and 56. In order to adjust for samples removed from each replicate, mortality was calculated as follows. Interval mortality, mortality occurring between sample dates (day 1–14, 15–28, 29–42, 43–56), was determined for each replicate group by dividing the number of oysters dying during an interval by the number of oysters that were alive at the beginning of the interval. Interval mortality was then multiplied by the proportion of survivors of the previous interval (1-cumulative mortality of preceding interval) to yield the adjusted interval mortality. Successive cumulative mortalities were then determined by summing adjusted interval mortalities and preceding cumulative mortalities.

Differences in mean cumulative mortality and mean prevalence between treatment groups and through time were determined by a two factor analysis of variance (ANOVA). Differences in mean cumulative mortality and mean prevalence between treatment groups on each sample date and on data collapsed across time were determined by a one factor ANOVA. When significant differences were found a Student-Newman-Keuls (SNK) test for multiple comparison among means was performed (Zar 1984). Prior to analysis the dependent variable was arcsine transformed and evaluated for compliance to the test assumptions. Normality was examined using a Komogorov-Smirnov goodness of fit test and homoscedasticity was evaluated with a Cochrans C test (Sokal and Rohlf 1981, Zar 1984).

A hierarchical log-linear test (log-likelihood ratio test) was utilized to detect differences between salinity treatments and through time in the distribution of oysters within the four *P. marinus* intensity categories (Sokal and Rohlf 1981). All tests were judged significant at an alpha level of 0.05. Computations were made on a Prime computer using a SPSSX statistical package.

RESULTS

The mean prevalence of *P. marinus* in oysters sampled at the initiation of the experiment was 80% (±8% s.d.) and infection intensity did not vary greatly between replicates (Figs. 1 and 2). Prevalence of *P. marinus* in oysters sampled from treatment groups on day 14, 28, 42, and 56 ranged from 76% to 100% (Fig. 1). A two factor analysis of variance indicated that the effect of salinity on prevalence was significant (P = 0.031), while the effect of time and the interaction of salinity and time were not significant (P = 0.285 and P = 0.915 respectively). Prevalence, however, did not significantly differ among treatment groups on any sample date (day 14 P = 0.3910, day 28 P = 0.9446, day 42 P = 0.1752, day 56 P = 0.1538). A significant difference in mean prevalence between treatment groups was observed when data was collapsed across time (P = 0.0235). A SNK test revealed a significant difference only between 9 ppt and 12 ppt treatments. *Perkinsus marinus* was not detected in control oysters sampled at the initiation of the experiment; however, the parasite was present at low prevalences in live control oysters sampled at the termination of the experiment (0% at 20 ppt, 4% at 12 ppt, 12% at 9 ppt, and 0% at 6 ppt).

The effect of salinity on infection intensity was significant (P = 0.0338). Oysters maintained at 6 and 9 ppt had a higher total number of negative and light infections and a lower total number

