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Comparison Of Infectivity And Pathogenicity Of Meront (Trophozoite) And Prezoosporangiae Stages Of The Oyster Pathogen Perkinsus-Marinus In Eastern Oysters, Crassostrea-Virginica (Gmelin, 1791)

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COMPARISON OF INFECTIVITY AND PATHOGENICITY OF MERONT (TROPHOZOITE) AND PREZOOSPORANGIAE STAGES OF THE OYSTER PATHOGEN *PERKINSUS MARINUS* IN EASTERN OYSTERS, *CRASSOSTREA VIRGINICA* (GMELIN, 1791)

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ABSTRACT Two experiments were conducted to compare the infectivity and pathogenicity of two life stages of the parasite *Perkinsus marinus*, meronts (trophozoites) and prezoosporangia (hypnospores), in eastern oysters, *Crassostrea virginica*. Oysters were inoculated with 5×10^4 meronts or prezoosporangia per oyster by injection into the shell cavity. Prevalence and intensity of *P. marinus* infections, condition index, serum protein concentrations, and lysozyme activities were measured in oysters after 15, 25, 40, and 65 days in experiment 1 and after 20, 40, 50, 65, and 75 days postchallenge by *P. marinus* cells in experiment 2. Controls were injected with filtered York River water. In the first experiment, *P. marinus* infections were initially detected in oysters exposed to prezoosporangia after 15 days postchallenge. In the second experiment, infection was not detected in oysters until 40 days postchallenge with either meronts or prezoosporangia. Intensity and prevalence of *P. marinus* infection were significantly higher ($p < 0.002$) in oysters challenged by meronts compared with prezoosporangia-challenged oysters at the end of both experiments. In experiment 1, a significant decrease ($p < 0.05$) was observed in serum protein in infected oysters challenged by prezoosporangia compared with uninfected oysters. Condition index was higher in uninfected oysters compared with infected oysters challenged by prezoosporangia. The differences in condition index and protein were insignificant between oysters infected by meronts or prezoosporangia. Lysozyme activities were significantly lower ($p < 0.05$) in infected oysters than in uninfected oysters challenged with meronts. No significant differences were observed in condition index, protein concentrations, and lysozyme activities between oysters challenged by meronts and prezoosporangia in experiment 2. Lower condition index and protein concentrations in the groups of oysters infected with prezoosporangia compared with the groups infected by meronts and nonchallenged at the end of experiment 1 suggest a higher energetic demand on these oysters.

KEY WORDS: *Perkinsus marinus, Crassostrea virginica,* oyster disease, lysozyme, condition index, protein

INTRODUCTION

The once-thriving oyster industry in the Chesapeake Bay and the East Coast of United States has been threatened by overfishing and diseases caused by two protistan parasites, *Haplosporidium nelsoni* (MSX) and *Perkinsus marinus* (Dermo). The effects of the diseases caused by the two protists have been well documented (Andrews 1988, Barber et al. 1988, Ford 1988, Ford and Figueras 1988, Chu et al. 1993, Chu and La Peyre 1993a and b, Paynter and Burreson 1991). Since 1986, *P. marinus* has reportedly caused greater oyster mortalities in lower Chesapeake Bay than *H. nelsoni* (Andrews 1988).

The life history of *P. marinus* was studied in detail by Perkins (1966). Three life stages were identified, namely, merozoites, prezoosporangia, and the biflagellated zoospores. Immature meronts (merozoites), usually found in the phagosomes of hemocytes are 2 to 4 μm in size and coccoid, with a fibrogranular wall. As the cells mature, they enlarge to about 10 to 20 μm with an eccentrically placed vacuole, which often contains a refringent vacuoplast. The mature meronts, on repeated karyokinesis and cytokinesis, yield sporangia (schizont, 10 to 40 μm in size), an 8 to 32 cell stage enclosed within a mother cell wall (Perkins 1966). Enlargement of meronts to form prezoosporangia is achieved by incubating the meronts in fluid thioglycollate medium (FTM) (Ray 1952). The prezoosporangia are characterized by an extremely large vacuole, which compresses the cytoplasm into a thin layer against the cell wall. On enlargement, the vaculoplast disappears and the nucleus attains a sausage shape, with numerous small lipoid droplets dispersed inside the cell.

Numerous field (Soniat 1985, Craig et al. 1989, Soniat and Gauthier 1989, Crosby and Roberts 1990, Gauthier et al. 1990, Burreson 1989 and 1990) and laboratory studies (Mackin 1951, 1956 and 1962, Andrews and Hewatt 1957, Perkins 1966, Chu and La Peyre 1989, Ragone 1991, Ragone and Burreson 1993) have investigated the effects of temperature and salinity on the disease processes of *P. marinus* in eastern oysters. Other previous laboratory experiments induced *P. marinus* infection through exposure of oysters to meronts, merozoites, and schizonts contained in unpurified or partially purified infected oyster tissue (Chu & La Peyre 1993a, Hewatt & Andrews 1956, Mackin 1962). For convenience, the cellular stages found in oyster tissue will hereafter be termed meronts, with the recognition that merozoites and schizonts are also present.

