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CHARACTERIZATION OF OVERWINTERING INFECTIONS OF *PERKINSUS MARINUS* (APICOMPLEXA) IN CHESAPEAKE BAY OYSTERS

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ABSTRACT To determine the nature and abundance of overwintering *P. marinus* infections, infected oysters (*Crassostrea virginica*) collected from the upper James River, VA, were placed in a tray and suspended from a pier in the lower York River, VA in November 1991. Every six weeks through May 1992 oysters (n = 25) were removed from the tray, examined for *P. marinus* by hemolymph culture in fluid thioglycollate medium (FTM), 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 reanalyzed for *P. marinus* by both hemolymph and tissue cultures in FTM. A second group of 25 oysters from the tray was sacrificed at the initiation of each incubation, diagnosed using FTM cultures of hemolymph and tissue, and examined for cryptic stages using immunoassays. On the basis of FTM assays, prevalence of *P. marinus* gradually declined from 100% in November 1991 to 32% in May 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 initial FTM cultures indicated. Immunoassay diagnosis revealed infections in many of the oysters diagnosed as negative by FTM cultures. Most infections detected by immunoassay were comprised of individual *P. marinus* meronts within hemocytes in the midgut epithelial lining. Previously unidentified cryptic stages were not observed. *Perkinsus marinus* appears to overwinter at very low intensities in a high proportion of oysters. Comparison of *P. marinus* prevalence and intensity in transplanted oysters maintained in the York River to that in oysters monitored at the original James River collection site suggests that salinity may greatly influence overwintering infections. Infection intensity and prevalence declined earlier and to a greater extent at the James River site (4–12 ppt) than at the York River location (19–23 ppt). It appears that the synergistic effect of low temperature and low salinity may be more important in regulating *P. marinus* epizootics than either factor acting alone.

KEY WORDS: immunoassay, parasite, prevalence, intensity, oyster, salinity, temperature

INTRODUCTION

Perkinsus marinus (Mackin, Owen and Collier 1950), a protozoan parasite of the eastern oyster, *Crassostrea virginica* (Gmelin 1791), is distributed along the southeastern coast of the United States and in the Gulf of Mexico (Andrews 1988, Andrews and Ray 1988). This subtropical distribution implies the importance of temperature as a regulating factor and temperature is believed to be the most important factor affecting the geographic distribution and activity of *P. marinus* (Ray 1954, Andrews and Hewatt 1957, Mackin 1962, Quick and Mackin 1971, Andrews 1988). The influence of temperature on the activity of *P. marinus* has been well documented. Multiplication and virulence of the parasite are highest at temperatures exceeding 20°C (Ray 1954, Andrews and Hewatt 1957, Mackin 1962, Quick and Mackin 1971, Andrews 1988). Temperatures below 15°C retard *P. marinus* infectivity and infection progression (Ray 1954).

Minimum winter temperature is believed to control the northern limit of the geographic distribution of *P. marinus* and variations in seasonal and annual temperature patterns between northern and southern waters result in important regional differences in the seasonal cycle of the pathogen. Typically, *P. marinus* is present in southern areas at high prevalences throughout the year, declining only slightly during winter months (Andrews and Ray 1988, Soniat and Gauthier 1989, Crosby and Roberts 1990). In contrast, epizootics in the Chesapeake Bay display a more dramatic seasonal periodicity in which peak prevalences in late summer and fall are followed by a precipitous decline during the late winter-early spring months. In late spring as temperature consistently exceeds 20°C the pathogen begins multiplying, new infections are acquired, and prevalence once again increases (Andrews 1988, Andrews and Hewatt 1957).

The scarcity of overwintering infections of *P. marinus* in the Chesapeake Bay has long puzzled investigators. Frequently, oyster grounds having 100% prevalence in the fall exhibit late winter prevalences as low as 0%. The lack of positive diagnoses for *P. marinus* using tissue cultured in fluid thioglycollate medium (FTM) in late winter prompted Andrews and Hewatt (1957) to place oysters collected in winter from an enzootic area having 0% prevalence at the time of collection into warm water aquaria (23 to 28°C) in an effort to detect overwintering infections. Three groups of 15 or fewer oysters were warmed for periods of 60 to 100 days. After warming, *P. marinus* was detected in nearly all of the oysters, but since the oysters were not isolated from one another it was impossible to separate overwintering infections from proximity infections which may have been acquired during the warming period. Based on the time-distribution of deaths the authors speculated that initially only three oysters were infected. This work suggests that some prepatent infections that are not easily disclosed by routine diagnosis using FTM may be present in oysters during the winter months. However, the nature and abundance of overwintering infections of *P. marinus* and their relative contribution to subsequent summer prevalences remains unknown. The objective of this investigation was first to determine the nature of overwintering infections, that is, to determine whether overwintering infections are comprised of typical *P. marinus* cell types or cryptic stages which do not respond to FTM. The second objective was to determine the proportion of oysters that carry overwintering infections and the intensity of such infections in individual oysters.

MATERIALS AND METHODS

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" control oysters (n = 275) were collected on 31 October 1991 from Ross' Rock, Rappahannock River, Virginia. This group served as a control for new infections acquired in either field or laboratory conditions during the investigation. Historically, *P. marinus* had not been detected in oysters sampled in spring and fall from this location, but in recent years Ross' Rock oysters maintained in the laboratory at high salinity and temperature have exhibited infections, suggesting that the parasite may have actually been present at low levels. While probably not parasite free, Ross' Rock has the lowest prevalence of any oyster bed in Virginia and hence was the best local source for native control oysters. 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 (0.1 g/oyster) daily.

On 18 November 1991, 50 oysters were randomly sampled from each group and diagnosed for *P. marinus* using both hemolymph and tissue FTM cultures. 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 were placed in separate mesh trays (120 × 60 × 14 cm) and suspended in the York River from a pier at the Virginia Institute of Marine Science (VIMS), Gloucester Point, Virginia. Transplantation of the oyster population to the York River made it logistically easier to deploy control oysters, and closely monitor oysters and environmental parameters. At the time of deployment water temperature was 12°C and the salinity was 24 ppt. Daily mean temperature and salinity data were obtained from the VIMS York River monitoring program. Means are based on measurements recorded at six minute intervals by a metering system.

Perkinsus marinus prevalence and intensity in the infected oyster population, James River tray group (JRT), was assessed approximately every six weeks from November through May. On each date (November 18, 1991; January 6, 1992; February 25, 1992; April 3, 1992; and May 19, 1992) two groups of 25 oysters were removed from the tray. The first group was sacrificed on the sample date, diagnosed using both hemolymph and tissue FTM cultures, and preserved in Davidson's AFA for later analysis using routine histology and immunoassay. Oysters in the second group were cleaned, individually labeled, notched, and a hemolymph sample was withdrawn for *P. marinus* diagnosis. The oysters were then placed in individual 1-L 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. Filtration of ambient water served to remove infective particles from the ambient water. While we cannot be certain that all infective cells were removed, we have successfully maintained uninfected oysters in previous investigations using the same filtration method. 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. Rappahannock River tray control oysters (RRT) (n = 25) were also sampled and warmed as described above.

As part of the VIMS oyster disease monitoring program native Point of Shoal, James River (JRN) oysters were monitored monthly for *P. marinus*. Each month 25 oysters were collected, sacrificed, and examined for *P. marinus* using tissue FTM cultures. Sample dates and ambient water conditions measured at the time of collection are shown in Table 1. While not originally intended to be a part of this investigation, observed differences in disease patterns between the JRN monitoring samples and JRT samples during the course of this investigation led us to further analyze JRN oysters collected in March and May for prepatent infections. In addition to diagnosis by FTM tissue culture, JRN oysters collected in March and May were preserved for immunoassay diagnosis of *P. marinus* and 25 additional oysters were collected, diagnosed for *P. marinus* using hemolymph assays, and warmed as described above.