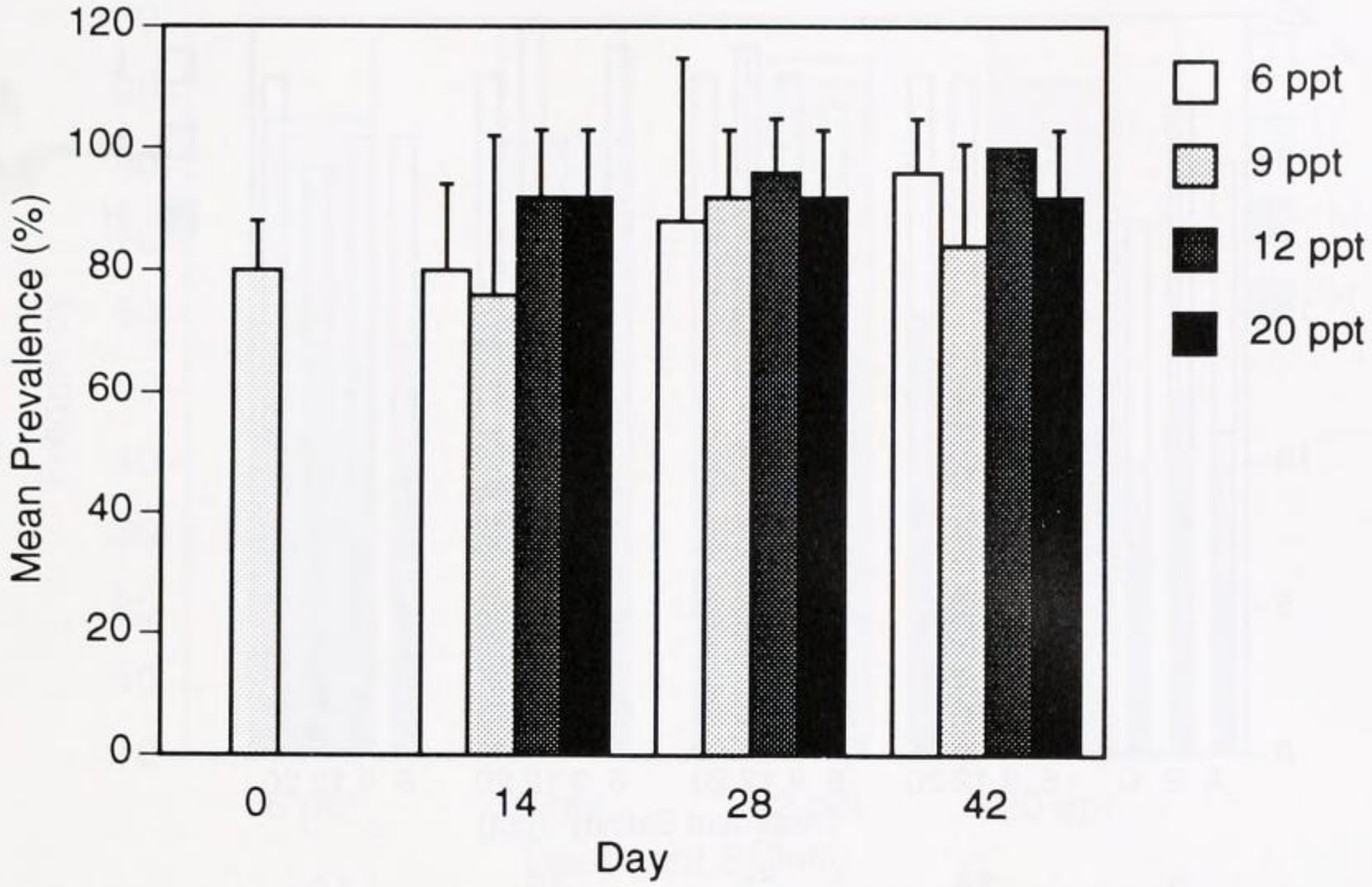

Figure 1. Mean prevalence (±1 standard deviation) of *P. marinus* in oysters sampled from each treatment group at the initiation of the experiment (day 0) and after 14, 28, 42, and 56 days of exposure to treatment salinities. Day 0 mean prevalence is based on three samples of 25 oysters, all other means are based on five replicate samples of 5 oysters.

of moderate and heavy infections than oysters held at 12 ppt and 20 ppt (Fig. 3). On day 14 there were relatively large differences between treatments in the number of light and heavy infections (Fig. 2). Differences in infection intensity between treatment groups were not as great as the experiment progressed and the number of oysters within each infection category (negative, light, moderate, and heavy) did not significantly differ through time ($P = 0.0624$). The interactive effect due to salinity and time was not significant ($P = 0.7087$).

Despite the high prevalence of *P. marinus* at all four salinity treatments a marked difference in mortality was observed. Mean cumulative mortality progressively increased with increasing salinity (Fig. 4). Mean cumulative mortalities at 6, 9, 12, and 20 ppt were respectively: 0.7%, 2.0%, 2.0%, and 7.3% on day 14; 1.5%, 6.8%, 10.1%, and 17.7% on day 28; 2.5%, 8.8%, 16.4%, 21.1% on day 42; and 9.1%, 11.6%, 21.1%, 27.8% on day 56. The effects of salinity and time on cumulative mortality were highly significant ($P < 0.0001$) while the interactive effect of salinity and time was not significant ($P = 0.8907$). Treatment means significantly differed on days 14 and 28 ($P < 0.0281$ and $P < 0.0037$, respectively) but did not significantly differ on days 14 and 56 ($P < 0.0956$ and $P < 0.0607$) (Fig. 4).