In nature, the meronts (3 to 15 μm) rarely enlarge to a size of 15 to 100 μm in moribund oysters and when enlarged are called prezoosporangia (Perkins 1966). Prezoosporangia, when placed in seawater, divide by successive bipartitioning and form biflagellated zoospores (Perkins 1966, Chu and Greene 1989). Whereas slightly enlarged cells, believed to be prezoosporangia, can be found in moribund oyster, such cells have never been isolated and induced to form zoospores. The presumption is that they have the capability to zoosporulate. Because the exposure of oysters to minced oyster tissue containing meronts or freshly isolated and partially purified meronts results in a high prevalence of *P. marinus* infection, Perkins (1966) suggested that meronts and merozoites may be the primary infective agents transmitting disease among oysters in the field, with the recognition that zoospores also can induce infections. However, similar infection rates were found by exposing oysters to prezoosporangia and biflagellated zoospores in our laboratory (Chu et al., unpublished results). These

results suggest that all three life stages, namely, meronts, prezoosporangia, and biflagellated zoospores, are capable of inducing infection in oysters, although some of the previous studies have used minced infected oyster tissue (Hewatt and Andrews 1956) or minced infected oyster tissue incubated in thioglycollate medium in 1 day (Mackin 1962). Therefore, the infective cells used in previous studies would mostly be meronts with some prezoosporangia. None of the previous studies have examined purified prezoosporangia as an infective agent, nor were the physiopathological effects investigated. This article reports the results of experiments in which the infectivity and pathogenicity of meronts and prezoosporangia were compared. The physiological responses of oysters challenged by these two infective stages were also determined.

MATERIALS AND METHODS

P. marinus *Diagnosis*

P. marinus infections were diagnosed using hemolymph and tissue assays (Gauthier and Fisher 1990, Ray 1952 and 1966). The hemolymph assay was as follows: 300 µl of hemolymph containing hemocytes was obtained and incubated in FTM containing antibiotics (penicillin and streptomycin) for 4 days. After incubation, the thioglycollate medium was separated by centrifugation at 800 × *g* and incubated with 1N NaOH for 1 hour to remove tissue debris and hemocytes. The suspension was then washed twice with water, and prezoosporangia were stained with Lugol's iodine and counted. Disease intensity was ranked from 1 to 5 (light to heavy). At the end of each experiment, infections were also diagnosed according to the method of Ray (1952) by incubating pieces of rectal and mantle tissue in FTM. Infection intensities were rated as light to heavy (1 to 5), and weighted indices were calculated based on Ray (1954) and Mackin (1962).

Lysozyme Activity

Lysozyme activity (L) was determined spectrophotometrically according to Shugar (1952) and modified by Chu and La Peyre (1989). Briefly, 0.1 ml of cell-free oyster serum was added to 1.4 ml of bacterial (*Micrococcus lysodiekticus*) suspension. The decrease in absorbance at 450 nm on a Shimadzu UV 600 spectrophotometer was measured after 1 minute. Results are expressed as units per ml of oyster serum. One unit is defined as decrease in absorbance of 0.001 in the bacterial suspension per minute at room temperature (22–23°C).

Serum Protein Concentration

The concentrations of serum protein (P) were measured spectrophotometrically according to Lowry et al. (1951) with bovine albumin as a standard.

Experiments

Two experiments were conducted to compare the pathogenic effects of meronts and prezoosporangia.

Experiment 1

Eastern oysters were collected from the Ross' Rock area of the Rappahannock River, Virginia (ambient salinity, 6 ppt; ambient temperature, 19°C). Oysters from this location have the lowest prevalence of *P. marinus* infection of any oyster bed in Virginia (Ragone Calvo and Burreson 1994). Oysters were gradually acclimated over a period of 6 weeks to the test conditions (temperature, 25.6 ± 1.3°C; salinity, 20.7 ± 1.04) in a 200 l tank. Ninety-six oysters were then randomly placed in aerated individual chambers with flowing 1 µ filtered York River water (YRW). Oysters were fed daily during the acclimation and the experimental period with algal paste (0.1 g per oyster, using a mixture of *Isochrysis galbana, Pavlova lutheri,* and Tahitian *I. galbana*), and water was changed every other day. Meronts were partially purified from infected oyster tissue according to Chu and La Peyre (1993a). Prezoosporangia were cultured on the basis of the method described by Chu and Greene (1989). One hundred microliters of filtered YRW containing 5×10^4 meronts or prezoosporangia cells (meronts cultured in FTM and enlarged to size range of >100 µm) was injected into the shell cavity of each oyster. Controls were injected with 1 µm filtered YRW. There were three treatments: control, meront-challenged, and prezoosporangia-challenged oysters. To monitor infection development, eight oysters were randomly sampled from each treatment at 15, 25, 40, and 65 days postchallenge. Hemolymph samples were withdrawn from the anterior adductor muscle of individual oysters with a syringe with a 27 gauge needle. Serum L and P concentration were measured. Hemolymph was also assayed to evaluated *P. marinus* infection (Gauthier and Fisher 1990). After withdrawal of hemolymph samples, oysters were sacrificed and condition index (CI) (dry meat weight/dry shell weight × 100; Lucas and Beninger 1985) was determined. *P. marinus* infections in oysters were also diagnosed using rectal and mantle tissue according to the tissue assay described by Ray (1952).