Diagnosis of P. marinus

Tissue diagnosis of *P. marinus* was by culture of rectal, mantle, and gill tissue in fluid thioglycollate medium (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 (Mackin 1962). The numerical values for all individuals examined were summed and divided by the total number of individuals examined for the determination of weighted prevalence. Weighted prevalence is the same measure as weighted incidence described by Mackin (1962). The terminology has been changed here based on definitions of prevalence and incidence presented by Margolis et al. (1982). Incidence relates to new cases of infection appearing in a population within a given time and prevalence relates to the number of individuals infected. Given this distinction, we believe that the term weighted prevalence is more appropriate than weighted incidence.

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 sinus using a 3-cc disposable syringe with a 23-gauge needle. The hemolymph was added to a microcentrifuge tube containing 1.0 mL of FTM fortified with 500 units of penicillin and streptomycin. Cultures were incubated in the dark at 27°C for 5–7 days. Following incubation the samples were centrifuged at 400 × g for 10 minutes. The supernatant was removed and the pellet was resuspended in 1.0 mL of 2 M NaOH. The samples were then incubated at room temperature for 30 minutes. Following incubation the samples were washed twice with distilled water and finally

TABLE 1.

James River native oyster (JRN) 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

resuspended in 1.0 mL distilled water and stained 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 light (1–200 cells/well), moderate (200–15,000 cells/well), and heavy (>15,000 cells per well). Weighted prevalence was determined as described above.

Immunoassays and histological examinations were conducted on selected individuals in an effort to detect prepatent infections and possible cryptic states that were not revealed by FTM cultures. It was assumed that immunoassays would highlight rare infections and that if cryptic stages of *P. marinus* were present, they would have been antigenically similar to known stages and recognizable by the polyclonal antibody. Since our main objective was to detect prepatent infections and possible cryptic stages, not to compare techniques, immunoassays were only conducted on oysters that were diagnosed as negative by both tissue and hemolymph FTM cultures. The oysters selected for analysis by immunoassay and histology included 4 JRT oysters sampled in April, 14 JRT oysters sampled in May, 21 JRN oysters sampled in March, and 19 JRN oysters sampled in May. The primary antibody used in the immunoassays was polyclonal, rabbit anti-*P. marinus* raised against hypnospores (supplied by C. F. Dungan, Cooperative Oxford Laboratory, Maryland). Production and specificity of the antibody is described by Dungan and Roberson (1993). Biocell goat anti-rabbit IgG gold probe and Biocell light microscopy silver enhancement 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. Three consecutive 5-μm sections were affixed to separate glass slides, dewaxed in xylene, and hydrated in a graded ethanol series to water. One of the three slides was stained with Harris' hematoxylin and eosin. The remaining two slides were washed in running tap water and phosphate buffered saline (PBS) and blocked for 30 min with 10% v/v normal goat serum in phosphate buffered saline containing 1.0% bovine serum albumin (PBSA). One of the two slides was incubated for 30 min in a 1:100 dilution of primary antibody in PBSA. The other slide, serving as a negative control, was incubated for 30 minutes in a 1:100 dilution of normal rabbit serum in PBSA. The slides were then washed 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, cleared in xylene and covered with a coverglass.

RESULTS

Perkinsus marinus prevalence in James River oysters examined at the initiation of the experiment was 100%. Tissue and hemolymph assays for the detection of *P. marinus* closely corresponded. Tissue diagnosis revealed 8 heavy, 9 moderate, and 33 light infections while diagnosis by hemolymph assay found 5 heavy infections, 6 moderate infections, 36 light infections, and 3 oysters diagnosed as negative. Initial prevalence of *P. marinus* in the Rappahannock River oysters was 8% based on tissue diagnosis and 2% based on hemolymph diagnosis. All infections were light.

During the course of the study, water temperature at the York River transplant site declined from 12°C in November, at the time of deployment, to a low of 3.8°C in mid-February (Fig. 1). Water temperature during the winter months was relatively warm, generally 1–3°C above the long term (1947–1991) average. Salinity ranged from 18.8–23.0 ppt and the mean weekly salinity was 22.1 ppt (S.D. = 1.66).

On the basis of tissue FTM diagnosis of sacrificed oysters, *P. marinus* prevalence in JRT oysters gradually declined during the winter months to 32% in May (Fig. 2). Weighted prevalence followed a similar trend declining from 2.0 in November to 0.56 in May (Fig. 2). Hemolymph assays for *P. marinus* prevalence closely corresponded with tissue diagnosis in November, January and May samples of sacrificed oysters, but were less sensitive in February and April analyses (Fig. 2). In most instances false negatives by hemolymph diagnoses were categorized as very light to light infections by tissue cultures.

In every sample of warmed JRT oysters, prevalence determined after the 30 day incubation period at 25°C was higher than that determined by hemolymph assays prior to incubation (Fig. 3). In November, January, and February *P. marinus* prevalence was >90% prior to warming and increased to 100% after warming. More striking differences in prevalence before and after incubation were observed in April and May. Prevalence increased from 36% to 52% in April and from 36% to 76% in May, based on hemolymph assays. Post warm-up prevalence based on tissue culture diagnosis was 71% in April and 100% in May. Post-incubation determinations of prevalence by hemolymph assays were similar to tissue diagnoses in November through February and lower than tissue diagnoses in April and May (Fig. 3). Weighted prevalence after warming was always higher than before warming. A decline in weighted prevalence, both initial and final, was observed from November through April (Fig. 3).

Some oyster mortality occurred during all five incubations. Mortalities during November, January, February, April, and May incubations were respectively, 36, 20, 12, 4, and 32 percent. Twenty-one of the 26 dead oysters were examined for *P. marinus*. Fifteen of those had moderate to heavy infections, 4 had light infections, and two were uninfected. Both uninfected oysters and one of the oysters with a light infection were found in May.

Rappahannock River tray (RRT) control oysters that were warmed in November exhibited a relatively high prevalence of *P. marinus*, 54%, after warm-up. Post warm-up *P. marinus* preva-