Oysters transferred to high salinity, 20 ppt, following a 28 day low salinity treatment experienced a much higher mortality rate than those remaining continuously at the original treatment salinity. The mortality began soon after the transfer and continued until the termination of the experiment (Fig. 5).

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. Three of the five dead control oysters were infected by *P. marinus*. All three infections were light.

Histological analysis revealed the presence of *H. nelsoni*, *B. cuculus*, and *N. ostrearum* in 3%, 4%, and 20%, respectively, of the total number of live oysters sampled. In general, as the experiment progressed the prevalence of all three parasites declined (Table 1). Prevalence of *H. nelsoni* at 6 and 9 ppt declined from an initial mean prevalence of 12% to 0% within the first 14 days of the investigation and remained below 4% for the remainder of the experiment. *Haplosporidium nelsoni* was present in only 3 of 73 gaping oysters that were examined histologically. In agreement with thioglycollate cultures, *P. marinus* was present in 100% of the dead oysters examined histologically. Ninety percent of the dead oysters had moderate to heavy *P. marinus* infections.

DISCUSSION

Previous investigations have indicated that low salinity suppresses oyster mortality caused by *P. marinus* (Andrews and Hewatt 1957, Ray 1954, Scott et al. 1985). This investigation substantiates their results and further extends our understanding of this relationship by defining 9–12 ppt as a critical range for *P. marinus* activity. Oyster mortality at 6 and 9 ppt was reduced by more than 50% compared to oysters maintained at 12 and 20 ppt. At the end of the experiment mean cumulative mortality of oysters at 6 ppt was 67% lower than at 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. An abundance of advanced infections in the dead oysters at 20 ppt indicates that infections were progressing during the course of the study. The pattern at 12 ppt was similar although the onset of mortality was slightly delayed relative to the 20 ppt group. Mortality of oysters at 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.