Experiment 2

The experimental conditions were similar to those of experiment 1, with the exception that oysters were collected from the Damarsicotta River, Maine, a region out of the range of *P. marinus* (ambient salinity and temperature, 32 to 35 ppt and 12 to 14°C, respectively). As in experiment 1, oysters were gradually adjusted to the test conditions (temperature, 21.78 ± 0.84°C; salinity, 20.5 ± 1.19 ppt) in 6 weeks, and then, 135 oysters were randomly placed in individual chambers with 1 µm filtered aerated YRW. Nine oysters from each treatment were sampled at the end of 20, 40, 50, 65, and 75 days after being challenged with infective particles. Measurements of CI and serum L and P were conducted in individual oysters as indicated above.

Statistical Analyses

A one-factor analysis of variance (ANOVA) followed by a Tukey-Kramer test was used to determine the differences in CI, L, and P among treatments. The data were first analyzed for differences among treatments and sampling times. Some of the oysters were not infected after they were challenged with meronts or prezoosporangia. CI, L, and P data from challenged oysters at all sampling times from experiment 1 were split into infected and uninfected oysters. Data from uninfected oysters from each treatment at all sampling times were pooled with the controls. This resulted in three groups, namely, uninfected, meront infected, and prezoosporangia infected. Data were then reanalyzed with one-way ANOVA to determine differences among groups. In experiment 2, CI, L, and P data were analyzed with one-way ANOVA without being split into infected and uninfected groups. Logistic regression (Agresti 1990) was used to determine differences in prevalence of infection between treatments and sampling times in both experiments.

