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PROJECT TITLE: Disease processes and transmission dynamics of *Perkinsus marinus* in American oysters (*Crassostrea virginica*).

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Project description:

Background:

The once thriving oyster industry in the Chesapeake Bay and east coast of the United States, has been threatened by overfishing, and diseases caused by two protistan parasites, *Haplosporidium nelsoni* (MSX) and *Perkinsus marinus* (Dermo). Three lifestages are recognized in the lifecycle of *P. marinus*, meronts (trophozoites), prezoosporangia, and the biflagellated zoospores. 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 LaPeyre 1993a, 1993b, Paynter and Burreson 1991). Since 1986, *P. marinus* has reportedly caused greater oyster mortalities in lower Chesapeake Bay than *H.nelsoni* (Andrews 1988).

Numerous investigators have performed field (Soniat 1985, Craig et al 1989, Soniat and

Gauthier 1989, Crosby and Roberts 1990, Gauthier et al 1990, Burreson 1989, 1990) and laboratory studies (Mackin 1951, 1956, 1962, Andrews and Hewatt 1957, Perkins 1966, Chu and LaPeyre 1989, Ragone and Burreson 1993) to investigate the effects of temperature and salinity on the disease processes of *P. marinus* in eastern oysters. Previous laboratory experiments were conducted by inducing *P.marinus* infection through exposure of oysters to meronts, merozoites and schizonts contained in suspension of partially purified meronts isolated from homogenised infected oyster tissue (Chu 1993a, 1993b). Since exposure of oysters to minced oyster tissue containing meronts or freshly isolated and partially purified meronts resulted in high prevalence of *P. marinus* infection, Perkins (1988) 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rates were found by exposing oysters to prezoosporangia and biflagellated zoospores in our laboratory (Chu et al., unpublished results).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 the three life stages, namely meronts, prezoosporangia and biflagellated zoospores are capable of inducing infection in oysters. None of the previous studies have examined purified prezoosporangia as an infective agent nor were the physiopathological effects investigated. High salinity and high temperature were positively correlated with *P. marinus* infection in the field (eg. Mackin 1951, 1956, Andrews and Hewatt 1957, Soniat 1985, Crosby and Roberts 1990). Low infection prevalnces of *P. marinus* infection in oysters at low salinity has led to the hypothesis that "dosage" of infective elements is important and that the flushing and diluting effect of the inflowing freshwater may be the reason for low infection rates (Mackin 1956). On the other hand, Scott et. al (1985) suggested that the physiological changes in the oyster due to changes in salinity are the reasons for the altered susceptibility of the oysters to *P. marinus* disease and their later survival. Although studies (Mackin 1962) were performed to investigate the dose dependent mortality in oysters exposed to oyster tissue containing *P. marinus* infective particles ($10-10^6$), the physiopathologic effects on the oyster were not examined. Despite all the studies, *P. marinus* disease processes in *C. virginica* are poorly understood and a lot of questions still remain unanswered regarding the disease processes and transmission dynamics of *P. marinus* in oysters. For example, (i) the principal infective stage of *P. marinus*, and the minimum number (dose) of infective particles required to initiate *P. marinus* infection in oysters is unknown, and (ii) synergetic effects of temperature and salinity on the dose response of *P. marinus* in oysters are unclear. To revive the oyster fishery, we need to understand the interaction between the host, oyster and the parasite, *P. marinus*. Gaining insight into the disease processes of Dermo and it's transmission dynamics among oysters will allow the development of management strategies to improve the east coast oyster resources.

Objectives

The objectives of this study are to 1) investigate which *P. marinus* lifestage (cell type), biflagellated zoospores, or meronts, or prezoosporangia (zoosporangia), is the most effective and primary agent for disease transmission among oysters; 2) asses the time required for each *P. marinus* lifestage to initiate infection; 3) determine the minimal dose of each lifestage to initiate *P. marinus* infection; and 4) determine the combined effects of temperature and salinity on *P. marinus* dose response in oysters.

Project activities

During the funding period, we have conducted experiments, 1) to compare the infectivity of the two lifestages, meronts and prezoosporangia, as disease agent in *P. marinus* infection in oysters and the time required for these two lifestages to initiate infection, 2) to study the responses of oysters to different doses of meronts and prezoosporangia and the pathological effects in oysters and, 3) to examine the synergetic effect of temperature and salinity on the dose response of *P. marinus* meronts in oysters.

Methodology:

Preparation of meront suspension

Meront suspension was prepared as follows: *P. marinus* infected oyster tissues were rinsed thoroughly with filtered (0.22 μm) York River Water (YRW) and subsequently homogenized in o.22 μm filtered YRW with a blender (Virtis, Model 23) at high speed for 2 minutes. The suspension was then passed through a series of 100, 50, 35 and 20 μm meshes to remove oyster tissue residues (La Peyre and Chu, 1994). The number of meronts in suspension was counted using a hemacytometer and adjusted to the desired concentration as described in the experiments.

Preparation of hemocytes and sera

Hemocytes and sera were prepared as follows: hemolymph was withdrawn from the adductor muscle sinus with a syringe through notches in the shell and hemolymph was placed in test tubes in an ice bath. Total hemocyte and number of granulocytes and agranulocytes were counted for five individual hemolymph samples using a hemacytometer. For humoral activity measurements, serum of each hemolymph sample was separated from hemocytes through centrifugation (400 x g at 4°C for 10 min). Sera were withdrawn and stored at -20°C for other analyses.

P.marinus diagnosis

P. marinus infections were diagnosed using hemolymph and tissue assays (Gauthier and Fisher 1990, and Ray 1952). The hemolymph assay was as follows: 300μl of hemolymph containing hemocytes were obtained and incubated in FTM containing antibiotics (penicillin and streptomycin) for 4 days. After incubation, the thioglycollate medium was separated by centrifugation at 800 x g and incubated with 1N NaOH for 1 hour to remove tissue debris and hemocytes. The pellet (prezoosporangia) was washed twice with water and stained with Lugol's iodine. The number of prezoosporangia was counted under an inverted microscope (Nikon). Disease intensity was ranked 0, 1, 3, 5 (negative, light, moderate, and heavy) based on the number of prezoosporangia present in the hemolymph sample. At the end of each experiment, infections were also diagnosed according to the method of Ray (1952) by incubating a piece of rectal and mantle tissue in FTM. Weighted indices were calculated according to Ray (1954) and Mackin (1962).

Lysozyme activity (L)

Lysozyme activity was determined spectrophotometrically according to Shugar (1952) and modified by Chu and LaPeyre (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 activity (units, one unit is described as a decrease in absorbance of 0.001 in the bacterial suspension at room temperature in 1 min) or concentration (μg/ml). