1-1993

Environmental control of Perkinsus marinus and elucidation of over wintering infections : Final report

Eugene M. Burreson
Virginia Institute of Marine Science,

Lisa M. Ragone Calvo
Virginia Institute of Marine Science,

ENVIRONMENTAL CONTROL OF *PERKINSUS MARINUS* AND ELUCIDATION OF OVER WINTERING INFECTIONS

Grant number NA16FL0401–01

FINAL REPORT

Submitted to

NOAA, NMFS
Northeast Regional Office

by

Eugene M. Burreson and Lisa M. Ragone Calvo
School of Marine Science
Virginia Institute of Marine Science
College of William and Mary
Gloucester Point, VA 23062

13 January 1993

ABSTRACT

Experiment 1: Temperature and salinity exposure

The role of low temperature and low salinity in controlling *P. marinus* was investigated under laboratory conditions which simulated typical and extreme winter and spring environmental conditions. Oysters (*Crassostrea virginica*) infected with *P. marinus* were collected from the upper James River, VA in December 1991, individually marked and analyzed for *P. marinus* by hemolymph assay. The oysters were then subjected to a sequential treatment of various temperature and salinity combinations. In the first phase oysters were placed in recirculating seawater systems at 10 ppt and low temperature (1°C and 4°C). Half of the oysters were treated at each temperature for 3 weeks and the other half were held for 6 weeks. In the second phase the oysters were gradually warmed to 12°C, adjusted to one of three salinities (3, 6, and 15 ppt), and held for 2 weeks. Finally, all oysters were gradually adjusted to 25°C and 20 ppt and maintained for 4 weeks to determine if any observed declines in prevalence or intensity resulting from prior treatment were permanent. At the end of each phase *P. marinus* prevalence and intensity was assessed using hemolymph assay. Control oysters were maintained at 15°C and 15 ppt during treatment phase 1 and 2 and adjusted to 25°C and 20 ppt in phase 3.

Low temperature exposure, alone, did not significantly effect *P. marinus* prevalence or infection intensity. However, declines in prevalence and intensity, relative to initial levels were observed after 2 weeks at 12°C and 3, 6, and 15 ppt. *Perkinsus marinus* prevalence and intensity in control oysters significantly increased as the experiment progressed. High mortality during phase 3 precluded assessment of the permanence of the observed prevalence and intensity declines. These results suggest that low winter temperatures have little effect on the annual abundance of *P. marinus* within an estuary, while springtime depressions in salinity are very important.

Experiment 2: Elucidation of overwintering infections

The scarcity of overwintering infections of *Perkinsus marinus* in Chesapeake Bay oysters has long puzzled investigators. Typically, prevalence of the pathogen declines in winter and infections are not easily disclosed by routine diagnosis using tissue cultured in thioglycollate medium (FTM). It is unknown whether cryptic stages of the parasite are harbored in the oyster during winter or whether elimination occurs; hence, the actual abundance and relative contribution of overwintering infections to subsequent summer prevalences is unclear.

The objective of this investigation was to determine the nature and abundance of overwintering *P. marinus* infections. Infected oysters were placed in a tray and suspended from a pier in the lower York River, VA in November 1991. Every six weeks from November 1991 through May 1992 oysters (n=25) were removed from the tray, examined for *P. marinus* by hemolymph analysis, gradually warmed in individual containers to 25°C and held for one month. After the incubation period, which permitted the development of very light and/or cryptic parasite stages to detectable levels, the oysters were re-analyzed for *P. marinus* by both hemolymph and tissue cultures in FTM. A second group of 25 oysters was sacrificed on each date, diagnosed using tissue FTM cultures, and examined for cryptic stages using immunoassays.

Prevalence of *P. marinus* gradually declined from 100% in November 1991 to 32% in April 1992. Incubation of oysters at 25°C always resulted in an increase of *P. marinus* prevalence and intensity, suggesting that the parasite was more abundant than FTM cultures indicated. Immunoassay did not reveal the presence of cryptic stages, although it was generally more sensitive than FTM diagnosis, especially during early spring. *Perkinsus marinus* appears to overwinter at very light intensities in a high proportion of oysters. These infections are likely to be an important cause of summer mortalities.

Temperature and salinity have long been recognized as the two most important physical parameters controlling the abundance and distribution of *Perkinsus marinus*. The influence of these two parameters on the occurrence of the parasite has been the focus of numerous studies in both the Gulf of Mexico and the Chesapeake Bay (Ray, 1954; Andrews and Hewatt, 1957; Quick and Mackin, 1971; Andrews, 1988; Soniat and Gauthier, 1989; Craig et al., 1989; Crosby and Roberts, 1990; Ragone and Burreson, 1993).

The tropical to subtropical world wide distribution of *Perkinsus* species (Perkins, 1988) implies that temperature is an important regulating factor. Historically, *Perkinsus marinus* has not occurred north of Chesapeake Bay along the east coast of the United States (although it has recently become abundant in Delaware Bay), and its northern limit appears to be limited by minimum winter temperature. If temperature is important in regulating long–term survival of *P. marinus*, then it is plausible to assume that temperature is also an important determinant of annual abundance of *P. marinus* in endemic areas. Most research has addressed the summer temperature required for parasite development and host mortality and has shown that parasite development is most rapid above 25°C and that parasite development and host mortality generally ceases below about 20°C (Andrews, 1988). Although it is known that *P. marinus* can persist at low levels at temperatures below 5°C (Andrews, 1988), few laboratory studies have been conducted that address the role of winter temperature regime as a regulating factor in subsequent *P. marinus* abundance.

Low temperature control of *P. marinus* was supported by the rapid, dramatic decline in prevalence and intensity of the parasite following the unusually cold December of 1989 (VIMS monitoring data). Water temperature averaged about 2.0°C for about two weeks and prevalence of *P. marinus* in the James River declined from 92.0% in December, 1989 to 24.0% in January, 1990. In addition, prevalence remained below 20.0% for five months even though temperature in January and February was unusually warm, and the typical summer increase was delayed about two months compared with the previous two years (Burreson, 1990,1991). For example, during the winter of 1989, prevalence of *P. marinus* declined gradually during February and March to a low of 20.0% in April and then increased rapidly during May and June. This pattern, typical of relatively warm winters, is in marked contrast to the rapid decline of *P. marinus* prevalence in January, 1990 and the delayed increase in prevalence in July and August, 1990

The influence of salinity on the activity of *P. marinus* is also important and several authors have documented a direct correlation between salinity values and infection intensity (Mackin, 1956; Andrews and Hewatt, 1957; Soniat, 1985; Craig et al., 1989; Soniat and Gauthier, 1989; Crosby and Roberts, 1990). The parasite persists in oysters at salinity as low as 6ppt; however, salinity below about 10–12ppt tends to delay development of infections and significantly reduces host mortality (Ray, 1954; Andrews and Hewatt, 1957; Scott et al., 1985; Ragone and Burreson, 1993).

The distribution of *P. marinus* in the Chesapeake Bay is extensive. Drought conditions during 1985–88 caused record high salinities in the Bay and allowed the pathogen to expand its range to previously disease free areas (Burreson and Andrews, 1988). In 1988 *P. marinus* was present on all oyster beds in Virginia and, despite above average runoff and a reduction of salinity during 1989 and 1990, the pathogen continues to persist tenaciously in all areas (Burreson, 1992). *Perkinsus marinus* is now also widespread in Delaware Bay. Such widespread distribution of the pathogen poses a serious threat to the middle Atlantic region's oyster industry and increases the need to enhance our capability to predict the environmental conditions required to eliminate *P. marinus* from areas that were previously disease free.

Although the effect of salinity and temperature on *P. marinus* has been extensively investigated, the relationship remains obscure. Most studies have been field studies that considered only one factor at a time and the numerous uncontrollable variables inherent in field studies could have had significant effects and confounded interpretation of results. In addition, no laboratory studies have focused on low temperature effects or the combined effects of low temperature and low salinity. One objective of this research was to gain, in closely controlled laboratory experiments, a better understanding of the relationships among

host, parasite and the combined effects of low temperature and low salinity. An hypothesis of particular interest was that a combination of temperature below 4°C for at least three weeks during winter and salinity below 6.0ppt during spring when oysters start pumping, is required to eliminate *P. marinus* from oysters.

The scarcity of over wintering infections has long puzzled investigators. Typically, thioglycollate tests show only rare cases of infection from January through April in oysters that had 100% prevalence the previous October. If oysters collected during the winter period are isolated and warmed in the laboratory, infections develop suggesting that there are cryptic over wintering stages not easily disclosed by thioglycollate tests (Andrews, 1988). The relative importance of over wintering infections and new infections to the progression of the disease during the subsequent summer is unknown. For example, at Deep Water shoal,. the uppermost oyster bed in the James River, prevalence of *P. marinus* increased from 8.0% in 1989 to 64.0% in 1990 although there were seven months from February through August 1990 that the parasite was undetectable. It is not known whether the increase in prevalence was the result of new infections, over wintering infections, or a combination of both. As Andrews (1988) states, "over wintering of the disease requires more study." A second objective of this research was to investigate the presence of cryptic over wintering infections.

METHODS

Experiment 1: Temperature and salinity exposure

Oyster collection

Oysters were collected from the upper James River at Point of Shoals on 9 December 1991. At the time of collection, the water temperature was 11.5°C and the salinity was 12 ppt. The oysters were transported to the Virginia Institute of Marine Science (VIMS), cleaned, individually labeled with a numbered tag and placed in two 400 l tanks containing 15°C York River water diluted to 10 ppt. The oysters, 450 in total, were notched and diagnosed for *P. marinus* (tl) using a modification of the method described by Gauthier and Fisher (1990) (see below).

Experimental design

The oysters were subjected to a three phase temperature and salinity treatment schedule (Figure 1, Table 1) designed to simulate various potential natural environmental conditions in the Chesapeake Bay during winter and early spring. Oysters were first exposed to low temperature, then gradually warmed at various salinity combinations, and finally maintained at a temperature and salinity optimal for *P. marinus* . The purpose of the last phase was to determine if any remission induced by the previous treatment phases was permanent.