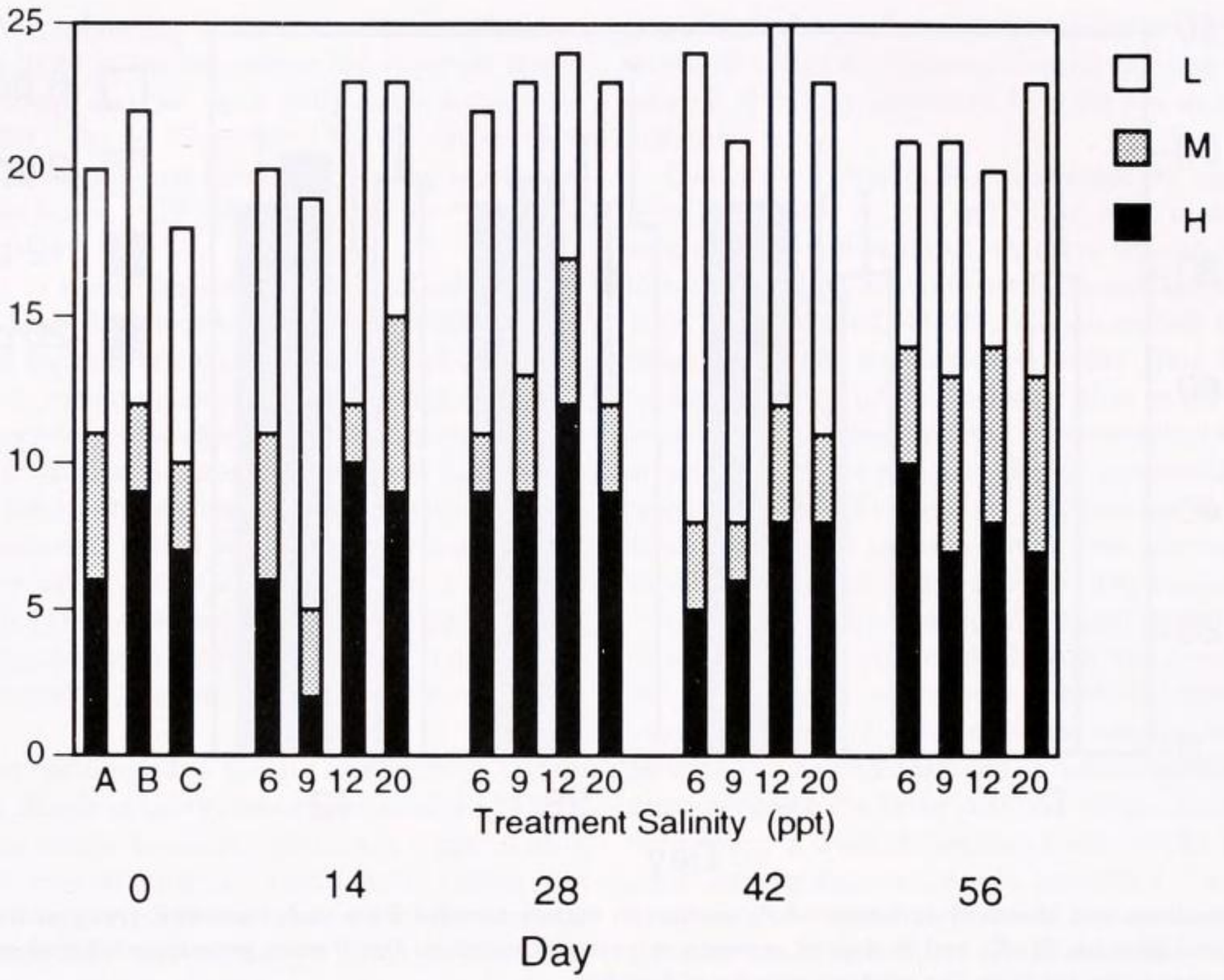

Figure 2. ***Perkinsus marinus*** **infection intensity (H = heavy, M = moderate, and L = light) in oysters sampled at the initiation of the experiment (day 0) and after 14, 28, 42, and 56 days exposure to treatment salinities (6, 9, 12, and 20 ppt). Day 0 replicates are designated as A, B, and C. Sample size for the 12 ppt treatment group on day 56 was 20, all other samples consisted of 25 oysters.**

Enhanced survival was not a permanent attribute of oysters exposed to low salinity. When transferred to high salinity, the oysters died at a relatively high rate compared to those continuously held at their original salinity. The sharp increase in mortality most likely reflects increased multiplication of the parasite in response to more favorable conditions. 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. Unlike *H. nelsoni*, which is readily eliminated from the oyster after a two week exposure to salinities less than 10 ppt (Ford 1985), *P. marinus*, once established in the eastern oyster, can tolerate salinities as low as 6 ppt for a period of at least 56 days at temperatures exceeding 20°C.

Infection intensities were also indicative of a lack of *P. marinus* expulsion. Had low salinity induced expulsion, a coincident decline in parasite intensity would have been observed in sampled oysters. A striking decrease in parasite intensity was not observed at any treatment; however, low salinity did prevent, or at least delay development of infections to pathogenic levels. Infections at 6 and 9 ppt did not significantly change during the experiment while infections at 12 and 20 ppt progressed and caused mortality within the first few weeks. Advanced infections were more numerous in oysters maintained at 12 and 20 ppt than in oysters held at 6 and 9 ppt. Statistical analysis suggest that infection intensity did not significantly change through time at any treatment. However, it is important to note that as the experiment progressed the number of oysters sampled from the high salinity groups having advanced infections is obscured by the high mortality of oysters having advanced infections. Many oysters from the 12 and 20 ppt groups perished early in the experiment, as a result of moderate to heavy infections, and were not included in subsequent samples. Hence, the actual number of advanced infections at 12 and 20 ppt is not reflected in the statistical analysis. Development of *P. marinus* in the Ross' Rock "uninfected" control oysters may be attributed to infections which were present but undetectable at the initiation of the experiment. *Perkinsus marinus* has been detected in subsequent samples of native oysters from Ross' Rock. However, it is also possible that the infections resulted from infective cells received in incoming water during the experiment. Since the number of "new" infections is relatively small, we do not believe their presence confounds the results of this investigation.