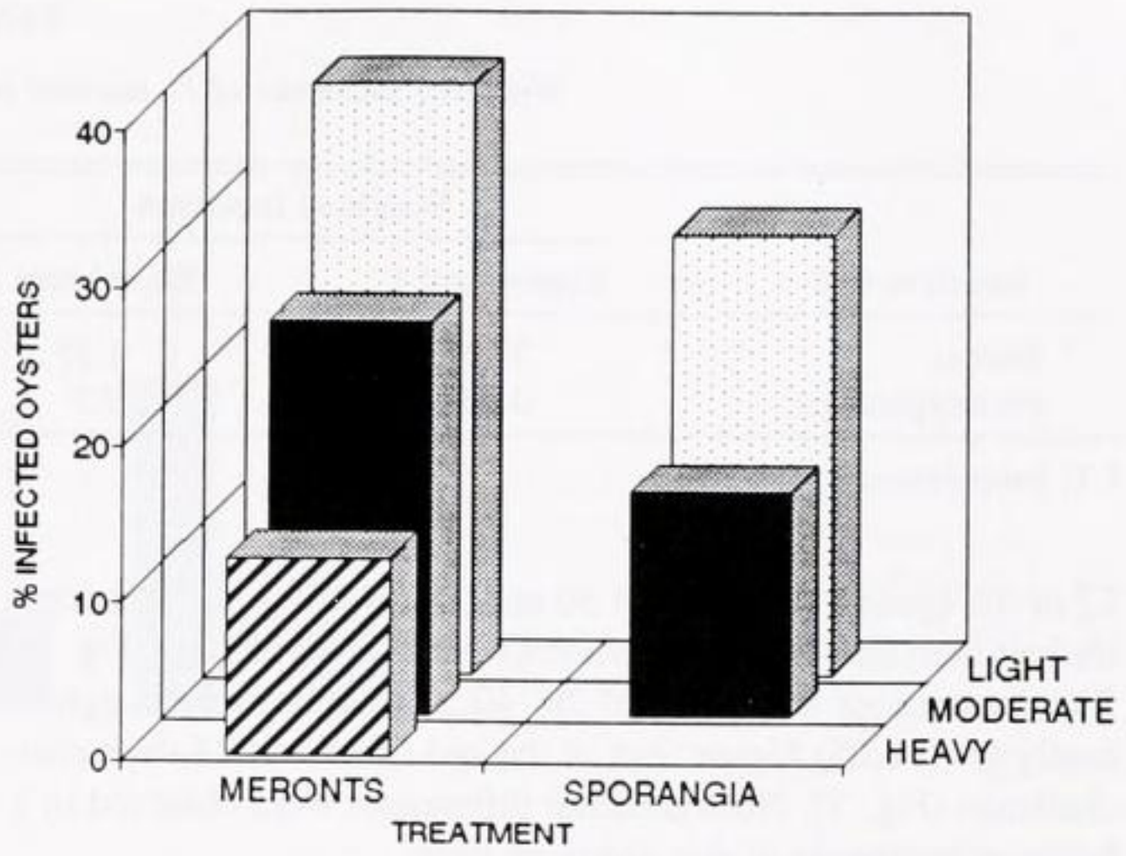
RESULTS

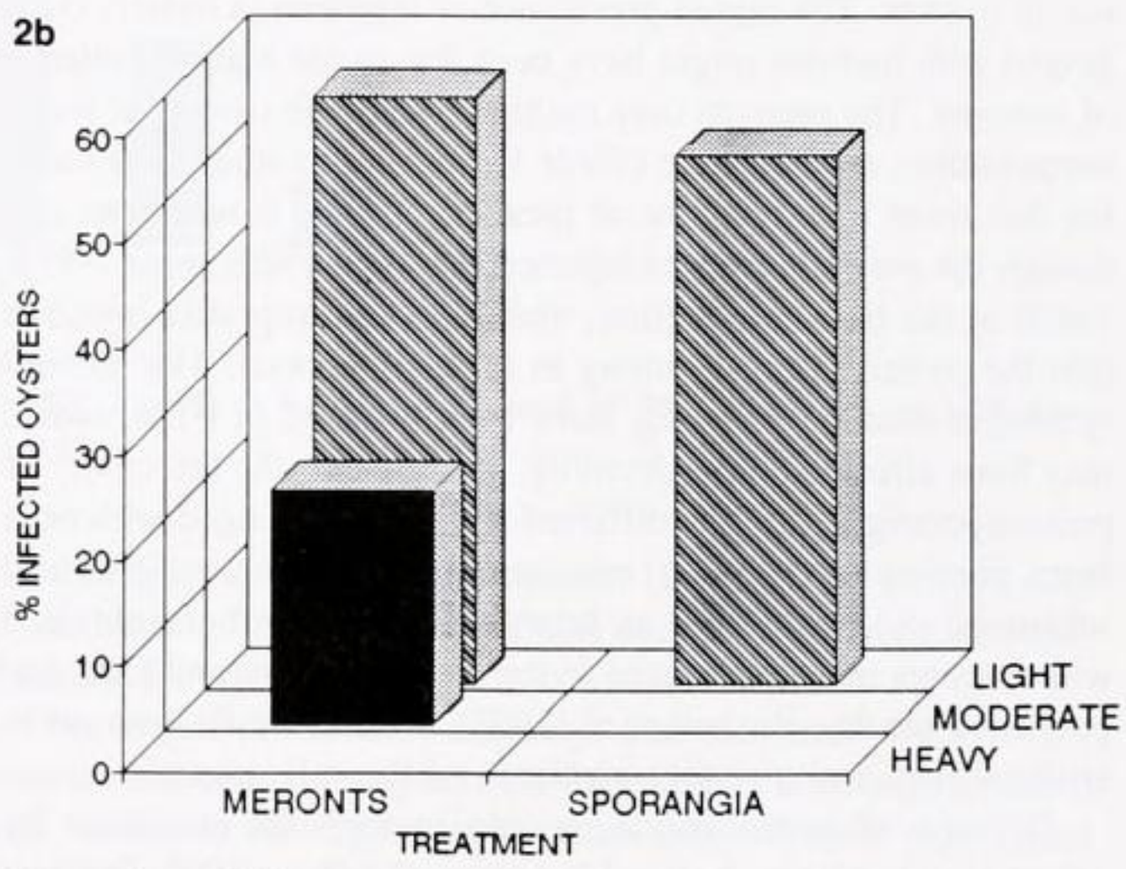
In experiment 1, infection first appeared in oysters 15 days after being challenged with prezoosporangia and 25 days after

challenge with meronts (Fig. 1a). Prevalence, at 65 days postchallenge, was higher in oysters challenged by meronts (87.5%) compared with oysters challenged by prezoosporangia (43%) (Fig. 1a). Prevalences of both groups significantly increased with time ($p < 0.05$). Prevalence was not significantly different between meront-challenged and prezoosporangia-challenged oysters. Intensities of infections ranged from light to heavy (1 to 5) in meront-challenged oysters, whereas no heavy infections were detected in prezoosporangia-challenged oysters (Fig. 2a). When intensity of infection was expressed as weighted incidence (sum of disease code number/total number of oysters examined), it showed a trend similar to that of prevalence. Weighted incidence (Table 1) at the end of the experiment was higher in oysters challenged with meronts (2.13) compared with oysters challenged with prezoosporangia (0.86).

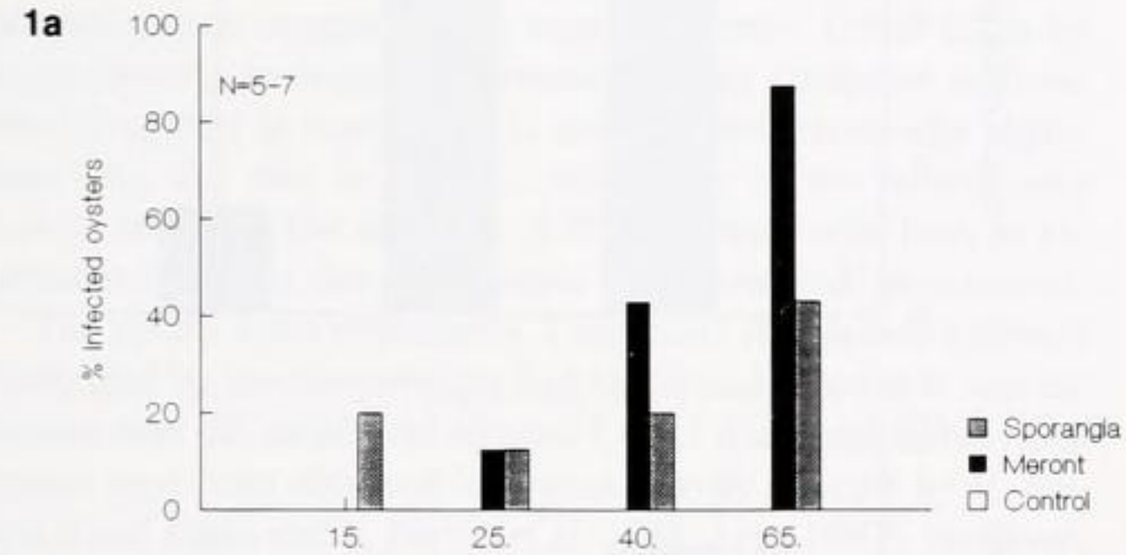
In experiment 2, the first infections appeared after 40 days in both meront- and prezoosporangia-challenged oysters. Prevalence (Fig. 1b) was significantly ($p < 0.002$) higher in meront-challenged oysters (77.5%) compared with prezoosporangia-challenged oysters (57.2%). As in experiment 1, infection in both groups increased with time ($p < 0.0001$). Intensities of infections ranged from light to moderate heavy (1 to 4) in oysters challenged with meronts, whereas only light infections (1) were observed in prezoosporangia-challenged oysters (Fig. 2b). Weighted incidence (Table 1) at the end of the experiment was higher in meront-challenged oysters (0.86) as compared with prezoosporangia-challenged oysters (0.5).