Phase 1. In the first phase, two hundred of the prebled, individually numbered oysters were placed in 200 l tanks at each of two temperatures—1°C and 4°C. The 4°C tank was established in a cold room maintained at treatment temperature and the 1°C system was maintained in a self-contained recirculating chiller system (Water Management Unit, Aquarium Systems, Mentor, Ohio). In both cases the water was maintained at 10 ppt and aerated. Half of the oysters at each treatment temperature were exposed to the low temperature condition for a period of three weeks (group 1), the other half were exposed to the condition for six weeks (group 2). Two groups of 25 oysters, serving as positive controls for each experimental group, were established at 15°C and 15 ppt. To limit cross infection, these oysters were placed in individual 1 liter plastic containers and aerated The water in each container was changed every other day with 1.0 µm–filtered York River water and the oysters were fed an algal paste suspension (0.1g/oyster) once daily.

After each prescribed low temperature exposure period hemolymph samples for *P. marinus* diagnosis were taken from the 100 oysters in each treatment group as well as from

the 25 positive control oysters (Figure 1, t2). Twenty five of the 100 re-bled oysters from each group were sacrificed, a sample of tissue was cultured in fluid thioglycollate media (FTM) for *P. marinus* diagnosis, and a section of tissue was preserved in Davidson's AFA for later histological processing and analysis by immunoassay.

Phase 2. In the second exposure phase the remaining 75 oysters from each treatment group were placed in individual 1 liter containers, gradually warmed (1°C per day) to 12°C and adjusted (1-2 ppt per day) to one of three salinity regimes. Twenty five oysters from each group—1°C and 4°C—were adjusted to 3, 6, and 15 ppt and maintained under these conditions for a two week period. Water temperature was controlled by immersion of the individual containers in a recirculating water bath connected to a chiller unit (Model CSP5, Aquanetics, San Diego, California). The water in the individual oyster containers was renewed every other day with 1.0 µm–filtered York River water previously adjusted to the proper temperature and salinity, and the oysters were fed an algal paste suspension (0.1g/oyster) each day. Positive control oysters remained at 15°C and 15 ppt. At the end of the two week period both treatment and control oysters were once again bled and the hemolymph was examined for *P. marinus* (Figure 1, t3).

Phase 3. Finally, during phase 3, the temperature and salinity of all groups including the controls, were gradually increased to 25°C and 20 ppt (1-2°C per day and 2-3 ppt every other day). The oysters were maintained in individual containers at 25°C and 20 ppt for a four week period. Water was changed every day with 1.0 µm–filtered York River water previously adjusted to the proper temperature and salinity, and food was added daily. At the conclusion of the experiment the oysters were re-examined for *P. marinus* using both hemolymph and tissue FTM culture techniques (Figure 1, t4).

Diagnosis of *P. marinus*

Tissue diagnosis of *P. marinus* was by culture of rectal, mantle and gill tissue in fluid thioglycollate media (FTM), as described by Ray (1954). Infections were categorized as negative, light, moderate, and heavy (Ray, 1954) and assigned numerical values of 0, 1, 3, and 5, respectively, for the determination of weighted prevalence (weighted incidence of Mackin, 1962). Infection category values were summed and the total divided by the total number of oysters in the sample.

A modification of the method described by Gauthier and Fisher (1990) was used for hemolymph diagnosis. Notches were cut in oyster shells posterior to the adductor muscle using a lapidary saw. A 300 µl hemolymph sample was withdrawn from the adductor muscle using a 3 cc disposable syringe with a 23 gauge needle. The hemolymph was added to a microcentrifuge tube and was centrifuged for 10 minutes at 403 x g. The supernatant was decanted and the pellet was resuspended in 1.0 ml of FTM fortified with 50 µl penicillin/streptomycin (25 units of each/ml). Cultures were incubated in the dark at 27°C for 5–7 days. Following incubation the samples were centrifuged at 403 x g for 10 minutes. The supernatant was removed and the pellet was resuspended in 1.0 ml of 2M NaOH and incubated at room temperature for 30 minutes. The samples were then washed twice with distilled water and finally resuspended in 1.0 ml distilled water with 50 µl Lugol's stain (1:6 dilution). The samples were gently mixed with a pipette and transferred to 24–well culture plates. A Zeiss inverted microscope was used to examine the samples and infections were categorized as very light (< 10 cells/well), light (10–200 cells/well), light–moderate (200–1000 cells/well), moderate (>1000 <15,000 cells/well), moderate–heavy (>300 cells/50X field, and heavy (cells cover entire bottom of well). These categories were assigned values of one through six respectively for computation of weighted prevalence.

When diagnosis of tissue samples using FTM revealed significant declines in prevalence relative to initial (t1) samples, the corresponding preserved sections were examined for cryptic stages using immunoassays and routine histology. The primary antibody used in the immunoassays was polyclonal, rabbit anti–*P. marinus* raised against meronts (supplied by C.F. Dungan, Oxford Cooperative Laboratory). Biocell goat anti-rabbit IgG gold probe and Biocell light microscopy silver enhancement kit reagents were utilized to detect and visualize

specific binding of the primary antibody (Goldmark Biologicals, Phillipsburg, New Jersey). Briefly, oysters preserved in Davidson's AFA were processed for paraffin histology following standard techniques. Four 5 μm sections were cut and affixed to glass slides, dewaxed in xylene, and hydrated in a graded ethanol series to water. One of the four slides was stained with Harris' hematoxylin and eosin. The remaining three slides were washed in running tap water and phosphate buffered saline (PBS), blocked for 30 min with 10% v/v normal goat serum in phosphate buffered saline containing 1.0% bovine serum albumin (PBSA). Two of the three slides were incubated for 30 min in a 1:100 dilution of primary antibody in PBSA. The third slide, serving as a negative control, was incubated for 30 min in a 1:100 dilution of normal rabbit serum in PBSA. All slides were rinsed thoroughly in PBS and incubated for 1 hr in a 1:100 dilution of affinity purified goat anti-rabbit IgG coated onto 5 nm colloidal gold particles in PBSA. After thorough washing in PBS and distilled water, the bound colloidal gold particles were visualized with silver enhancement reagents that produced a brown black color. The slides were then washed in distilled water, counter stained with fast green, dehydrated in ethanol and cleared in xylene.

Statistics

Differences in *P. marinus* prevalence among treatments and within treatments through time were tested for significance using chi square tests; the contingency table columns were the various treatments, the contingency table rows were the number of infected and uninfected oysters.

Differences in weighted prevalence between groups on each sample date were tested for significance using a factorial ANOVA. Differences in intensity of *P. marinus* in hemolymph through time were tested with a repeated measures ANOVA. Levels of α less than or equal to 0.05 were considered significant.

Experiment 2: Over wintering infections

Oyster collection and experimental design

Perkinsus marinus –infected oysters (n=275) were collected from Point of Shoals, James River, Virginia on 13 November 1991. Uninfected oysters (n=275) were collected on 31 October 1991 from Ross' Rock, Rappahannock River, Virginia. Following collection the oysters were transported to VIMS, cleaned of fouling organisms and placed in separate 400 l aerated tanks containing 15°C filtered (1.0 μm) York River water that was diluted to 15 ppt with fresh tap water. The oysters were maintained at 15°C and 15 ppt until the initiation of the experiment on 18 November 1991. Water was changed once a week and the oysters were fed algae paste (0.1g/oyster) daily.

On 18 November 1991 50 oysters were randomly sampled from each group and diagnosed for *P. marinus* using both hemolymph and tissue assays. This sample was taken for the purpose of comparing diagnostic techniques and determining *P. marinus* prevalence and intensity at the initiation of the investigation. The remaining 225 oysters from each group (infected and uninfected) were placed in separate mesh trays (120 x 60 x 14 cm) and suspended in the York River from a pier at VIMS, Gloucester Point, Virginia. At the time of deployment water temperature was 12°C and the salinity was 24 ppt. Mean weekly temperatures at the site during the study are shown in Figure 2. Salinity at the VIMS pier ranged from 18.8-23.0 ppt and the mean weekly salinity was 22.1 ppt (S.D. = 1.66).

Perkinsus marinus prevalence and intensity in the Point of Shoals tray group (PST) was assessed approximately every six weeks from November through May (Table 2). On each date two groups of 25 oysters were removed from the tray. The first group was sacrificed on the sample date, diagnosed using tissue FTM cultures, and preserved in Davidson's AFA for later analysis using routine histology and immunoassays. Oysters in the second group were cleaned, individually labeled, notched, and a hemolymph sample was withdrawn for *P. marinus* diagnosis. The oysters were than placed in individual 1 liter plastic containers

containing aerated, ambient 1.0 µm–filtered York River water, gradually warmed (1-2°C per day) to 25°C and incubated for 30 days. During the warm up period water was changed and the oysters were fed daily. The incubation presumably permitted the development to detectable levels of very light and/or cryptic parasite stages which might not be detected by tissue or hemolymph assays. Isolation of oysters in individual containers removed the possibility of parasite transmission between oysters during the warm up period. After the incubation period the oysters were diagnosed for *P. marinus* using both hemolymph and tissue assays.

Ross' Rock tray oysters (RRT) (n=25) were also sampled and warmed up as described above. The uninfected group served as a control for infections acquired in both field and lab conditions during the investigation.

In addition to the York River tray groups, native Point of Shoal, James River (PSJR) oysters were monitored monthly for *P. marinus* during the study period. Sample dates and ambient water conditions are shown in Table 3. On each date 25 oysters were collected, sacrificed, and examined for *P. marinus* using tissue FTM. The PSJR oysters collected in March and May were also examined for *P. marinus* using immunoassay. In March and May an additional 25 oysters were also collected, diagnosed for *P. marinus* using hemolymph assays and warmed up as described above.

Diagnosis of *P. marinus*

FTM, hemolymph and immunoassay diagnoses were identical to those described above for Experiment 1. In experiment 2, weighted prevalence of tissue and hemolymph samples was based on four categories, negative (0), light (1), moderate (3), and heavy (5). The number of hemolymph intensity categories was reduced from 6 to 4 by designating very light and light infections as light, light–moderate and moderate infections as moderate, and moderate–heavy and heavy infections as heavy.