Differences in survival between *P. marinus* infected oysters maintained at high and low salinity have been attributed to differences in infective cell densities associated with estuarine circulation dynamics (Mackin 1961, Andrews and Hewatt 1957) and to physiological differences in oysters exposed to different salinities (Scott et al. 1985). Since infective cell density was not variable in this investigation, it is presumed that the salinity effect observed reflects a physiological response of host and/or parasite. The effect of salinity on physiological aspects of *P. marinus* and *C. virginica* has been the focus of few studies. Perkins (1966) and Chu and Greene (1989) have shown that salinities from 5–10 ppt exert a direct effect on the parasite by inhibiting zoosporulation. Other studies have indicated that the oyster's defense mechanisms may be altered by environmental conditions such as temperature and salinity. The salinities examined here are well within the range of tolerance of *C. virginica* (Castagna and Chanley 1973) but may

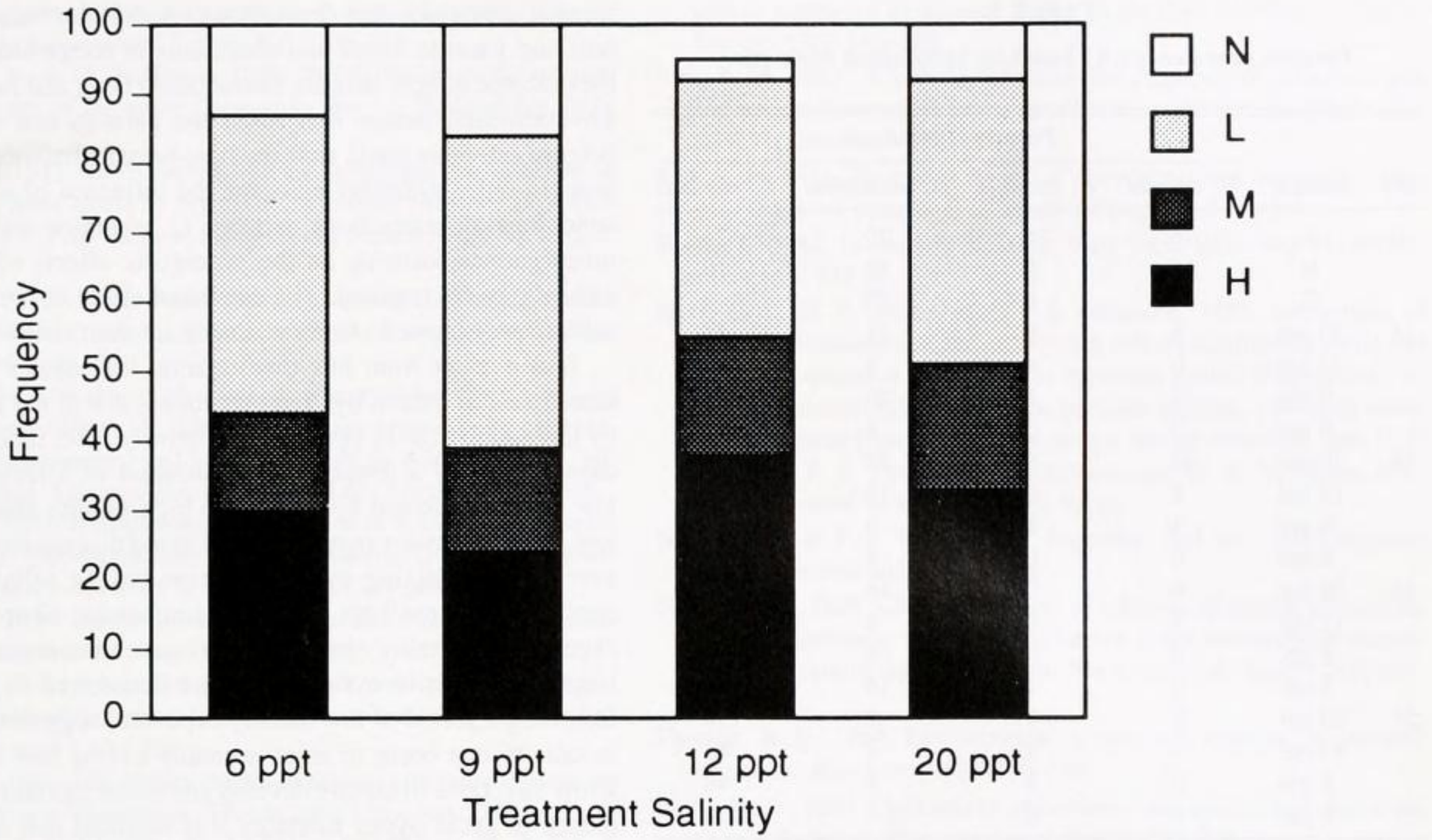

Figure 3. Infection intensity of *P. marinus* in oysters exposed to 6, 9, 12, and 20 ppt. Frequencies are based on the total number of live oysters sampled during the experimental period (day 14, 28, 42, and 56 samples are pooled). Infection levels are designated as negative (N), light (L), moderate (M), and heavy (H).

significantly effect hemocyte activities. 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. While the role of hemocytes in the oyster's defense of *P. marinus* has not been clarified, it seems apparent that salinity influences their activity.

Whether a physiological response of host, parasite, or a combination of both, the results of this investigation indicate that salinity has a significant effect on *P. marinus* pathogenicity at temperatures exceeding 20°C. While this discussion has focused on salinity effects the importance of temperature should not be neglected. Temperature is believed to be the most important environmental factor regulating the geographic distribution and seasonal activity of *P. marinus* in the Chesapeake Bay (Andrews and Hewatt 1957, Ray 1954). This experiment was conducted at temperatures within the range most favorable to multiplication of the parasite (Andrews and Hewatt 1957, Ray 1954). Dissimilar results may be observed at other temperatures. Kinne (1964) states that

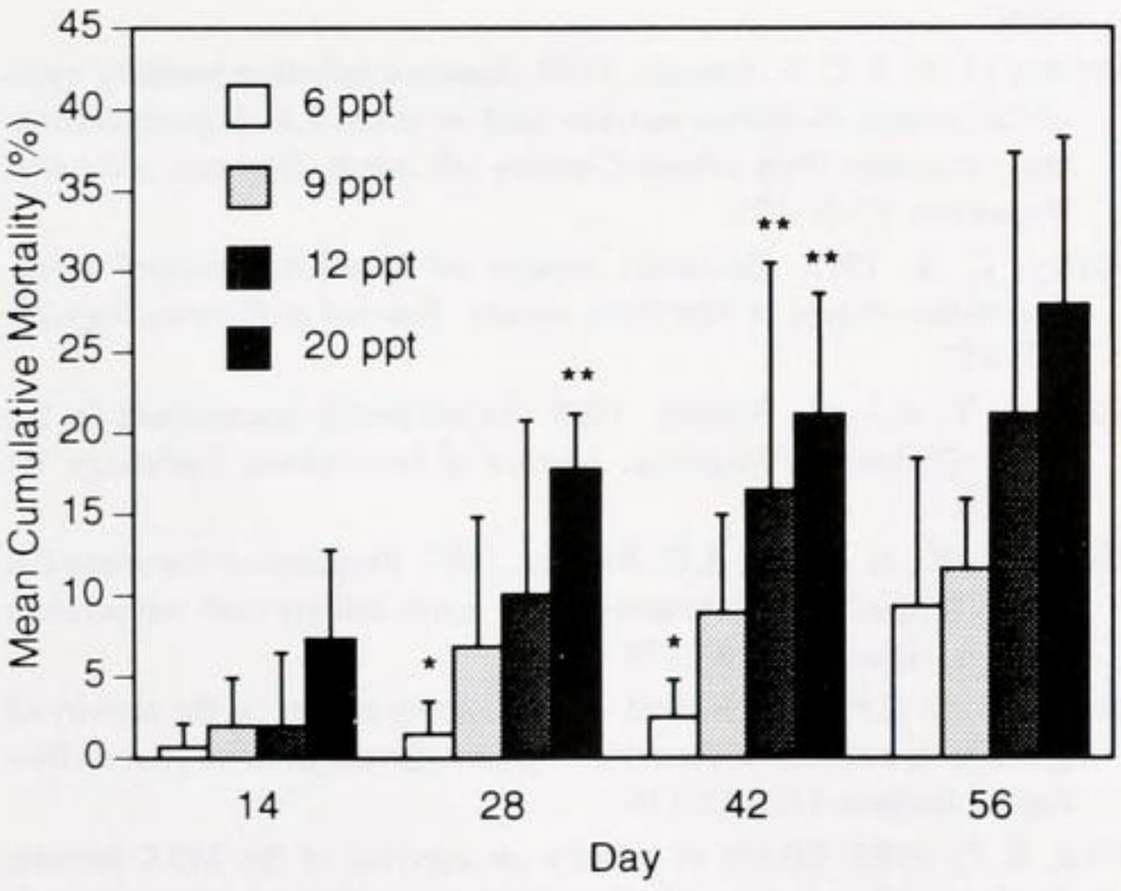

Figure 4. Mean percent cumulative mortality (±1 standard deviation) of oysters exposed to 6, 9, 12, and 20 ppt following 14, 28, 42, and 56 days of treatment (means are based on five replicate oyster groups). Treatment means denoted by a single asterisk significantly differ ($P < 0.05$) from those denoted by a double asterisk.