There were no differences in CI, L, and P among treatments at different sampling times in experiment 1 ($p > 0.05$). In experiment 1, within the prezoosporangia-challenged group, CI of infected oysters was lower than that of uninfected oysters (Fig. 3). The CI of infected oysters from the group challenged by meronts was not different from that of infected oysters from the group challenged by prezoosporangia ($p > 0.05$). Serum P concentrations in infected





Figures 2a and 2b. *P. marinus* infection intensity in oysters from experiment 1 (a) after 65 days and experiment 2 (b) after 75 days postchallenge by meronts and prezoosporangia.



Figures 1a and 1b. *P. marinus* prevalence in oysters after 15, 25, 40, and 65 days (a) and 20, 40, 50, 65, and 75 days postchallenge (b) by meronts or prezoosporangia.

oysters challenged with prezoosporangia were significantly lower ($p < 0.05$) than those in the uninfected oysters (Fig. 4). However, no significant difference in P concentrations was observed between infected and uninfected oysters in the group of oysters challenged with meronts. No differences ($p > 0.05$) were observed in serum P concentrations between meront- and prezoosporangia-challenged oysters. Also, no significant difference in P concentrations was observed between infected oysters challenged with meronts or with prezoosporangia. In oysters challenged by meronts, L was significantly higher ($p < 0.05$) in uninfected than in infected oysters (Fig. 5). No such differences were observed between infected and uninfected oysters challenged with prezoosporangia.

In experiment 2, CI and serum P concentrations significantly decreased ($p < 0.05$) in all treatments with time. The CI of oysters at the end of 20 days was significantly higher than the

TABLE 1.

Weighted incidence of *P. marinus* infection and experimental conditions.

	Weighted Incidence		Experimental Conditions[a]	
Infective Cell	**Experiment 1**	**Experiment 2**	**Experiment 1**	**Experiment 2**
Meront	2.13	1.33	T = 25.6 ± 1.33°C	T = 21.78 ± 0.84°C
Prezoosporangia	0.86	0.5	S = 20.7 ± 1.04 ppt	S = 20.5 ± 1.19 ppt

[a] T, temperature; S, salinity.

CI of the oysters at the end of 50 and 75 days (Fig. 6). P concentrations in oysters from all treatments decreased with time (Fig. 7). P concentrations at the end of 20, 40, and 50 days were significantly ($p < 0.05$) higher than at the end of 65 and 75 days postchallenge (Fig. 7). No significant differences were observed in L between treatments at any sampling time.

DISCUSSION

Results of this study show that both meronts and prezoosporangia infect oysters, with meronts being more infective than prezoosporangia. This supports the hypothesis (Perkins 1966) that meronts are the primary agents of disease transmission of *P. marinus* in oysters. The higher prevalence of infection in oysters challenged with meronts might have been due to the higher virulence of meronts. The meronts may multiply rapidly in oysters at warm temperatures, such as those (Table 1) used in this study. The cause for the lower infection rate of prezoosporangia is not clear. Although the prezoosporangia injected into the oysters were >95% viable at the time of infection, viability may drop after injection into the oyster tissue, resulting in lower infections. The prezoosporangia used in this study have been cultured in FTM, which may have affected their infectivity. In the field, the infectivity of prezoosporangia could be different. Oysters challenged with cells from pure cultures of *P. marinus* (meronts, merozoites, and schizonts) did not exhibit as heavy infections as those obtained with meronts in homogenized oyster tissue (Volety and Chu, unpublished results, Bushek et al. 1993). Culture of *P. marinus* in artificial media may reduce virulence of the cell stages.

Division of prezoosporangia into meront-like structures by schizogony has been observed in culture (La Peyre 1993; Perkins, personal communication). Although sporangia divide and release biflagellated zoospores in seawater (Perkins 1976, Chu and Greene 1989), the production of zoospores by meronts or prezoosporangia in oyster tissue or in cells isolated from oyster tissue without FTM treatment has not been documented. Indeed, the production of biflagellated zoospores and their subsequent release into seawater may not take place in oyster tissue. Furthermore, the fate of inoculated prezoosporangia in oyster tissue is not known. The lower prevalence in oysters challenged with prezoosporangia may be the result of a long lag time in the division of sporangia into meronts and/or the high mortality rate of cells induced to form prezoosporangia.

Dittman (1993) reported insignificant differences in CI between lightly infected and uninfected oysters. However, in the same study, significantly lower CI values were observed in heavily

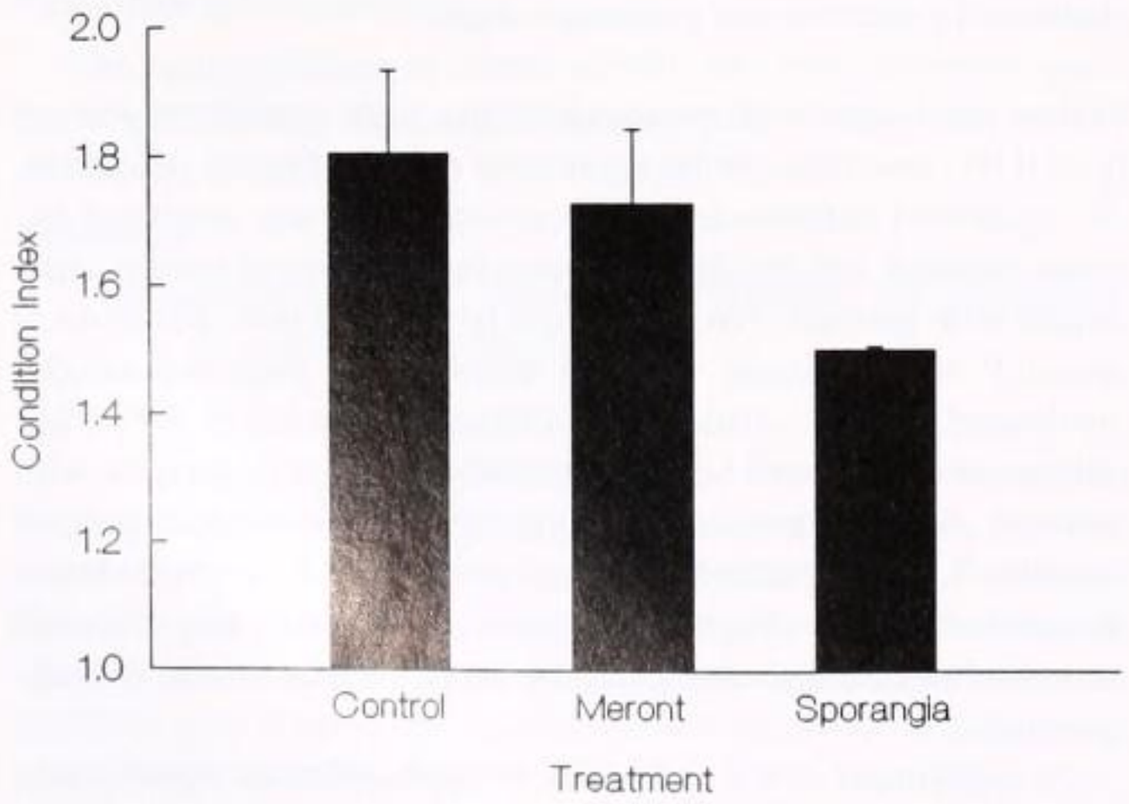


Figure 3. Mean CI (±SE) in uninfected, meront-, and prezoosporangia-challenged oysters.

Fig 4

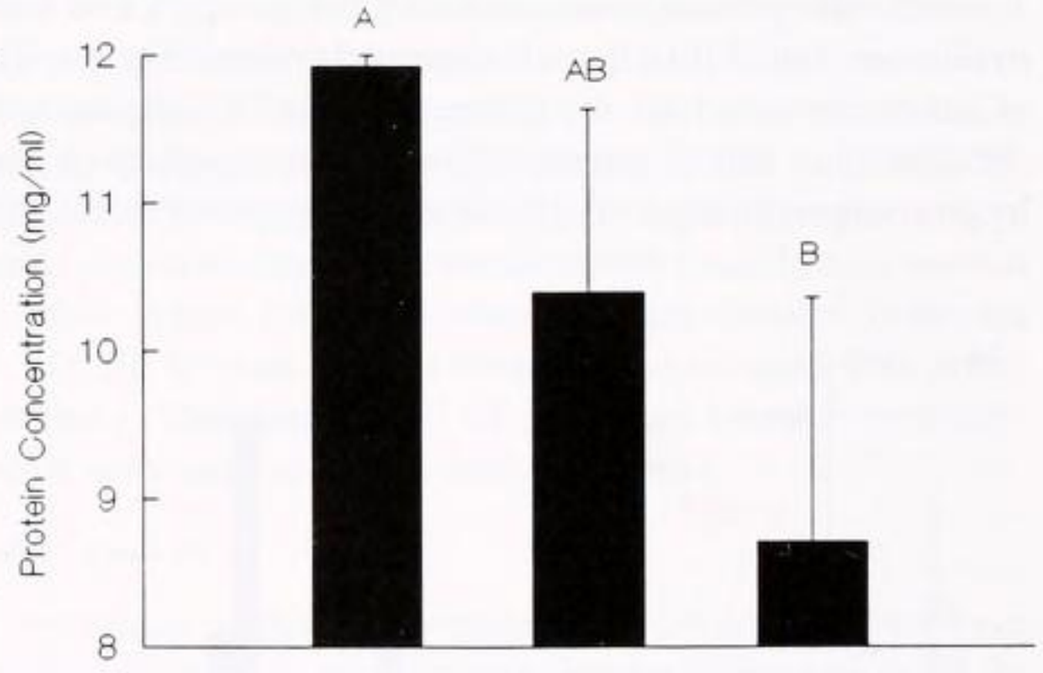
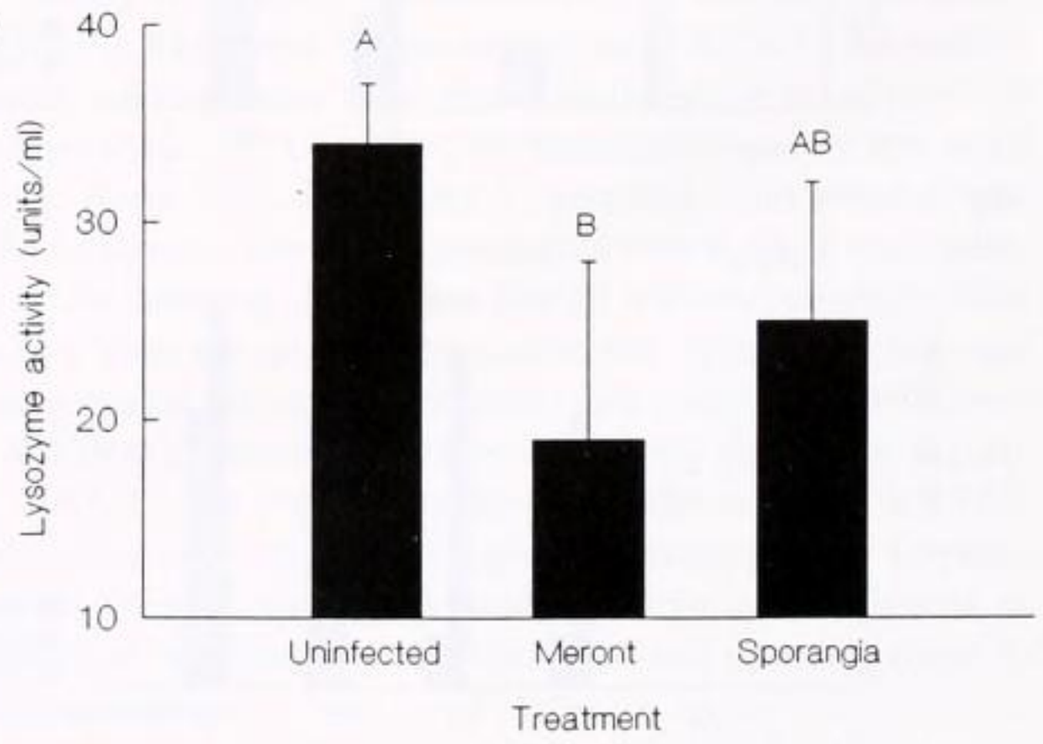
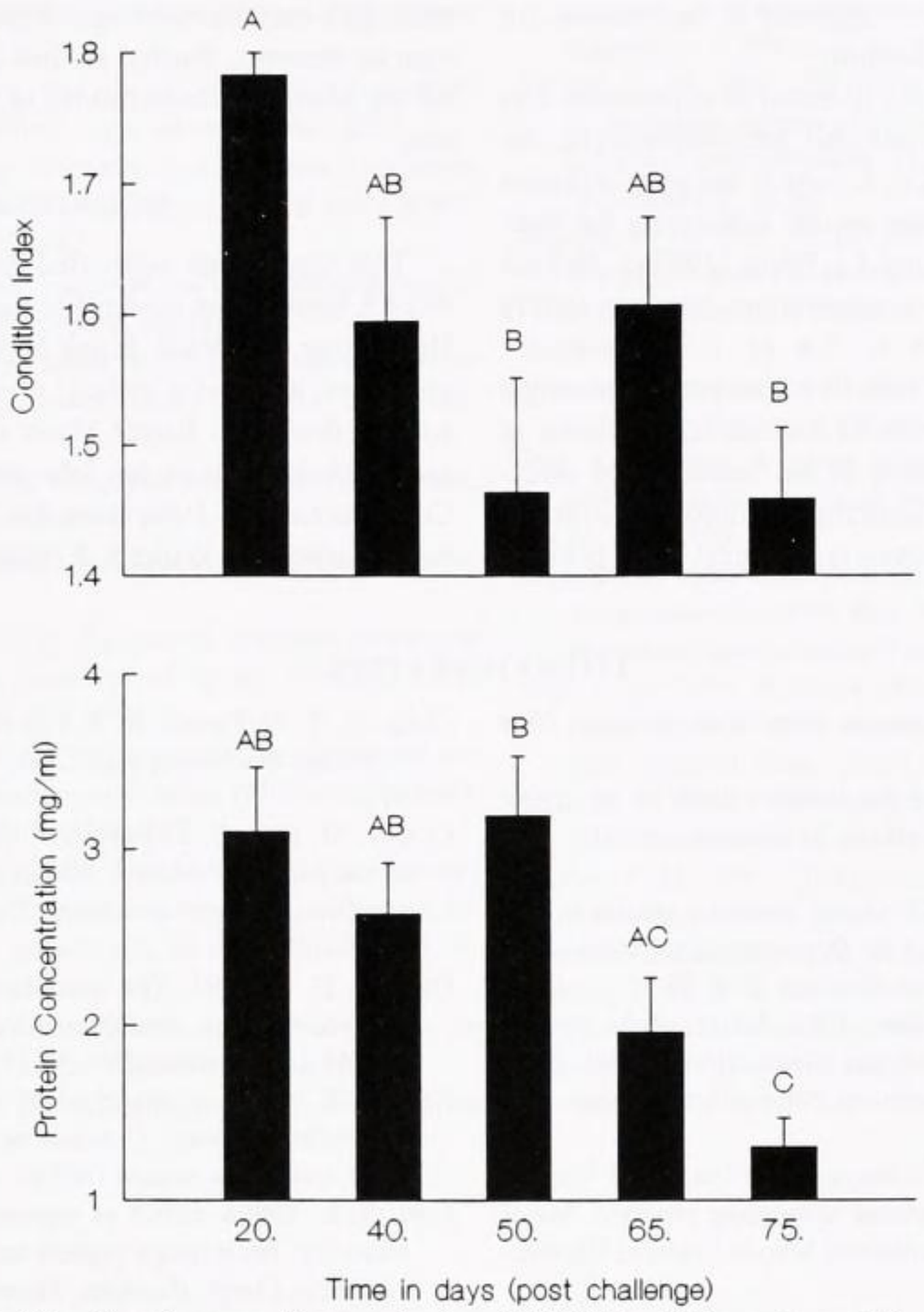