RESULTS

Experiment 1: Temperature and salinity exposure

Prevalence

At the initiation of the experiment (t1) prevalence of *P. marinus* in the various groups ranged from 60 to 91% (Figure 3). Prevalence of *P. marinus* did not significantly change after either 3– or 6–week exposure to either 1°C or 4°C (Phase 1, t2) (Figure 3) and prevalence in experimental groups was not significantly different from the controls at the end of Phase 1 (t2). After two weeks at the various salinity treatments at 12°C (Phase 2) prevalence decreased in oysters in all experimental groups (Figure 3). At t3, Oysters exposed to 3 ppt, regardless of length or degree of cold exposure, exhibited the greatest decline in *P. marinus* prevalence and prevalence differences were significant ($P<0.001$)at both temperature and exposure treatments. The mean decline in prevalence during Phase 2 in oysters exposed to 3 ppt was 41%; prevalence declined an average of 17% and 22% in oysters exposed to 6 and 15 ppt respectively. Decline in prevalence of *P. marinus* during Phase 2 in oysters held at 6 ppt was significant for all treatments ($P<0.05$) except six weeks at 4°C (Figure 3). Differences in *P. marinus* prevalence during Phase 2 for oysters held at 15 ppt were significant for 3 and 6 weeks at 4°C ($P<0.005$) , but not at 1°C (Figure 3).

In contrast to the experimental groups, prevalence of *P. marinus* in oysters in the control group held at 15°C and 15 ppt increased at the end of Phase 2. This increase was statistically significant in group 1 ($P<0.01$), but not in group 2.

At the end of exposure to the various salinity treatments at 12°C (Phase 2) for group 1 oysters (3 weeks cold exposure), *P. marinus* prevalence was significantly lower than that of the control groups in all experimental groups (Figure 3) ($P<0.001$ for 3 ppt. $P<0.007$ for 6 ppt, $P<0.05$ for 15 ppt). Within the experimental groups, prevalence differences were

significant only between 3 and 15 ppt at 1°C ($P<0.05$); prevalence differences between experimental groups at 4°C were not statistically significant. At the end of Phase 2 for group 2 (6 weeks cold exposure) at 1°C, *P. marinus* prevalence was significantly lower than the control group in both the 3 and 6 ppt group ($P<0.006$), but not in the 15 ppt group; within the experimental groups prevalence differences were significant only between 3 and 15 ppt ($P<0.05$). In the 4°C groups prevalence was significantly lower than the control group in both the 3 and 15 ppt groups ($P<0.01$), but not in the 6 ppt group (Figure 3); differences between the experimental groups were not significant.

Infection intensity

Distribution of infection intensity categories of *P. marinus* for each treatment group at each sample time are shown in Table 4. Weighted prevalence (a measure of infection intensity) of each group at each sample time is shown in Figure 4. There was no significant difference in weighted prevalence between experimental groups and control groups at the initiation of the experiment or after cold temperature exposure (Phase 1, t2). Weighted prevalence did not change from t1 to t2 (cold temperature exposure) for either experimental temperature or duration (Figure 4). Mean values ranged from 1.0 to 1.31 at the initiation of the experiment and from 1.03 to 1.37 at the end of Phase 1 (t2), indicating mostly light infections. Weighted prevalence of group 1 controls was significantly higher after Phase 1 (t2) than at the initiation of the experiment (t1); intensity increase in group 2 controls was not significant.

After two weeks at experimental salinities at 12°C (Phase 2, t3), weighted prevalence declined in all experimental treatments and increased or remained the same in control groups. In the oysters that had previously been held for 3 weeks at either 1 or 4°C (group 1), mean infection intensity at all three salinity regimes (3, 6 and 15 ppt) was significantly lower than in the control group ($P<0.05$), but there were no significant differences among experimental groups. The results of the repeated measures ANOVA, to test for differences in infection intensity through time, indicated that significant declines in intensity occurred in group 1, 1°C and 4°C oysters that were exposed to 3 ppt ($P<0.001$) and 6 ppt ($P<0.03$) during phase 2 (t3). Intensity differences in oysters held at 15 ppt were not significantly different between t2 and t3.

In oysters that had previously been held for 6 weeks at either 1 or 4°C (group 2), significant differences from the control group after Phase 2 (t3) were observed for oysters held at 3 and 6 ppt (1°C) and 3 ppt (4°C) (Figure 4). The repeated measures ANOVA for group 2 indicated significant intensity decreases in. 1°C oysters exposed to 3 ppt and 6 ppt ($P<0.003$) and 4°C oysters exposed to 3 ppt and 15 ppt ($P<0.02$)(Figure 4).

Mortality

Few oysters died during the cold temperature exposure or subsequent two week salinity treatments at 12°C (Phases 1 and 2); however, oysters from all experimental groups began to die soon after hemolymph sampling following the 12°C exposure (Figure 5). Mortality was high in all treatment groups as oysters were being warmed to 25°C, but the oysters held at 15 ppt at either temperature or duration had the lowest mortality. Mortality was much lower in the control group than in any treatment group. All dead oysters containing meat (gapers) were examined for *P. marinus*; greater than 90% of these oysters were uninfected or had light infections.

The high mortality during the final warm up period (Phase 3) prevented adequate evaluation of whether infection remission was permanent. Results for both hemolymph and tissue analysis of oysters that did survive to the termination of the experiment are shown in Table 5.

Experiment 2: Over wintering infections

The Point of Shoals tray (PST) oysters sacrificed for tissue FTM culture diagnosis had a *P. marinus* prevalence of 100% at the initiation of the experiment (Figure 6A). A gradual decline in prevalence to 32% was observed during the winter months. Weighted prevalence, a measure of infection intensity, also declined from 2.0 in November to 0.56 in May (Figure 6B). In contrast, Point of Shoals oyster sampled monthly from the James River (PSJR) exhibited a more rapid decline of both prevalence and weighted prevalence (Figure 6A, B). Oysters collected from Ross' Rock in the upper Rappahannock River and held in trays at VIMS (RRT) had *P. marinus* prevalence of 8.0% when collected in November; prevalence declined to zero by April (Figure 6A, B).

Both prevalence and weighted prevalence of *P. marinus* determined by hemolymph assay in the separate groups of PST oysters designated to be warmed (Figure 7) closely corresponded to tissue diagnoses of sacrificed oysters (Figure 6) on all sample dates except April. In April both prevalence and weighted prevalence based on hemolymph assay was 50% lower than that determined by tissue FTM culture (Figure 7A, B). In all five months during which PST oysters were warmed, final prevalence and weighted prevalence after 30 days at 25°C were greater than initial values prior to warm up (Figure 7A, B). During April and May, post–warm up diagnosis by tissue FTM culture resulted in higher prevalences than diagnosis by hemolymph assay; tissue FTM culture resulted in higher weighted prevalence than hemolymph diagnosis every month except February (Figure 7A, B). Immunoassay diagnosis of the same oysters sampled by tissue FTM culture in April revealed that three of the five oysters diagnosed as uninfected by FTM culture were actually infected with *P. marinus*. Immunoassay revealed *P. marinus* cells scattered in the epithelium of the stomach. However, similar immunoassay diagnosis of the oysters sampled by FTM culture in May failed to reveal *P. marinus* cells in the stomach epithelium, or elsewhere, in any of the 17 oysters diagnosed as uninfected by FTM culture. There was, however, a strong positive reaction in the cytoplasm of most hemocytes in these oysters, although intact *P. marinus* cells could not be discerned. There was a similar hemocyte reaction, although much less intense, in all the negative control slides.

Hemolymph diagnosis of oysters collected from Point of Shoals in the James River (PSJR) (Figure 8) closely corresponded to tissue diagnoses of sacrificed oysters in March and May (Figure 6) for both prevalence and weighted prevalence. Both prevalence and weighted prevalence increased after 30 days at 25°C in both March and May, although increases in March were small. Diagnosis after warm up by tissue FTM culture resulted in greater prevalence and weighted prevalence than diagnosis by hemolymph assay, especially in May. Immunoassay diagnosis of the same PSJR oysters sampled by tissue FTM culture in March revealed that 13 of the 21 oysters diagnosed as uninfected by FTM culture were definitely infected with *P. marinus*. In every case, *P. marinus* cells were found only in the stomach epithelium. Another six oysters had localized concentrations of antibody signal in the stomach epithelium although no intact *P. marinus* cell could be discerned. However, in May, none of the 21 oysters diagnosed uninfected by tissue FTM culture was found to be infected by immunoassay. As with the PST oysters in May, there was a strong positive reaction in hemocytes of some oysters, but similar, although reduced, reaction also occurred in the negative control slides.

Hemolymph diagnosis of Ross' Rock tray (RRT) oysters yielded a higher prevalence (20%) (Figure 9) in November than a separate group of oysters from the same location diagnosed with tissue FTM culture (Figure 6). After 30 days at 25°C, prevalence was similar to the initial value using hemolymph diagnosis, but was much higher using tissue FTM culture diagnosis. Subsequent samples exhibited very low prevalences and weighted prevalences even after warm up. In May, no *P. marinus* infections were detected in initial samples, but prevalence was 8.0% in both hemolymph and tissue samples after 30 days at 25°C (Figure 9).

DISCUSSION

Experiment 1: Temperature and salinity exposure

Contrary to the original hypothesis, which was based on the world–wide distribution of *P. marinus* and seasonal field observations in Chesapeake Bay, there was no immediate effect of low temperature exposure on either *P. marinus* prevalence or infection intensity under the experimental conditions chosen. During the time course of this experiment, *Perkinsus marinus* cells did not appear to be affected by either 3 or 6 week exposure to either 1 or 4°C. Unfortunately, oyster mortality was high during Phase 3 of the experiment and prevented analysis of any subsequent delay in the increase of *P. marinus* prevalence or intensity as was observed in the James River during 1990.

In contrast to temperature effects, there was a dramatic effect of salinity on *P. marinus* infections. Both prevalence and intensity of *P. marinus* infections decreased in all experimental treatments during Phase 2, although not all declines were statistically significant. Generally, after two weeks at 12°C and experimental salinities (Phase 2, t3)both prevalence and intensity of oysters held at 3 and 6 ppt were significantly lower than either control oysters at t3 or the same oysters after low temperature exposure (t2). These results suggest that salinity is the primary controlling factor for *P. marinus* in nature, and also suggest that as little as two weeks at 3 ppt is sufficient to significantly reduce, but not eliminate, *P. marinus* infections.

The experimental design does not allow us to determine the contribution of the low temperature exposure to the observed salinity effects. For example, if there had been no low temperature exposure prior to the various salinity treatments, would the same results have been obtained, or would there have been a reduced salinity effect? If the prior low temperature exposure was an important factor in the salinity–effect results, one might expect different prevalence and intensity decreases at t3 between 3 weeks at 4°C and 6 weeks at 1°C. However, the decline in prevalence and weighted prevalence between t1 and t3 was not consistently different for the two temperature treatments; at 3 ppt the declines were similar, at 6 ppt the decline was greater at 1°C than at 4°C and at 15 ppt the decline was greater at 4°C than at 1°C. A thorough investigation of the contribution of prior low temperature exposure to the observed salinity effect would have required much larger control groups exposed to similar salinity treatments as the other oysters. Such an increase in effort would have been logistically impossible given the facilities and personnel available. However, with the results of the present experiment, it is now possible to design a reduced experiment to answer these questions.

Because of the high mortality during Phase 3, we were not able to determine if observed remission after salinity treatment was permanent or even resulted in a delay in disease progression during subsequent return to optimal conditions for *P. marinus*. The cause of the high mortality during Phase 3 was not clear. However, it was not the result of repeated bleeding for hemolymph analysis because control oysters were bled on the same schedule as experimental oysters and mortality was low in control oysters. The mortality seems to be related to the low salinity exposure because mortality was higher at 3 and 6 ppt than it was at 15 ppt. It is possible that the combined stress of bleeding, increasing temperature and decreasing salinity during Phase 2, even though very gradual, were responsible for the mortality. In addition, the shell of all oysters was notched at the initiation of the experiment to allow hemolymph analysis and this notch prevented oysters from isolating themselves from the external environment, which is necessary for adjustment of internal osmotic pressure during salinity change. Thus, oysters may not have been able to compensate for the change in salinity and eventually died (Kennedy T. Paynter, personal communication).

Experiment 2: Over wintering infections

The prevalence in oysters collected at Point of Shoals in the James River and moved to a tray at VIMS in the lower York River was much different than prevalence in oysters at Point of Shoals during the winter. The prevalence declined much more rapidly in oysters at Point of Shoals (PSJR) than in tray oysters (PST) (Figure 6A). This difference appears to be salinity related. The salinity in the James River declined from 12 ppt to 4 ppt, while the salinity in the York River where the tray was held varied between 18.8 to 23 ppt. This observed difference in prevalence in the field supports the results of experiment 1 in which prevalence decline was greatest at the lowest salinity.

Both prevalence and intensity of *P. marinus* always increased in oysters that were warmed from ambient temperature to 25°C for 30 days. Because warmed oysters were held in individual containers in 1.0 µm–filtered water it seems unlikely that observed increases were the result of new infections during the warming period. However, it is possible that infective stages of *P. marinus* may pass through a 1.0 µm filter, and the greatest increase in prevalence after warming in both VIMS tray and James River oysters occurred during May when water temperature is increasing and infective stages may be present in the water. It is also possible that oysters warmed in May may have recently acquired new infections in nature that developed during the warming period in the laboratory. However, prevalence remained very low after warming in Ross' Rock oysters and these results suggest that if new infections were being acquired in tray oysters they accounted for less than 10% of the observed increase in prevalence. In addition, prevalence and intensity of *P. marinus* also increased after warming during January and February when infective stages are probably not present in the water. The increase in intensity clearly indicates that *P. marinus* cells are multiplying, as would be expected during 30 days at 25°C. Thus, it seems most probable that the increase in prevalence can be explained by an increase in intensity to detectable levels during warming of infections that were too light to diagnose initially. This conclusion is supported by the detection of a small number of cells by immunoassay in oysters that were thought to be uninfected after diagnosis by tissue FTM culture. Immunoassay did not reveal any previously unrecognized stages or cell types, but the technique did identify typical, individual *P. marinus* cells that were not detected by other methods apparently because the infections were so light. Although the infections were light, the prevalence was actually high; thus the contribution of overwintering infections to subsequent mortality may be higher than previously believed.

It is interesting that many undiagnosed infections were detected by immunoassay during March and April, but not during May. Cells detected by immunoassay only were always restricted to the stomach epithelium. Perhaps as development of *P. marinus* stops during winter, oyster hemocytes are able to remove them from most areas and transport them to the gut for elimination. Thus hemolymph or tissue diagnosis would be negative even though the oyster is still infected. The transport of foreign particles by oyster hemocytes to epithelial layers, including the lumen of the gut, and their subsequent elimination has been well documented (Stauber, 1950; Tripp, 1958, 1960; Feng and Feng, 1974) Tripp (1958) suggested that *P. marinus* may be eliminated from the host in this manner. The influence of temperature on such defense mechanisms has been discussed by Feng and Feng (1974). The authors found phagocytosis and hemocyte migration to occur at temperatures as low as 6°C; however, oysters were more effective in the disposal of foreign particles at 15–19°C. These observations support the speculation that the presence of *P. marinus* in the stomach lumen of oysters sampled in April may be related to parasite expulsion.

During May there appeared to be a strong positive antibody reaction in hemocytes, but negative control slides exhibited a less intense reaction in hemocytes so it is difficult to interpret these results. A positive reaction in negative control slides indicates a non–specific reaction in normal rabbit serum and a similar reaction with the anti–*P. marinus* antibody cannot be interpreted as a specific reaction for *P. marinus*.

Our results suggest that hemolymph assay is as sensitive as tissue FTM culture for the diagnosis of *P. marinus* during fall and early winter, but that tissue FTM culture is more sensitive than hemolymph analysis during spring. After warming in March, April or May, tissue FTM culture always yielded higher prevalence and weighted prevalence than hemolymph analysis. These results suggest a seasonal pattern to the abundance of *P. marinus* cells in the hemolymph and support the immunoassay results that demonstrate a restricted location for *P. marinus* cells during spring.

These results are contrary to those of Gauthier and Fisher (1990) who found hemolymph assay more sensitive than tissue FTM assay. However, their study was conducted in late spring and summer in the Gulf of Mexico. In addition, we used 0.3 ml of hemolymph and large (approximately 5.0 mm^2—1.0 cm^2) pieces of mantle and gill, as well as rectal tissue. Gauthier and Fisher (1990) used 1.0 ml of hemolymph and 4.0 mm^2 pieces of mantle, so it appears that sensitivity of the techniques depends on season and the amount of tissue or hemolymph utilized. Immunoassay was more sensitive than either of the other techniques during early spring, but not consistently, and it is a much more expensive and time consuming technique than the other methods. Immunoassay has definite research applications, but is not recommended for routine diagnosis, at least with paraffin sections.

ACKNOWLEDGMENTS

Juanita Walker was responsible for paraffin histology and tissue FTM culture diagnoses. Chris Dungan, Oxford Cooperative Laboratory, Oxford, MD provided the rabbit anti–*P. marinus* antibody.

LITERATURE CITED.

Andrews, J. D. 1988. Epizootiology of the disease caused by the oyster pathogen *Perkinsus marinus* and its effects on the oyster industry. Pgs. 47-63 In: Fisher, W. S. (Ed.) Disease Processes in Marine Bivalve Molluscs. Amer. Fish. Soc. Spec. Publ. 18. 315p.

Andrews, J. D., and W. G. Hewatt. 1957. Oyster mortality studies in Virginia. II. The fungus disease caused by *Dermocystidium marinum* in oysters of Chesapeake Bay. Ecological monographs 27: 1-25.

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 Pt., VA. 13p.

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 Pt., VA. 16p.

Burreson, E. M. 1992. Status of the major oyster diseases in Virginia–1991. A summary of the annual monitoring program. Marine Resource Report 92–1, Virginia Institute of Marine Science, Gloucester Pt., VA. 16p.

Burreson, E. M. and J. D. Andrews. 1988. Unusual intensification of Chesapeake Bay oyster disease during recent drought conditions. Proc. Oceans '88: 799-802.

Craig, A., E. N Powell, R. R. Fay and J. M. Brooks. 1989. Distribution of *Perkinsus marinus* in Gulf coast oyster populations. Estuaries 12: 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. Dis. Aquat. Orgs. 9: 149-155.

Feng, S. Y. and J. S. Feng. 1974. The effect of temperature on cellular reactions of *Crassostrea virginica* to the injection of avian erythrocytes. J. Invert. pathol. 23: 22–37.

Gauthier, J. D. and W. S. Fisher. 1990. Hemolymph assay for diagnosis of *Perkinsus marinus* in oysters *Crassostrea virginica* (Gmelin, 1791). J. Shellf. Res. 9(2): 367–372.

Mackin, J. G. 1956. *Dermocystidium marinum* and salinity. Proc. Nat. Shellfish. Assoc. 46: 116-128.

Mackin, J. G. 1962. Oyster diseases caused by *Dermocystidium marinum* and other microorganisms in Louisiana. Publications of the Institute of Marine Science, University of Texas 7: 132–229.

Perkins, F. O. 1988. Structure of protistan parasites found in bivalve molluscs. Pgs. 93–111 In: Fisher, W. S. (Ed.) Disease Processes in Marine Bivalve Molluscs. Amer. Fish. Soc. Spec. Publ. 18. 315p.

Quick, J. A. and J. G. Mackin. 1971. Oyster parasitism by *Labyrinthomyxa marina* in Florida. Florida Dept. Nat. Res. Mar. Lab., Professional Pap. Ser. 13. 55p.

Ragone, L. M. and E. M. Burreson. 1993. Effect of salinity on infection progression and pathogenicity of *Perkinsus marinus* in the eastern oyster, *Crassostrea virginica* (Gmelin). J. Shellfish. Res. (In Press).

Ray, S. M. 1954. Biological studies on *Dermocystidium marinum*, a fungus parasite of oysters. Rice Institute Pamphlet. The Rice Institute, Houston, TX. 114p.

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 Sci. 7(2): 171-174.

Soniat, T. M. and J. D. Gauthier. 1989. The prevalence and intensity of *Perkinsus marinus* from the mid northern Gulf of Mexico, with comments on the relationship of the oyster parasite to temperature and salinity. Tulane Stud. Zool. Bot. 27(1): 21-27.

Stauber, L. A. 1950. The fate of india ink injected intracardially into the oyster, *Ostrea virginica* Gmelin. Biol. Bull. 98: 227–241.

Tripp, M. R. 1958. Disposal by the oyster of intracardially injected red blood cells of vertebrates. Proc. Nat. Shellf. Assoc. 48: 143–147.

Tripp, M. R. 1960. Mechanisms of removal of injected microorganisms from the American oyster, *Crassostrea virginica* (Gmelin). Biol. Bull. 119: 273–282.

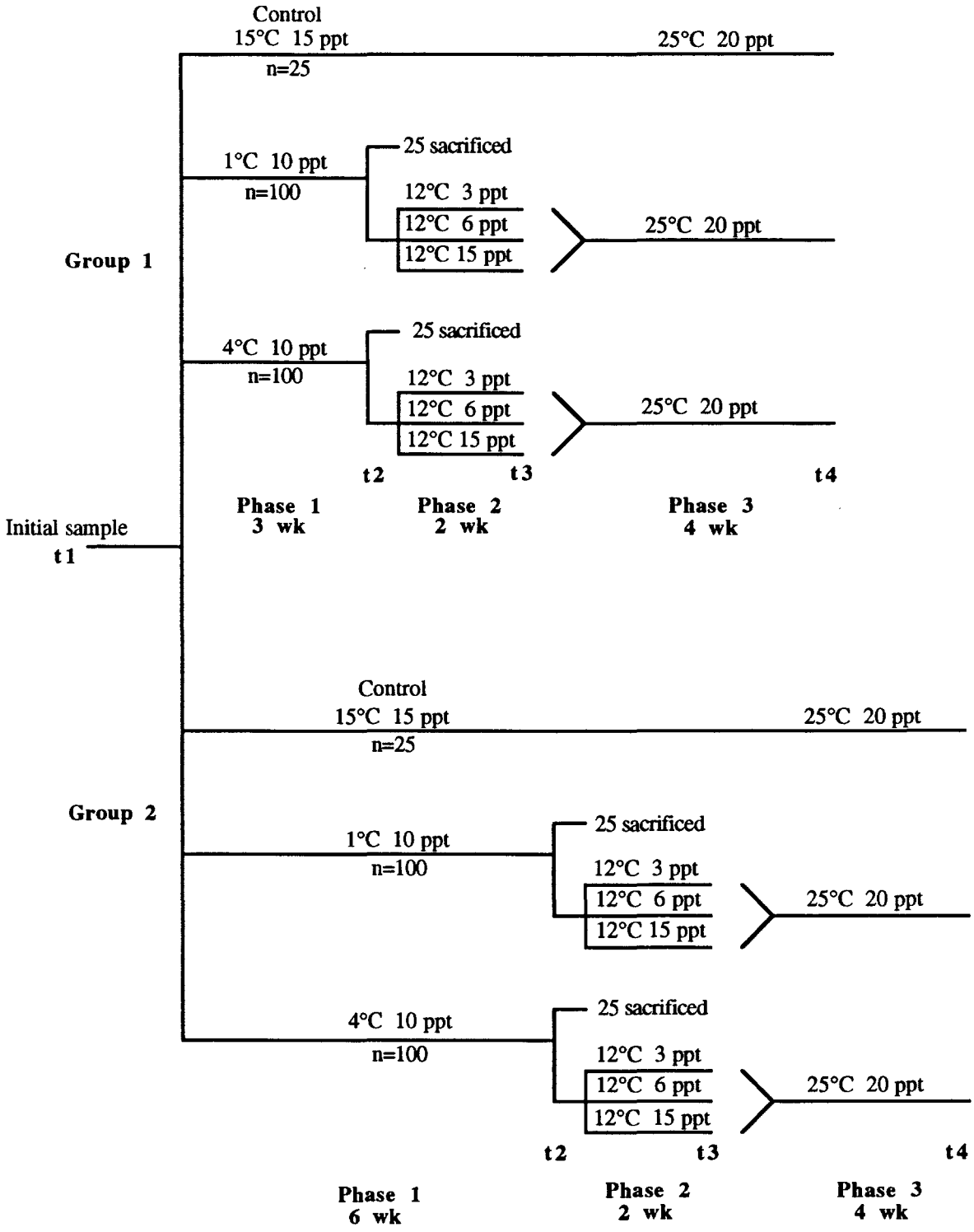

Figure 1. Schematic of experimental design. Treatment temperatures and salinities during each treatment phase are indicated. The duration of each phase for each treatment group is represented in weeks (wk). The number of oysters (n) at each teatment during phase 1 is indicated on the chart. Twenty-five oysters were established at each treatment during phase 2. Oyster hemolymph was analyzed for *P. marinus* at the initiation of the experiment (t1) and after each treatment phase (t2, t3, and t4). At the termination of the experiment (t4) oysters were also analyzed using tissue FTM cultures. Oysters sacrificed at time 2 (t2) were examined using both tissue and hemolymph assay, and preserved for immunoassay analysis.

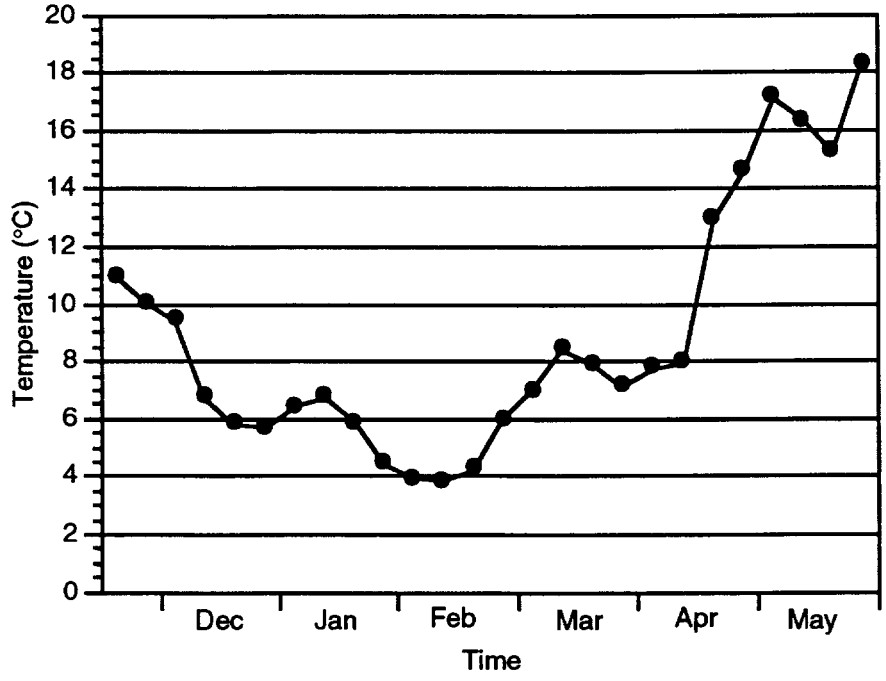

Figure 2. Mean weekly temperature at the VIMS pier site.

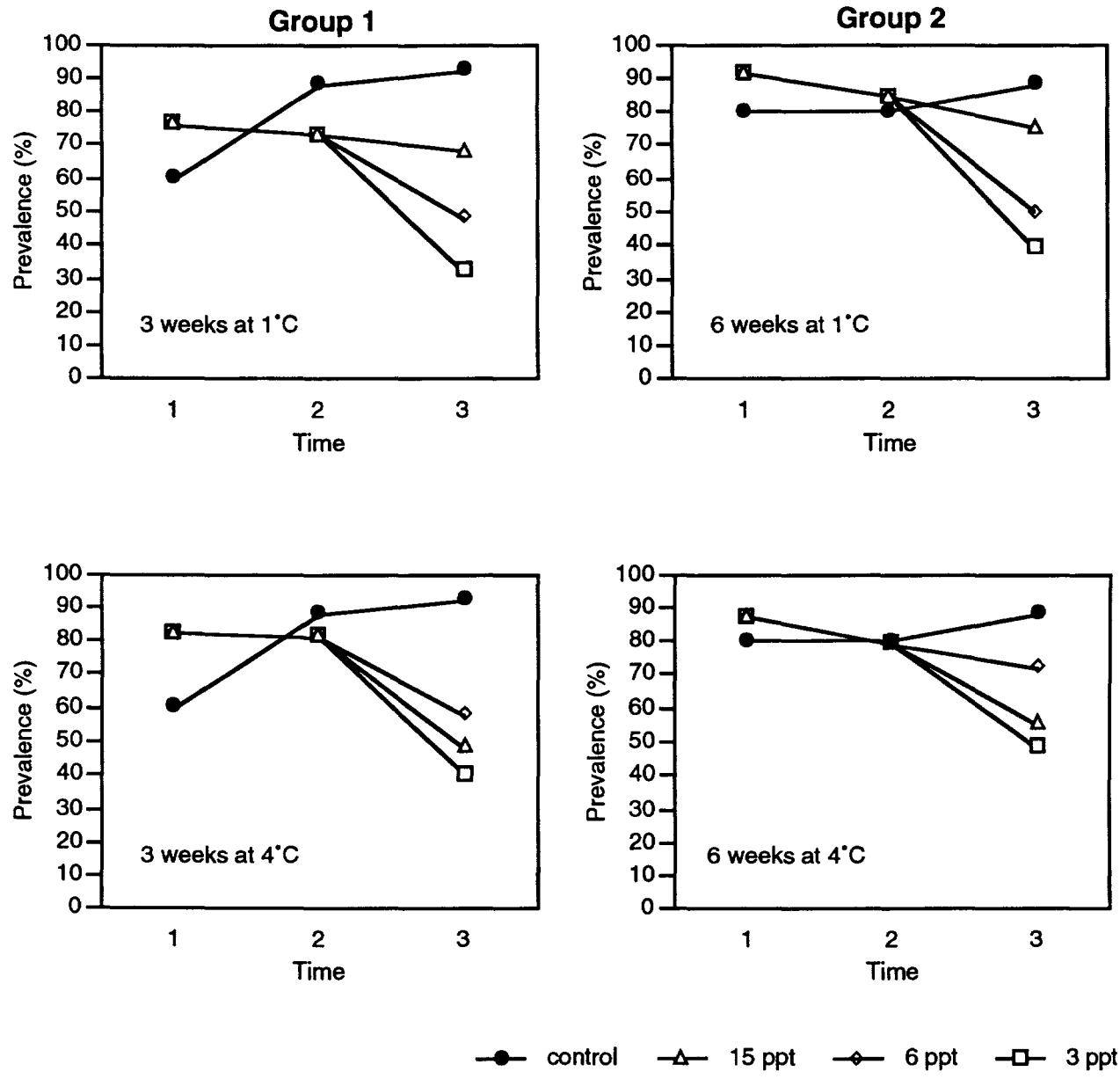

Figure 3. *Perkinsus marinus* prevalence in control and treatment oysters at the initiation of the experiment (time 1), after low temperature exposure (time 2), and after exposure to 12°C and treatment salinities (time 3). Control oysters were continuously maintained at 15°C and 15 ppt. Prevalence was determined using hemolymph assays.

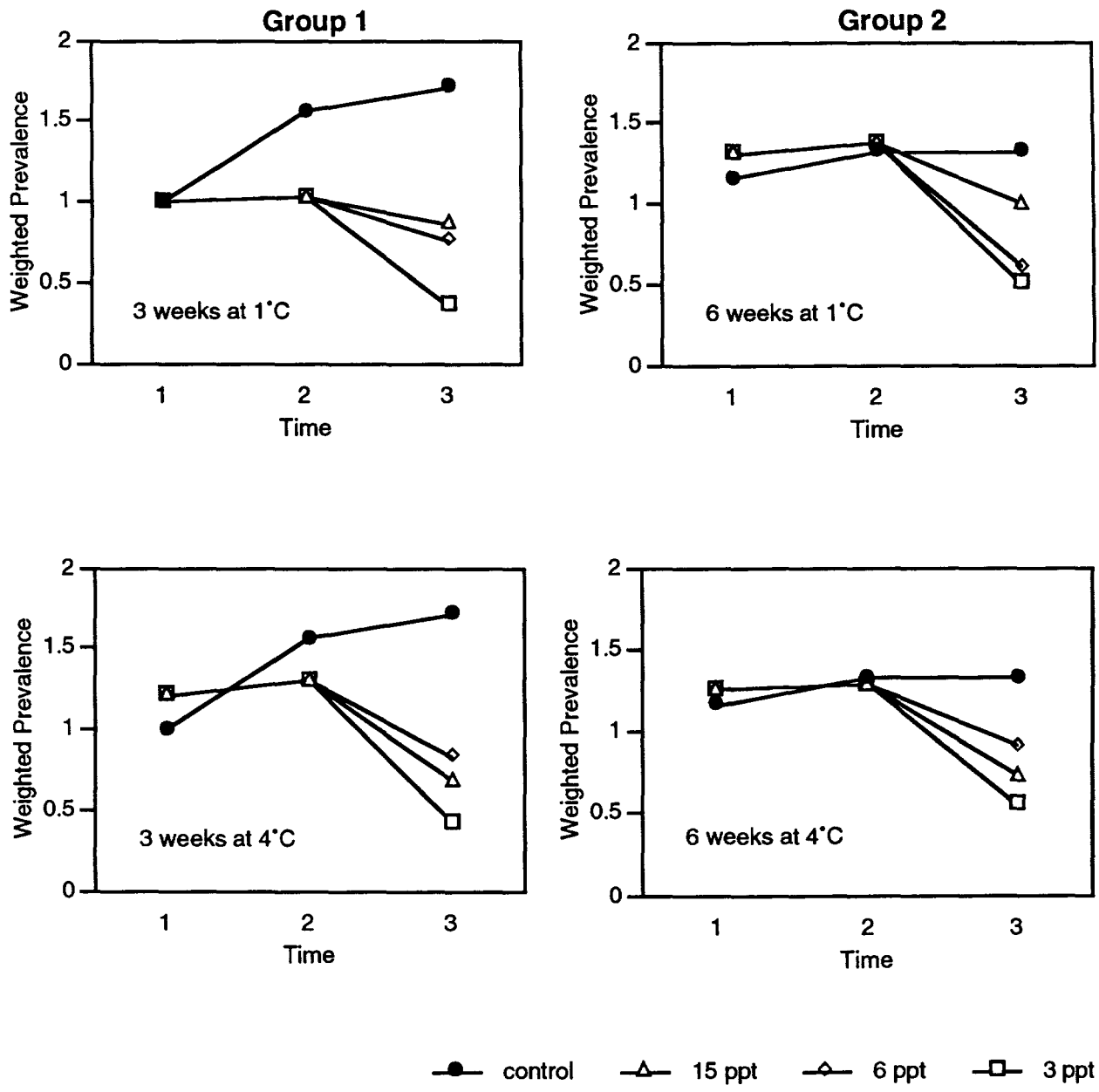

Figure 4. Weighted prevalence of *P. marinus* in control and treatment oysters sampled at the initiation of the experiment (time 1), after low temperature exposure (time 2), and after exposure to 12°C and treatment salinities (time 3). Control oysters were continuously maintained at 15°C and 15 ppt. Diagnosis is based on hemolymph assays.

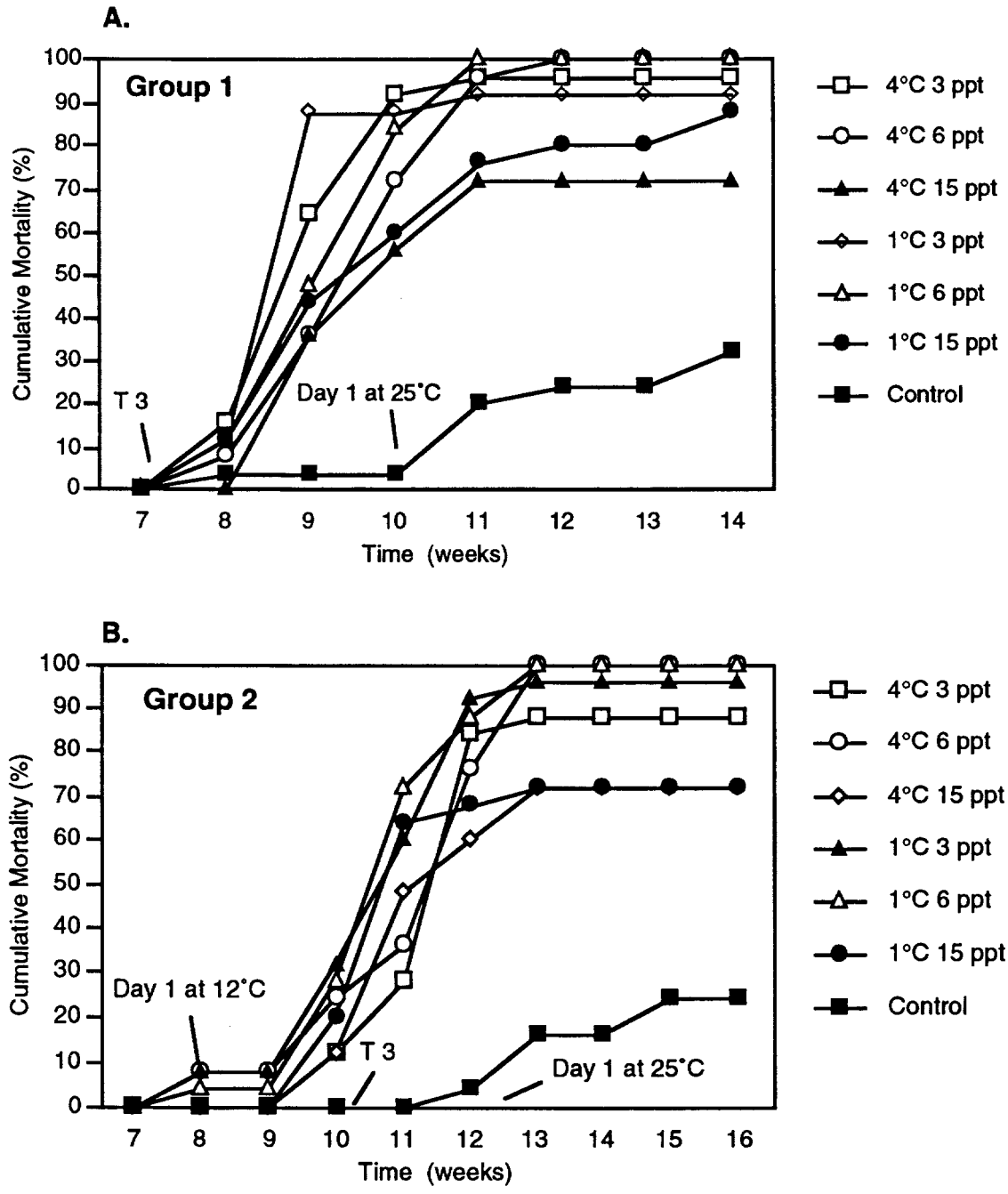

Figure 5. Cumulative mortality of group 1 (A) and group 2 (B) oysters from time after exposure to 12°C at various salinities, phase 2 (t 3), to the end of the experiment. Time is weeks from the initiation of the experiment.

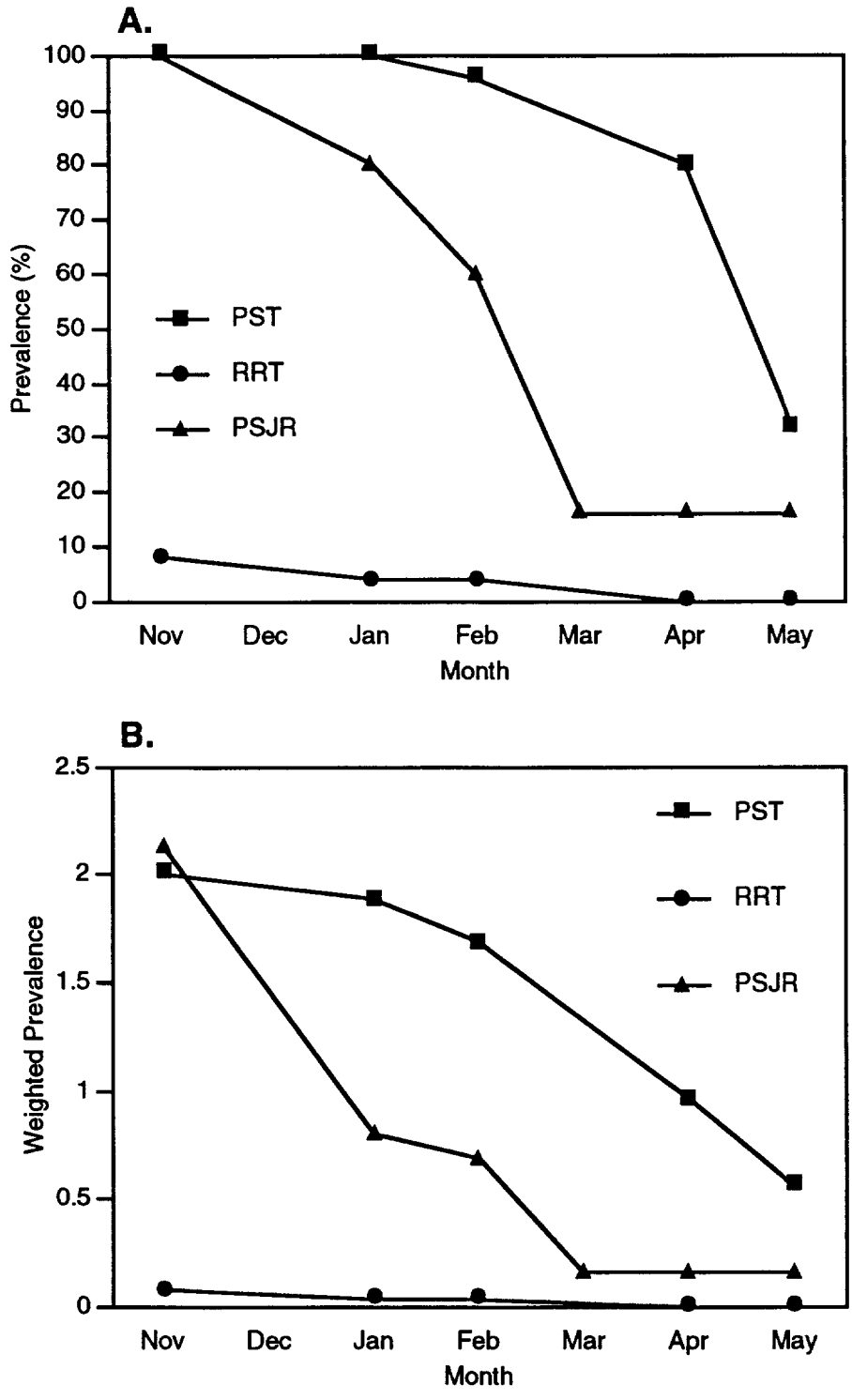

Figure 6. *Perkinsus marinus* prevalence (A) and weighted prevalence (B) in oysters sampled from Point of Shoals, York River tray (PST), Ross' Rock, York River tray (RRT), and Point of Shoals, James River. All PST and PSJR oysters were diagnosed using tissue cultures. RRT oysters were diagnosed by tissue culture in November and by hemolymph assays on all subsequent dates. Diagnosis was made on samples of 25 oysters on all occasions except for November PST and RRT samples (n=50).

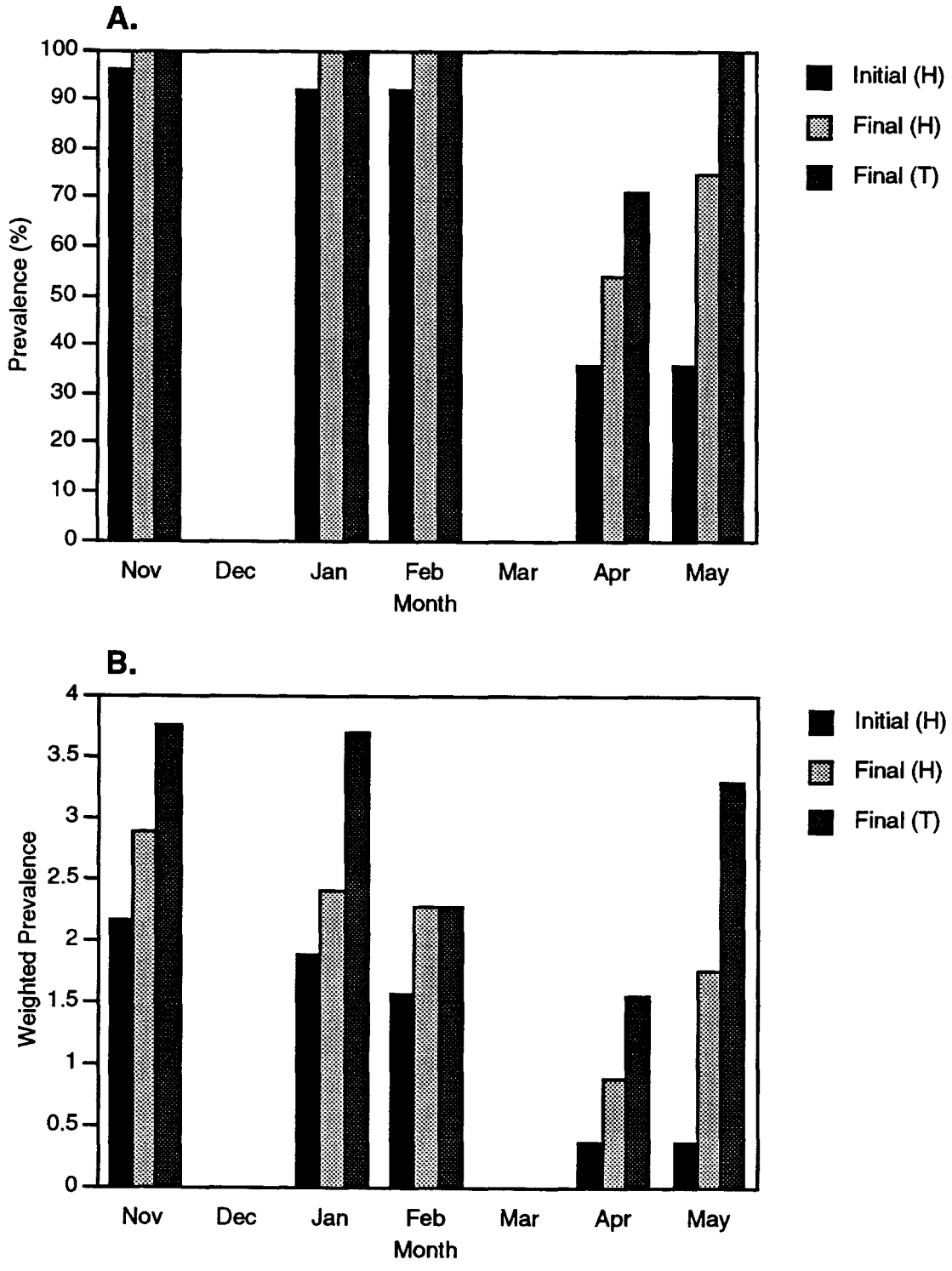

Figure 7. *Perkinsus marinus* prevalence (A) and weighted prevalence (B) in Point of Shoals tray oysters (PST) before (initial) and after warmup (final). Diagnosis before warmup was by hemolymph assay (H). Post–warmup diagnosis was by both hemolymph assay (H) and tissue culture (T).