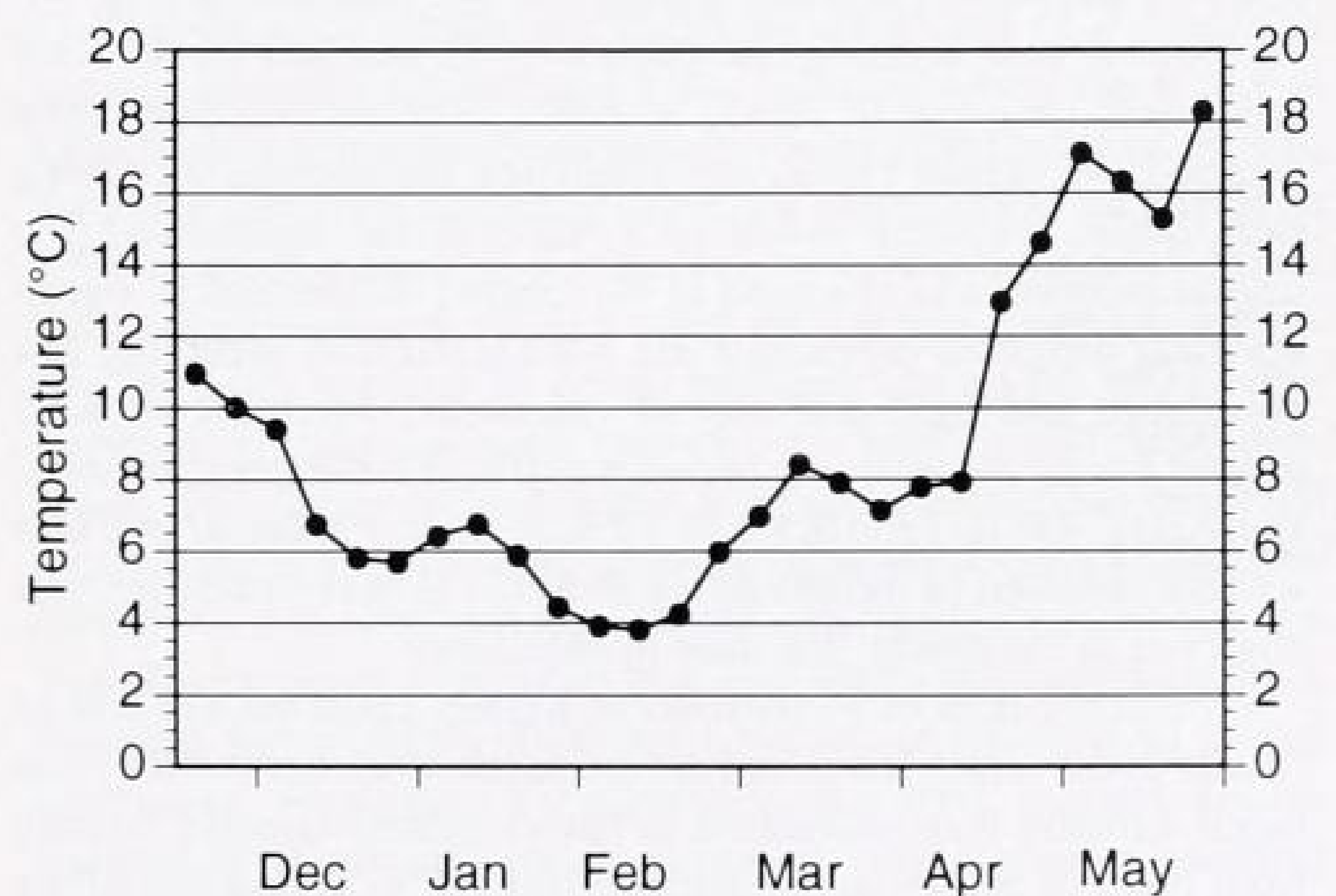


Figure 1. Mean weekly temperature at the VIMS pier York River site from mid-November 1991 through May 1992.

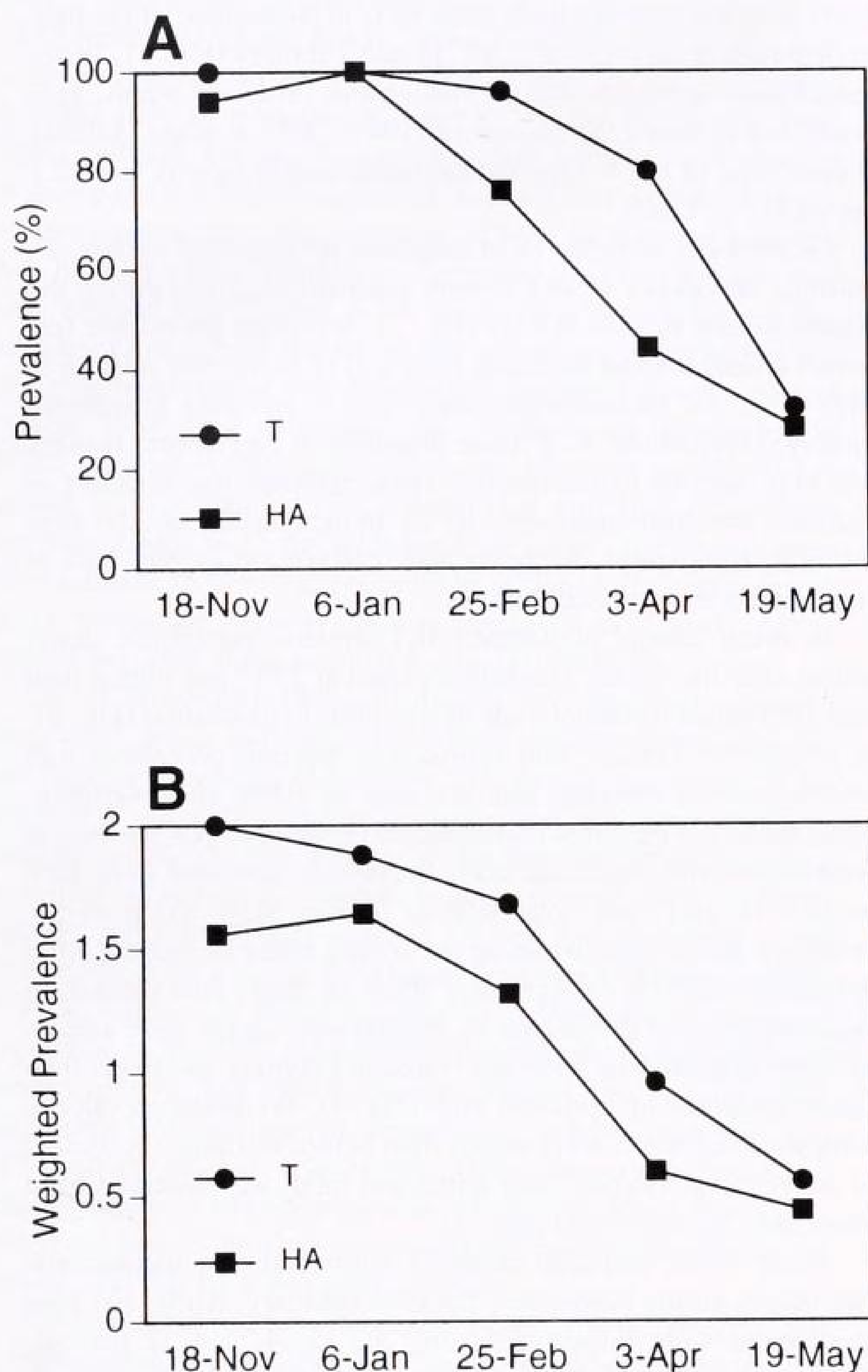


Figure 2. ***Perkinsus marinus*** **prevalence (A) and weighted prevalence (B) in sacrificed James River tray oysters (JRT). Diagnoses were made using both hemolymph (HA) and tissue culture (T) FTM assays. Sample dates (November 1991–May 1992) are shown on the *x*-axis. Sample size = 25.**

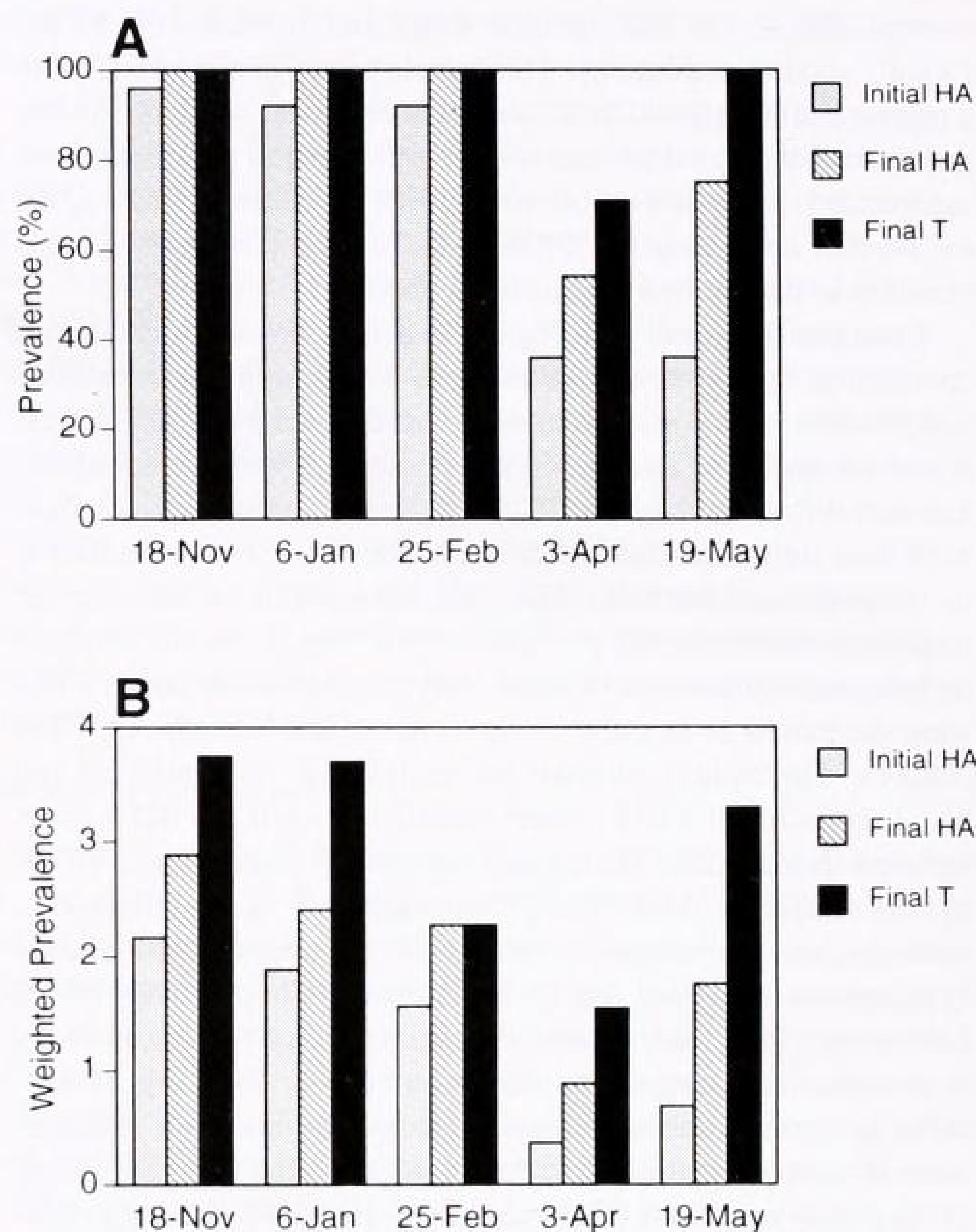


Figure 3. ***Perkinsus marinus*** **prevalence (A) and weighted prevalence (B) in incubated James River tray oysters (JRT) before (initial) and after (final) warm up. Diagnosis before warming was by hemolymph assay (HA). Post warm-up diagnosis was by hemolymph assay (HA) and tissue culture (T). Dates shown indicate date initial samples were taken and warming was initiated.**

lence in subsequent warmed groups did not exceed 9% (Fig. 4). The lowest post warm-up prevalence of *P. marinus* in the RRT group was observed in April, at which time no infections were detected. Weighted prevalence paralleled prevalence, increasing from initial pre-warm levels as a result of the incubation. The largest increase was observed in November; in subsequent warm-ups final weighted prevalence did not exceed 0.4, reflecting low prevalence and light intensity of infections. Mortality of RRT oysters during the warming period was 0% in November, January, and April, 4% in February and 12% in May. *Perkinsus marinus* was not detected in oysters dying in May. A heavy infection was observed in the oyster that died in February.

The prevalence of *P. marinus* in oysters collected at Point of Shoals in the James River and moved to the York River (JRT) was much different than prevalence in native oysters sampled directly from Point of Shoals in the James River (JRN) during the winter (Fig. 5). The prevalence declined much more rapidly in oysters at Point of Shoals than in the tray oysters. Water temperatures were similar at the two locations but salinity greatly differed. Point of Shoals salinity ranged from 4 ppt to 12 ppt while salinity at the York River site varied from 18.8 ppt to 23 ppt. Prevalence in JRN oysters that were warmed in March increased from 16%, based on pre-warm hemolymph assay, to 30.5% after warming. In May prevalence in warmed JRN oysters increased from 12% to 88%.

Immunoassays disclosed infections in many oysters that were diagnosed as negative by both tissue and hemolymph FTM cultures (Table 2). Immunoassays did not reveal a previously unrecognized cryptic stage of the parasite nor did they reveal high intensities of *P. marinus* cells overwintering in the oysters. Infections detected by immunoassays were typically comprised of single or small clusters of *P. marinus* cells within phagosomes of hemocytes in the epithelial lining of the digestive tract, usually the stomach (Fig. 6A,B). Often only one or two cells were observed in an entire preparation. Although the colloidal gold/silver stain assay obscures internal morphology to some degree, *P. marinus* cells appeared to be typical small, coccoid meronts as described by Perkins (1993). Occasionally, a localized positive antibody reaction was observed in stomach epithelium where no identifiable *P. marinus* cell was present. The reaction appeared to be a concentration of the silver stain precipitate; similar areas were not observed in negative control slides. Also observed was a positive antibody reaction inside hemocytes located in connective tissue, usually adjacent to the digestive tract (Table 2). Intact *P. marinus* cells were rarely observed in such instances, rather hemocytes contained a more or less diffuse aggregation of silver stain pre-

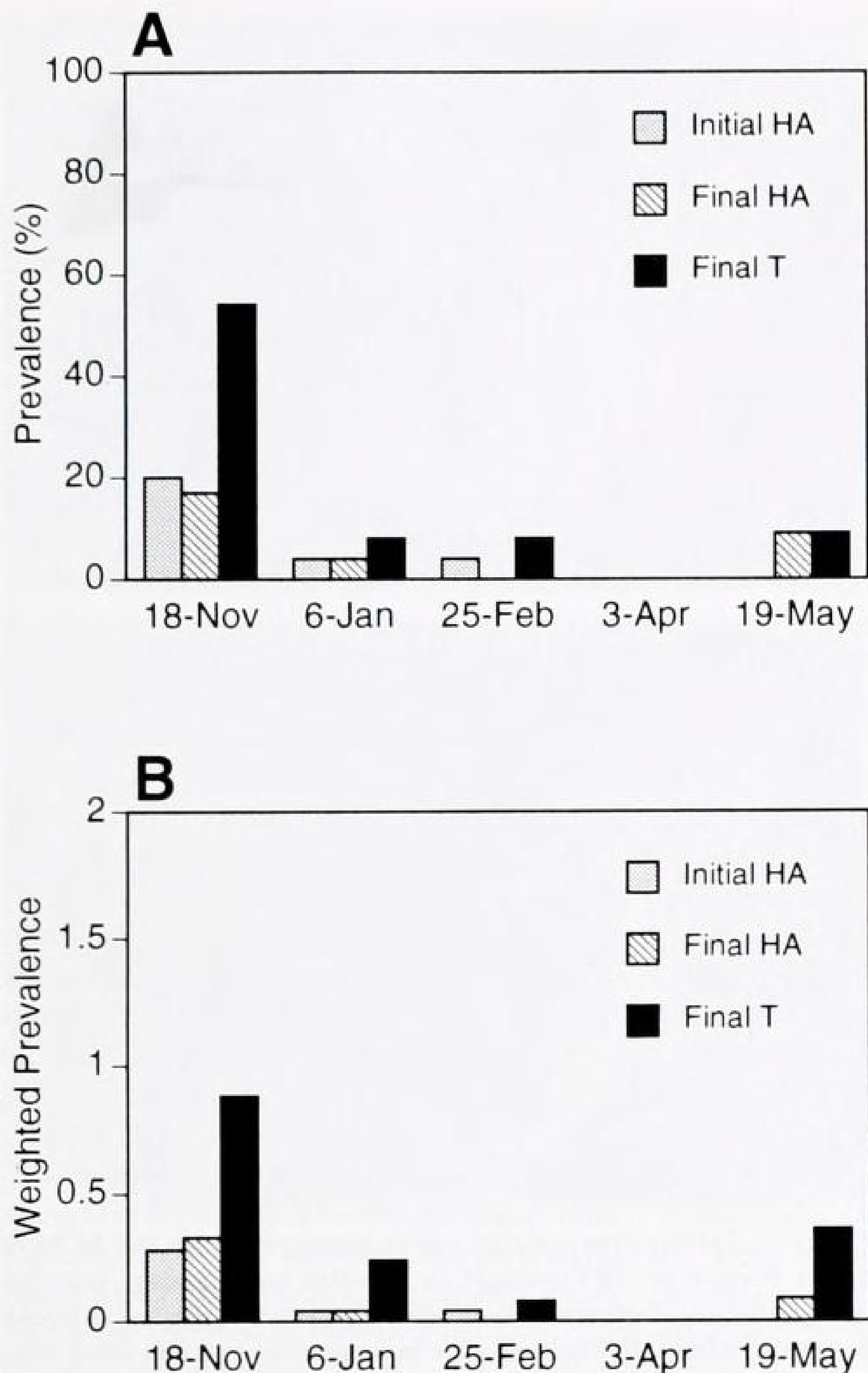


Figure 4. ***Perkinsus marinus*** **prevalence (A) and weighted prevalence (B) in incubated Rappahannock River tray oysters (RRT) before (initial) and after (final) warm up. Diagnosis before warming was by hemolymph assay (HA). Post warm-up diagnosis was by hemolymph assay (HA) and tissue culture (T). Dates shown indicate date initial samples were taken and warming was initiated.**

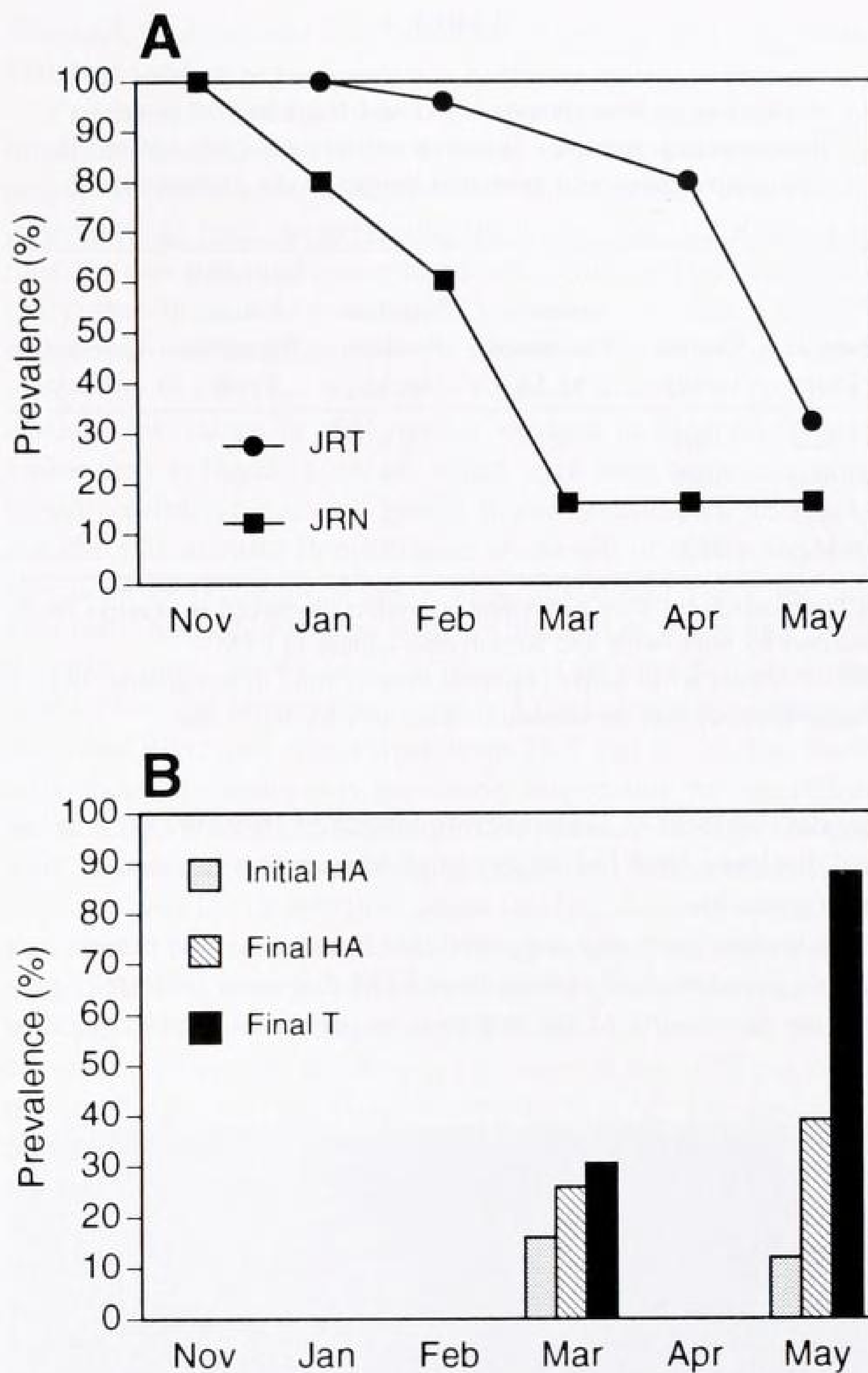


Figure 5. ***Perkinsus marinus*** **prevalence in James River native oysters (JRN) sampled directly from oyster grounds in comparison to that in James River tray oysters (JRT) maintained at the York River (A). Prevalence is based on tissue cultures of 25 oysters. Prevalence of JRN warmed oysters before (initial) and after warm up (final) is shown in B. Diagnosis before warming was by hemolymph assay (HA). Post warm-up diagnosis was by hemolymph assay (HA) and tissue culture (T).**

cipitate (Fig. 7A). In most oysters where this type of positive reaction was observed, a similar, but much less intense reaction was evident in the negative control section (Fig. 7B).

DISCUSSION

Annual epizootics of *P. marinus* in the Chesapeake Bay are believed to be initiated by oysters carrying overwintering infections from the previous fall. These overwintering cases develop into severe infections by August and cause a second generation of infections that are fatal by September (Andrews 1988). Andrews (1988) suggested that most light infections disappear in early winter while oysters carrying advanced infections are likely to die during the winter stress period. He speculated that rare survivors of advanced infections may be the carriers of overwintering infections. The results of the present investigation indicate that the proportion of oysters carrying overwintering infections is not limited to the rare survivors of advanced infections and may actually include 70–100% of the surviving oysters that were infected in the fall.

The ability to detect overwintering infections of *P. marinus* was enhanced by incubating oysters sampled during the winter at temperatures favorable to the multiplication of the parasite prior to diagnosis. Routine diagnosis based on oyster tissue incubated in FTM revealed a gradual decline in *P. marinus* during the winter and spring. However, all incubated JRT groups, with the exception of those warmed in April, had prevalences of 100% after warming. Because warmed oysters were held in individual containers in 1.0-μm filtered water it seems unlikely that observed increases in infections were the result of newly acquired infections during the warming period. Since *P. marinus* is enzootic at the York River site it is possible that new infections may have been acquired during the field exposure period and intensified during incubation. However, prevalence in the RRT control groups warmed in winter and spring remained very low after warming suggesting that new infections, if acquired, accounted for less than 10% of the observed increase in prevalence. Thus, it is most probable that the increase in prevalence during incubation reflects

TABLE 2.

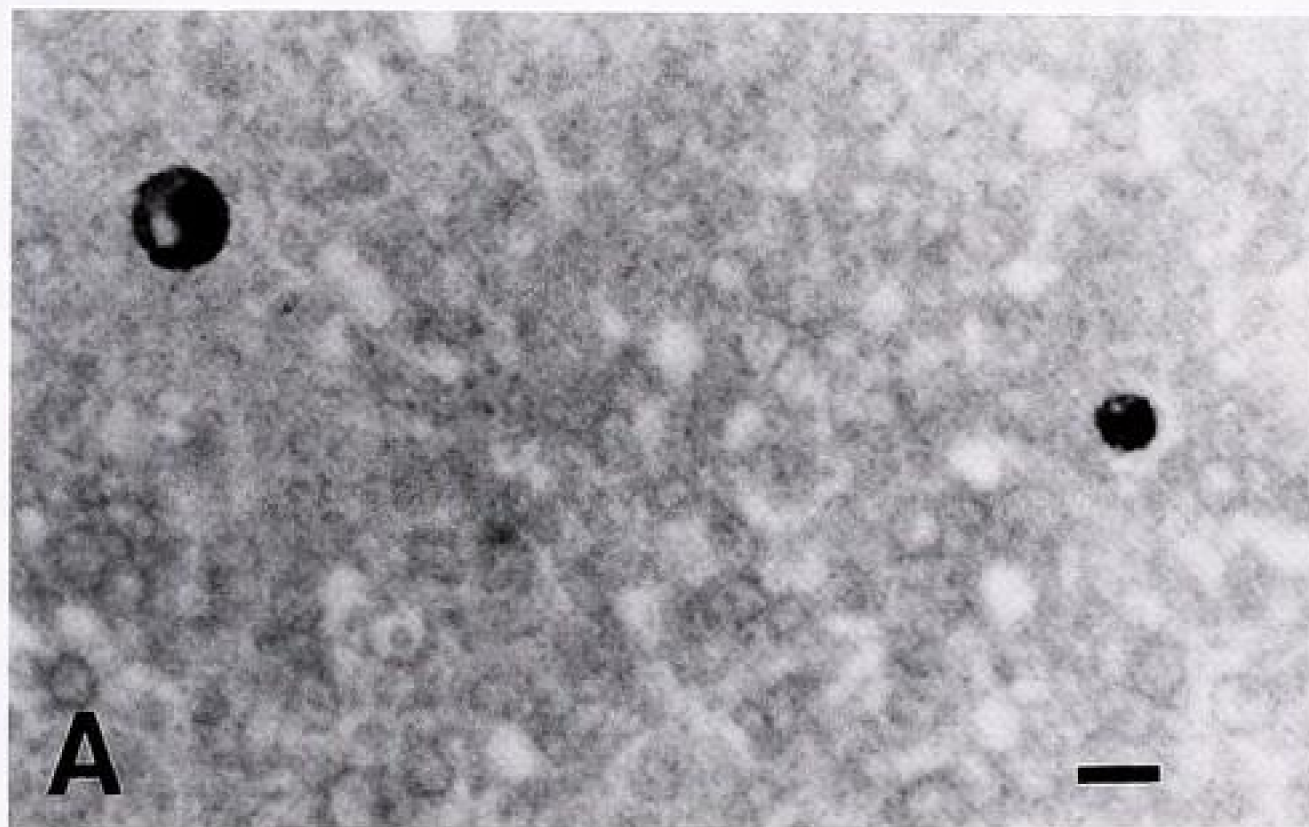
Number of oysters examined and diagnosed as positive for *P. marinus* by immunoassay (IA) and frequency of positive immunoassay reactions observed within hemocytes located in connective tissue and epithelial linings of the digestive tract.

Sample Date	Sample Group	Number Examined by IA	Number Positive by IA	Epithelial Lining Digestive Tract	Connective Tissue
17 Mar	JRN	21	21	18	3
19 May	JRN	19	3	0	3
3 Apr	JRT	4	3	3	0
19 May	JRT	14	7	0	7

All immunoassays were performed on oysters diagnosed as negative for *P. marinus* by both tissue and hemolymph culture in FTM.
JRN = James River natives sampled directly from oyster ground; JRT = James River oysters maintained in trays at York River site.

the development of overwintering infections that were present but not disclosed by FTM assays prior to warming because of their light intensity.

Immunoassays also suggested that *P. marinus* was present in a higher proportion of oysters than FTM diagnosis indicated, supporting the results of the warming experiments, and suggesting



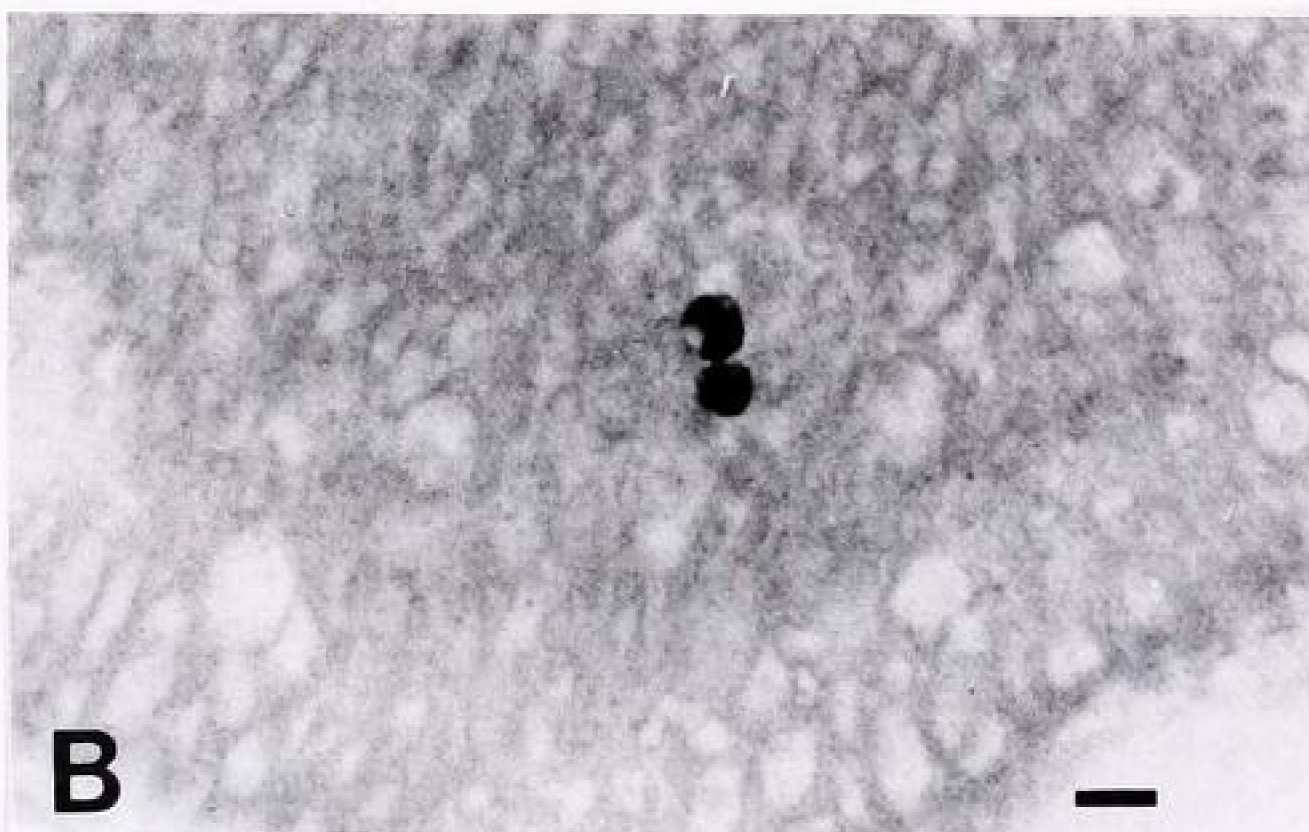
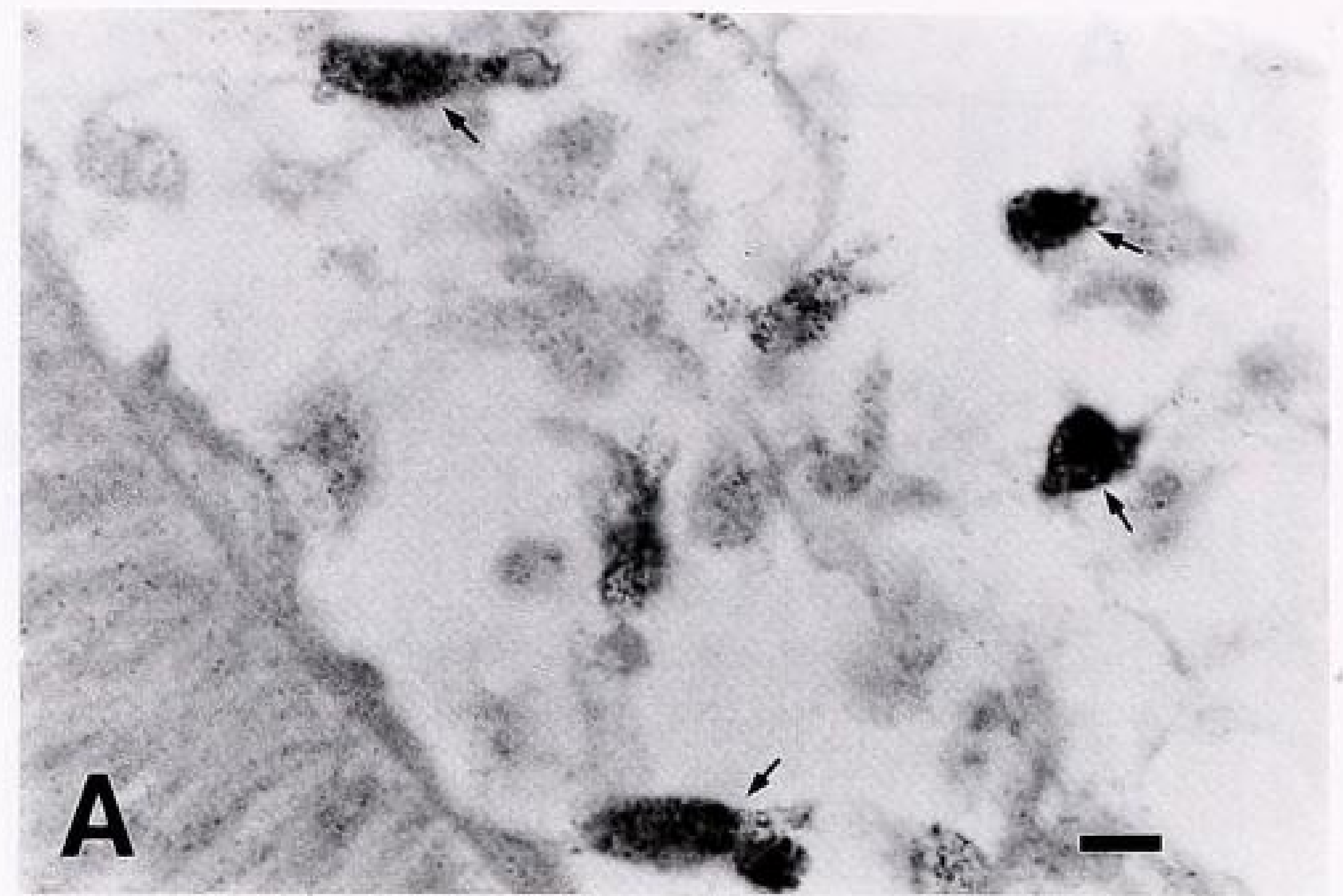


Figure 6A, B. Photomicrographs of *P. marinus* meronts within hemocytes in the gut epithelium of separate oysters visualized with colloidal gold immunoassay using anti-*P. marinus* antibody and fast green counter-stain. Oysters are from the 17 March James River native (JRN) sample. Scale bars = 5 μm.



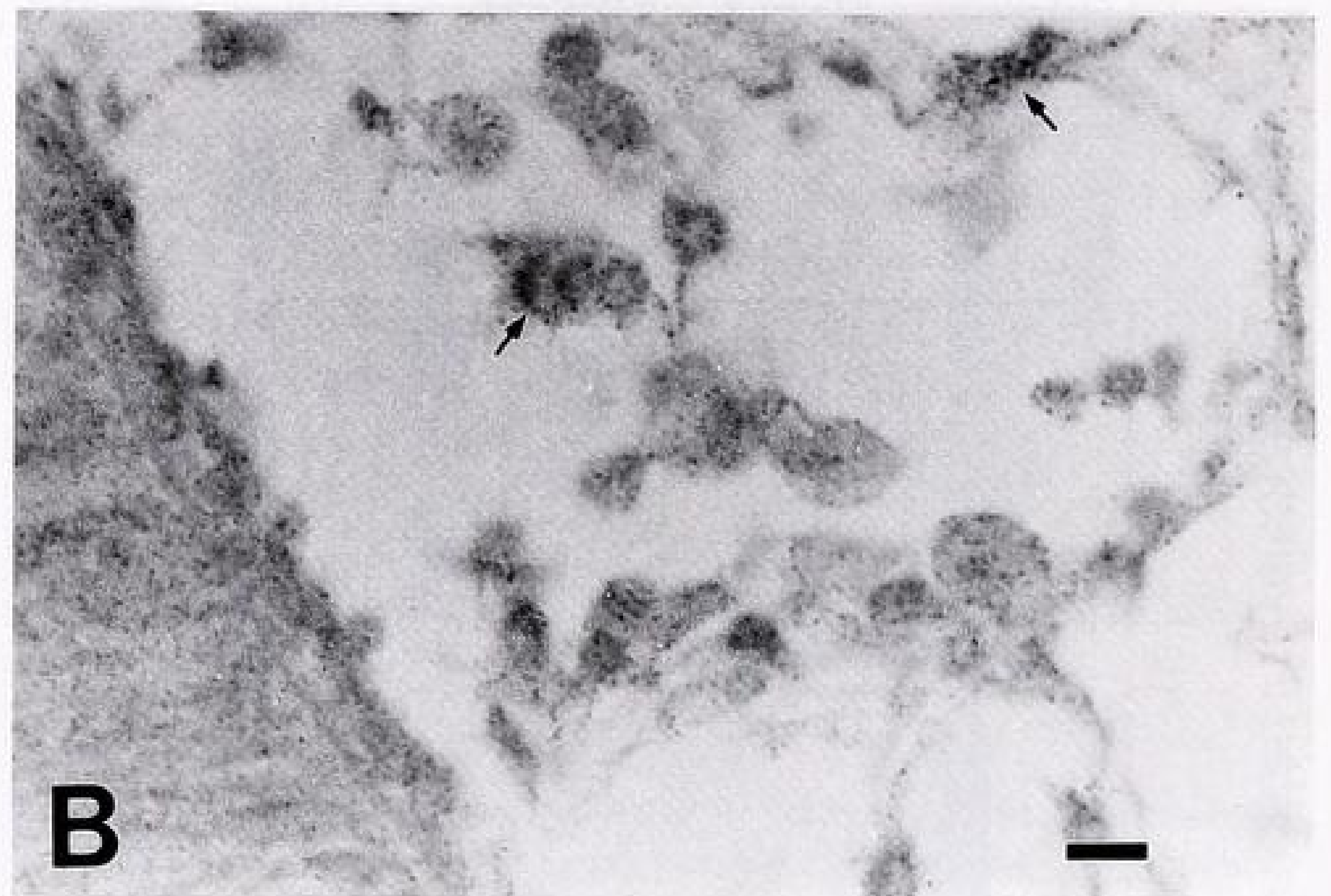


Figure 7. Photomicrographs of oyster hemocytes from the 19 May James River tray (JRT) sample. A: Positive immunoassay reaction using anti-*P. marinus* antibody in oyster hemocytes (arrows) adjacent to gut epithelium. B: Weak positive immunoassay reaction using normal rabbit serum (negative control) in oyster hemocytes (arrows) from the same oyster and area as in A. Scale bars = 5 μm.

that *P. marinus* overwinters at very low intensities in a high proportion of oysters. Immunoassays did not reveal the presence of previously unrecognized *P. marinus* cell types or "cryptic stages" but did detect very light infections of *P. marinus* in many oysters that were diagnosed as negative by FTM cultures of tissue and hemolymph. In most instances, particularly in March and April samples, typical *P. marinus* meronts were observed within hemocytes located in epithelial cells of the midgut.

The restriction of overwintering *P. marinus* meronts to hemocytes in the gut epithelium may be related to parasite expulsion involving the transport of parasite cells to the gut for elimination. Andrews and Hewatt (1957) and Ray (1954) suggested that at temperatures below 20°C, the metabolic activity of the parasite slows enabling the host to combat infections more effectively. 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 oysters may eliminate *P. marinus* in this manner. Feng and Feng (1974) observed phagocytosis and migration of oyster hemocytes at temperatures as low as 6°C so it would be possible for elimination of *P. marinus* to occur during the winter months. The continuous decline in *P. marinus* intensity in the oysters sacrificed in this

study from November through April may reflect the elimination of *P. marinus* via host defense processes. In May a strong positive reaction to the antibody was observed in hemocytes located in connective tissue, although few intact *P. marinus* cells were observed. It is possible that the antibody was reacting with soluble antigen or noncellular particulate matter from degraded *P. marinus* cells. The antibody utilized is known to react with soluble and noncellular particulate substances in and around *P. marinus* lesions (Dungan and Roberson 1993) and intracellular killing of *P. marinus* meronts by hemocytes at low temperature (15°C) has been observed (La Peyre et al. 1992). Perhaps by May each year hemocytes have eliminated or degraded most of the *P. marinus* cells. Unfortunately, hemocytes in negative control slides, in which the primary antibody was substituted with normal rabbit serum, also reacted to the silver stain, but to a lesser degree. Although the reaction in the control slides was less intense than in the treatment sections, the presence of any positive reaction at all in negative control slides prevents us from concluding with certainty that the reaction observed in the section exposed to the antibody was a specific reaction for *P. marinus*. Additional research on the nature of the positive antibody reaction in oyster hemocytes is needed.

While expulsion or degradation of the pathogen by the host may have been occurring during the winter months, high *P. marinus* prevalences after warming indicate that the pathogen was not completely eliminated. The increase in weighted prevalence during the incubation period suggests that even very light infections can increase to lethal levels in a 30 day period when temperature exceeds 25°C.

Correspondence between hemolymph and tissue assays for *P. marinus* was variable. The two techniques closely corresponded in November, January, and May; however, during late winter when lighter infections prevailed, hemolymph assays were less sensitive than tissue cultures. In February and April, 20% and 45% respectively of the examined oysters were falsely diagnosed as uninfected using the hemolymph assay. This result suggests that there may be a seasonal pattern associated with the abundance of *P. marinus* in oyster hemolymph and contradicts the results of Gauthier and Fisher (1990) which suggested that the hemolymph assay technique is more sensitive than the tissue culture method. Differences in technique and season may account for the lower assay sensitivity found in the present investigation. Gauthier and Fisher (1990) conducted their comparison using oysters collected from the Gulf of Mexico in the late spring and summer when *P. marinus* infection intensities are relatively high. In addition, they used only mantle for tissue analysis and 1.0 ml of hemolymph. In this investigation a smaller volume of hemolymph (0.3 ml) and a combination of mantle, gill, and rectal tissue was utilized.

Perkinsus marinus prevalence in oysters collected at Point of Shoals in the James River and moved to the lower York River greatly differed from prevalence in Point of Shoals oysters collected directly from the site during the winter. The prevalence and intensity declined much more rapidly in oysters at Point of Shoals (JRN) than in the tray oysters (JRT). Further, prevalence of JRN oysters warmed in March did not exceed 40%, remaining much lower than JRT oysters warmed in February and April. *Perkinsus marinus* prevalence in JRN oysters warmed in May (88%) was higher than in March; however, since there were no in situ uninfected controls at Point of Shoals it is impossible to determine whether this increase in prevalence is a result of newly acquired infections or prepatent overwintering infections. There are undoubtedly many differences between the James River and York River locations, but the most prominent is salinity. Salinity at the James River site ranged from 4 ppt to 12 ppt, while the salinity at the York River tray site varied from 18.8 ppt to 23 ppt. Such differences in salinity may be largely responsible for the differences in prevalence and intensity of *P. marinus* between the two sites. Salinity at the James River location from November through February was 10–12 ppt. Prevalence steadily declined during this time, but a more pronounced decrease was observed from February to March as increased river flow caused a reduction in salinity to 5 ppt. Ragone and Burreson (1993) did not see a decline in prevalence in oysters exposed to salinities as low as 6 ppt for a period of eight weeks at temperatures above 20°C. The decline in prevalence observed in James River oysters in the present investigation may be a result of the synergistic effect of low temperature and low salinity. Although some degree of parasite expulsion may occur at cold temperature and salinities of 18–23 ppt, as observed in the JRT oyster population, elimination was enhanced at salinities below 12 ppt as observed in the JRN population. Although this study was not designed to examine the effect of temperature and salinity on prevalence and intensity of *P. marinus* infections, results suggest that a combination of low temperature and low salinity has more effect on reducing the abundance of *P. marinus* than either factor acting alone.

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