Fig 5



Figures 4 and 5. Mean serum P concentration (±SE) (Fig. 4) and mean serum L (±SE) (Fig. 5) in uninfected and infected oysters challenged by meront and prezoosporangia. Bars with similar letters are not significantly different ($p > 0.05$).



Figures 6 and 7. Mean CI (±SE) (Fig. 6) and serum P concentration (±SE) (Fig. 7) in oysters at the end of 20, 40, 50, 65, and 75 days postchallenge. Bars with similar letters are not significantly different ($p > 0.05$).

infected oysters compared with uninfected ones. Lower CI in infected oysters challenged by prezoosporangia compared with uninfected oysters in experiment 1, although not statistically significant (Fig. 3), may be because only a few of the oysters were heavily infected. The decrease in CI of oysters with time in experiment 2 may be due to the stress in the confined environment.

The results from experiment 1 indicated that infected oysters challenged by prezoosporangia had significantly lower P concentrations than did uninfected oysters. Lower tissue and hemolymph protein have been observed in oysters heavily infected by *H. nelsoni* (Ford 1986a and b, Barber et al. 1988, Ling 1990). However, no significant differences in P concentrations were observed in oysters lightly infected by *P. marinus* as compared with uninfected oysters (Chu and La Peyre 1993a).

Lysosomal enzymes are believed to play a role in defense in both vertebrates and invertebrates (Ingram 1980, Jolles and Jolles 1984), including molluscs (McDade and Tripp 1967a and b, Cheng 1981 and 1983, Huffman and Tripp 1982, Moore and Gelder 1985, Chu 1988). L in oysters was observed to be negatively correlated with *P. marinus* infection and temperature (Chu and La Peyre 1993a). L of uninfected oysters in experiment 1 had significantly higher activities than infected oysters challenged with meronts (Fig. 5). Lysozyme is hypothesized to be an important enzyme in resistance to *P. marinus* infection (Chu et al. 1993). The absence of *P. marinus* infection in some of the oysters may have been as a result of higher serum L, which may explain the significantly higher L in uninfected oysters. However, no difference in L was observed between meront-challenged and prezoosporangia-challenged oysters.

The higher prevalence, intensity, and weighted indices of *P. marinus* infections in experiment 1 compared with experiment 2 may be due to the higher temperature in the former experiment (Table 1). Temperature is one of the two most important factors (the other being salinity) influencing the geographic distribution of *P. marinus* in oysters. Chu and La Peyre (1993a) reported that prevalence and intensity of *P. marinus* infection increased with increasing temperature. In their study, the prevalence of *P. marinus* in oysters was 23, 46, 91, and 100% at 10, 15, 20, and 25°C respectively. *P. marinus* infection is positively correlated with temperature in the field (Soniat 1985, Craig et al. 1989, Soniat and Gauthier 1989, Crosby and Roberts 1990, Gauthier et al. 1990). The batches of *P. marinus* meronts used for challenging the oysters in the two experiments were isolated from different infected oysters. Their relative infectivity and virulence could differ, contributing to the different infection rates. The difference in the source of oysters may also have been one of the factors for the lower incidence of *P. marinus* infection. Differences in the susceptibility of oysters from different populations to *P. marinus* infection have been reported (Chu and La Peyre 1993b, La Peyre 1993). Their studies have shown differences in the prevalence of *P. marinus* infection in oysters from three locations in the Chesapeake Bay and between Chesapeake Bay and Gulf oysters. Hab-

itat and genetic dissimilarities were suggested as the reasons for the differences in prevalence of infection.

Because only light infections were detected in experiment 2 in both oysters challenged with meronts and prezoosporangia, the insignificant differences noted in CI, L, and P between different treatments were not surprising. These results agree with the findings by Dittman (1993) and Chu and La Peyre (1993a). Neither found differences in CI, L, and P concentrations between lightly infected and uninfected oysters.

In summary, meronts are more infective than prezoosporangia and are possibly the principal agents of disease transmission in the field. The lower CI and P values in the treatment of infected oysters challenged with prezoosporangia, compared with uninfected and meront-challenged oysters, suggest that prezoosporangia may be exerting a higher energetic demand on the host than do meronts. Further studies are needed to examine the causes for the lower P concentrations in prezoosporangia-challenged oysters.

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