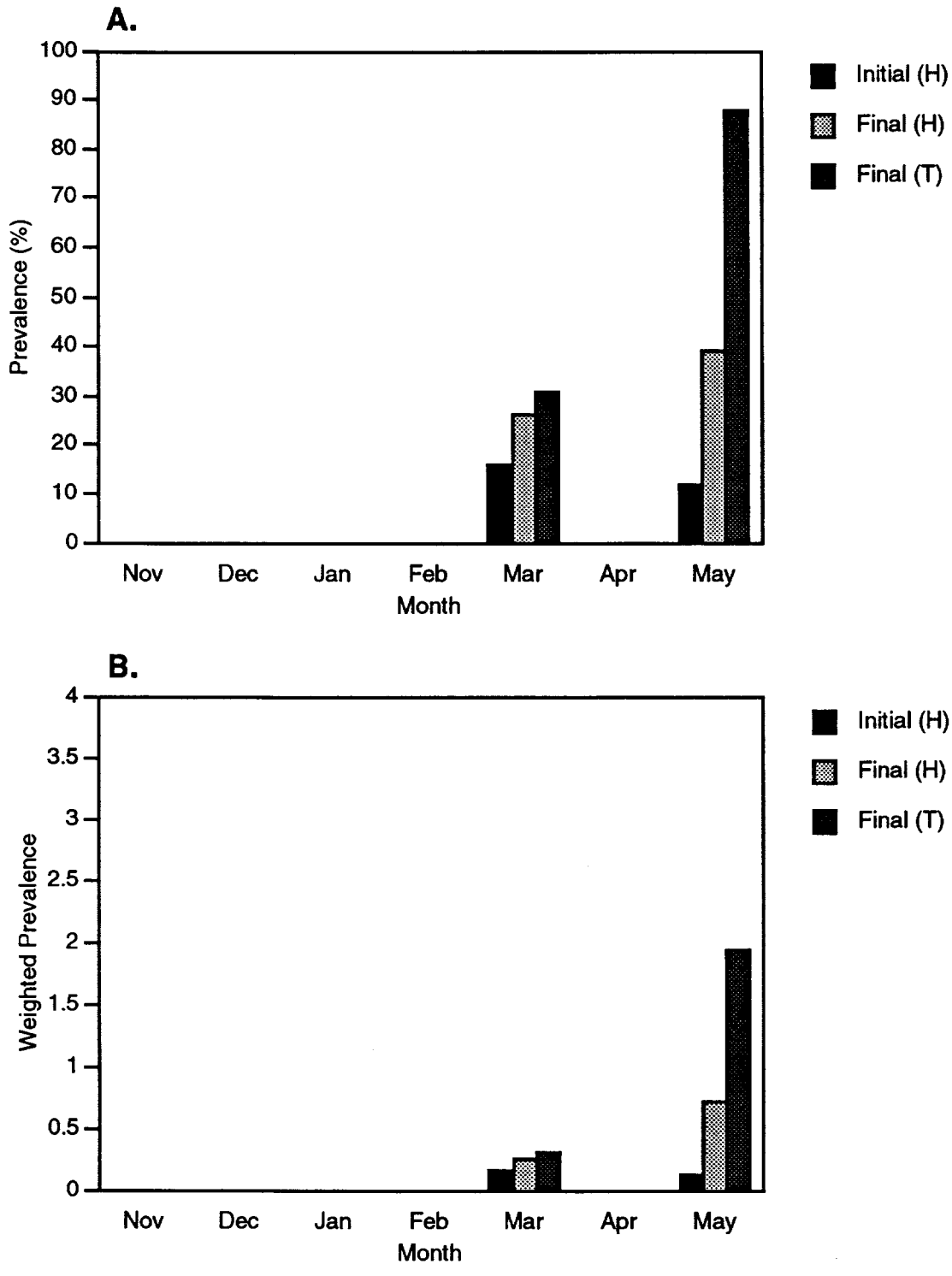

Figure 8. *Perkinsus marinus* prevalence (A) and weighted prevalence (B) in Point of Shoals James River oysters (PSJR) before (initial) and after warmup (final). Diagnosis before warmup was by hemolymph assay (H). Post-warmup diagnosis was by both hemolymph assay (H) and tissue culture (T).

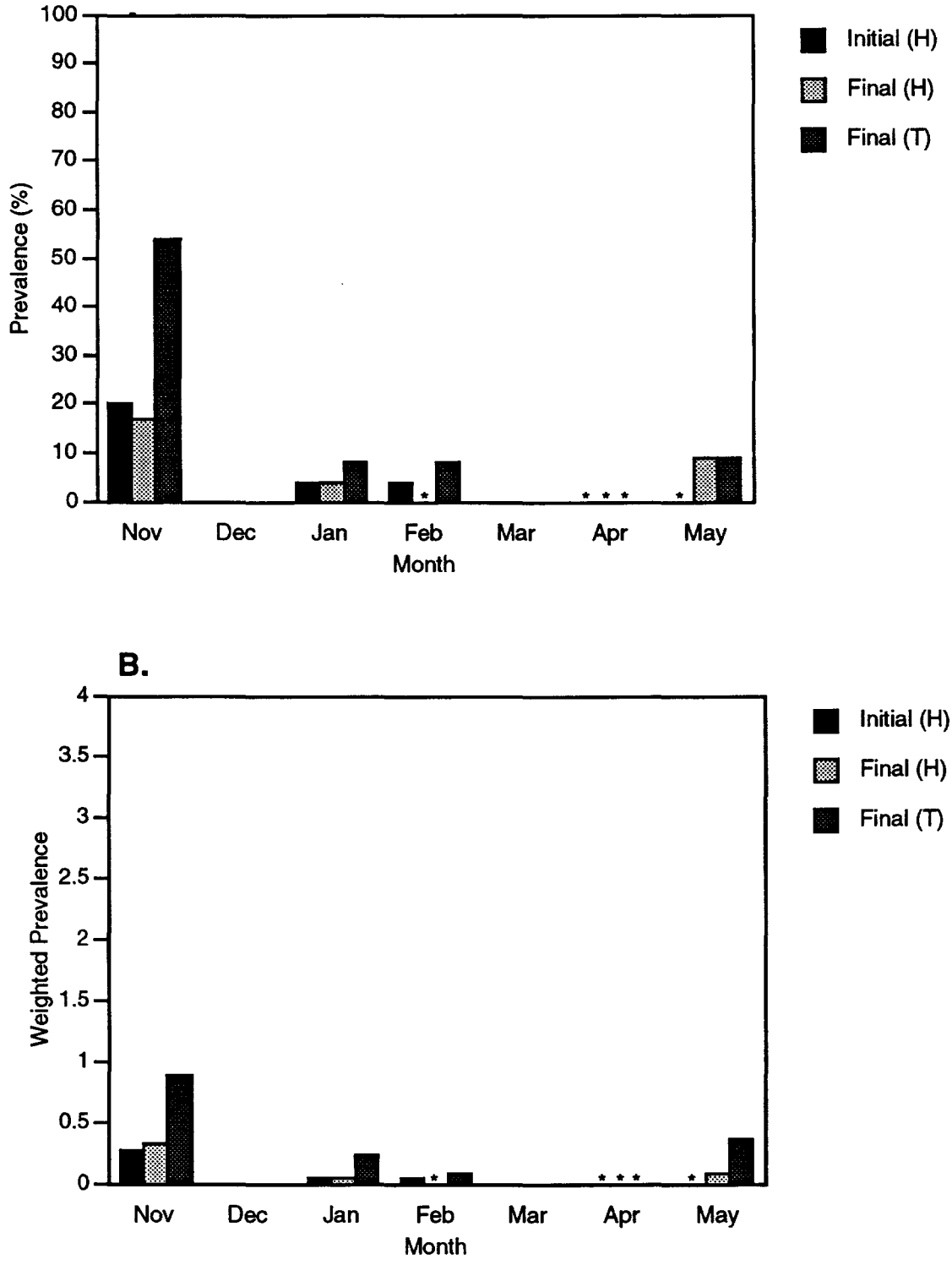

Figure 9. *Perkinsus marinus* prevalence (A) and weighted prevalence (B) in Ross' Rock tray oysters (RRT) before (initial) and after warmup (final). Diagnosis before warmup was by hemolymph assay (H). Post-warmup diagnosis was by both hemolymph assay (H) and tissue culture (T). Asterisks indicate 0% prevalence or weighted prevalence.

Table 1. Experiment 1 sampling schedule. Group 1 oysters were exposed to low temperatures for 3 weeks and group 2 oysters were exposed to low temperatures for 6 weeks.

Treatment and Sample Schedule	Group 1	Group 2
Oysters collected	12/9/91	12/9/91
Initial (t 1) sample	12/12/91	12/12/91
Phase 1		
Day 1 at treatment temperatures (1°C and 4°C)	12/23/91	12/23/91
Time 2 (t 2) sample (hemolymph)	1/14/92	2/3/92
Phase 2		
Day 1 at 12°C and salinity treatments	1/27/92	2/17/92
Time 3 (t 3) sample (hemolymph)	2/11/92	3/2/92
Phase 3		
Day 1 at 25°C	3/2/92	3/20/92
Final (t 4) sample (hemolymph and tissue)	3/30/92	4/17/92

Table 4. Infection intensity categories for experiment 1. Categories 1 (very light) and 2 (light) have been grouped as L, categories 3 (light–moderate) and 4 (moderate) have been grouped as M, and categories 5 (moderate–heavy) and 6 (heavy) have been grouped as H.

	Infection Intensity			
Treatment	Time 1 N-L-M-H	Time 2 N-L-M-H	Salinity	Time 3 N-L-M-H
Group 1				
1°C	18-54-2-1	20-51-3-1	3 ppt	17-8-0-0
			6 ppt	13-11-0-1
			15 ppt	7-14-1-0
4°C	13-58-0-4	13-53-8-1	3 ppt	15-9-0-0
			6 ppt	10-13-1-0
			15 ppt	12-13-0-0
control	10-12-3-0	3-19-3-0		2-17-5-0
Group 2				
1°C	7-65-3-0	12-57-4-2	3 ppt	11-7-0-0
			6 ppt	9-9-0-0
			15 ppt	5-15-0-0
4°C	10-60-4-1	16-50-8-1	3 ppt	11-10-0-0
			6 ppt	6-15-1-0
			15 ppt	10-12-0-0
control	5-18-2-0	5-18-0-2		3-21-1-0

Table 2. Experiment 2: Tray oyster (PST and RRT) sample dates.

Sample date / Warm up initiated	Warm up terminated
18 November 1991	20 December 1991
6 January 1992	14 February 1992
25 February 1992	3 April 1992
3 April 1992	14 May 1992
19 May 1992	17 June 1992

Table 3. Experiment 2: James River oyster (PSJR) sample dates and temperature and salinity at time of collection.

Sample date	Temperature (°C)	Salinity (ppt)
13 November 1991	11.0	12
22 January 1992	4.5	10
18 February 1992	7.5	12
17 March 1992	10.5	5
14 April 1992	16.0	7
14 May 1992	21.0	4

Table 5. Perkinsus marinus infection intensity and weighted prevalence in oysters that were alive (final sample n) at the termination of the experiment (t4). Also shown is the weighted prevalence of the total number of oysters at each treatment (n=25), which includes diagnosis of oysters that died during the experiment and those alive at the termination of the experiment. All diagnosis were by tissue FTM culture.

Treatment		Final sample n	Infection intensity N-L-M-H	Weighted prevalence final sample	Weighted prevalence total
Group 1					
1°C	3 ppt	0			0.76
	6 ppt	0			1.76
	15 ppt	3	0-1-1-1	3.0	1.72
4°C	3 ppt	0			1.12
	6 ppt	0			1.60
	15 ppt	5	0-0-2-3	4.2	2.08
control		17	0-10-1-6	2.47	2.92
Group 2					
1°C	3 ppt	0			0.64
	6 ppt	0			0.56
	15 ppt	6	0-2-0-4	3.67	1.64
4°C	3 ppt	2	0-2-0-0	1.00	0.76
	6 ppt	0			0.80
	15 ppt	8	1-3-2-2	2.65	1.72
control		19	0-5-7-7	3.20	3.56