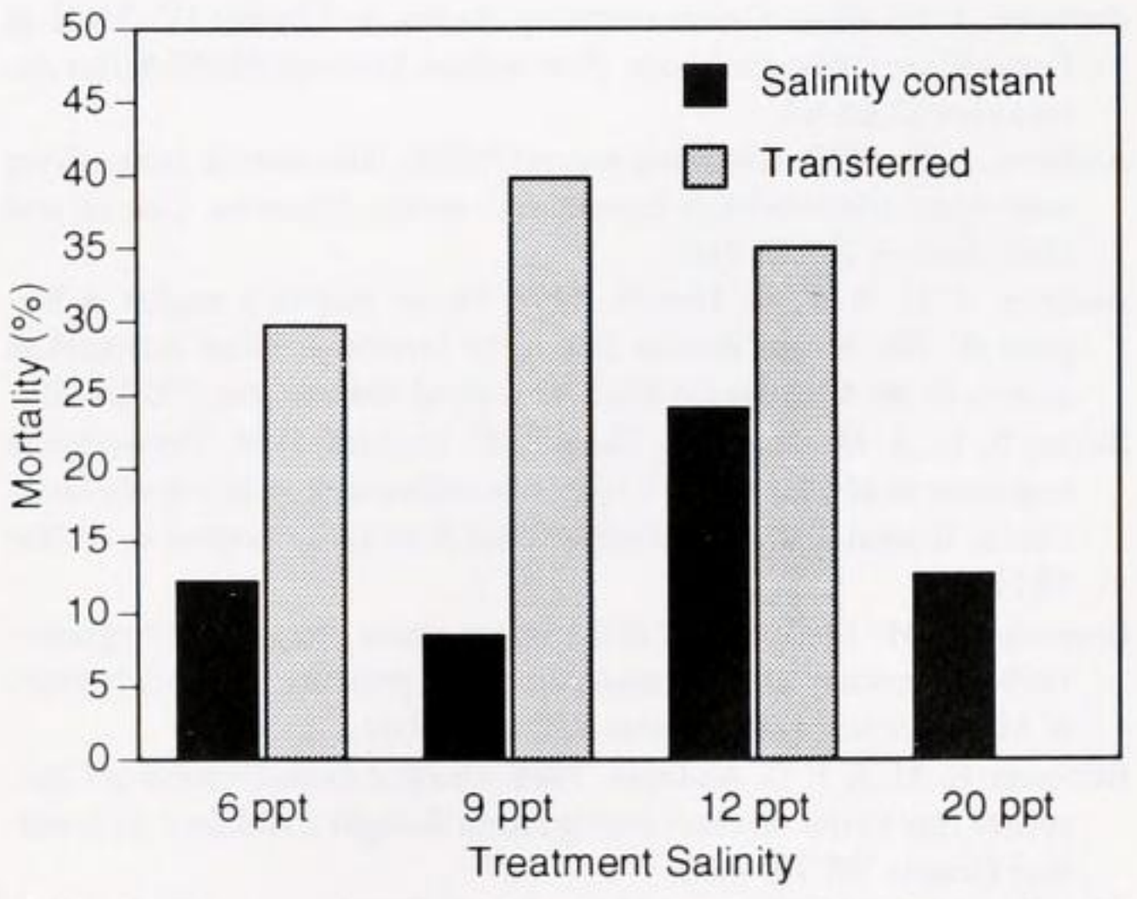

Figure 5. Percent mortality of oysters transferred to 20 ppt after 28 days exposure to 6, 9, and 12 ppt in comparison to those maintained at a constant salinity of 6, 9, 12, and 20 ppt. Percent mortality for each group is calculated for day 29–56. Values shown for groups in which the salinity was constant represent the means of five replicate groups. Transferred groups were not replicated.

TABLE 1.

Parasite prevalence (%) based on histological diagnosis.

Day	Sample	Parasite Prevalence			
		H. nelsoni	*B. cuculus*	*N. ostrearum*	*P. marinus*
0	A	16	24	20	24
	B	16	8	24	20
	C	4	8	20	8
14	20 ppt	8	8	28	44
	12 ppt	0	4	8	24
	9 ppt	0	8	20	16
	6 ppt	0	0	8	16
28	20 ppt	12	8	12	32
	12 ppt	8	0	16	48
	9 ppt	4	8	0	16
	6 ppt	0	0	8	8
42	20 ppt	0	0	16	48
	12 ppt	8	8	4	40
	9 ppt	0	0	8	56
	6 ppt	0	4	16	44
56	20 ppt	8	4	0	56
	12 ppt	0	8	4	44
	9 ppt	0	4	4	48
	6 ppt	0	0	4	48

Day 0 replicates are designated as A, B, and C. N = 25 for all data sets except day 56 12 ppt where n = 20.

temperature can enlarge, narrow, or shift the salinity range of an individual. While some organisms tolerate subnormal salinities better at the lower part of their temperature range others exhibit a reciprocal salinity/temperature tolerance in which the range of salinity tolerated is widest at optimal temperatures and vice versa (Kinne 1964). Additionally, Feng and Stauber (1968) suggested that host defense activities as well as parasite multiplication, metabolite production, and nutritional requirements can be altered by thermal elevations and depressions. A delicate balance between host and parasite exists and alterations in temperature can affect the outcome of host-parasite interactions (Feng and Stauber 1968). Environmental factors that stress the parasite and enhance host defense activities could shift the host-parasite balance to favor the host. In order to further elucidate the influence of environmental conditions on interactions between *C. virginica* and *P. marinus* investigations focusing on the synergistic effects of temperature and salinity are required. The combined effect of temperature and salinity may prove to be more important than either factor alone.

It is evident from this investigation that once *P. marinus* is established in natural oyster populations, it will not be eradicated by low salinity (6–12 ppt) over 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. Oyster growers may be able to avoid disease mortality, however, by maintaining oysters in areas having salinities that are consistently below 9 ppt. Oysters maintained at 12 ppt are likely to experience mortality comparable to oysters maintained at 20 ppt. High mortalities in oysters that were transferred to high salinity following a period of low salinity exposure, suggest that increases in salinity that occur in areas normally having low salinities will allow infections to rapidly develop and cause mortality. In order 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 harvesting and management strategies.

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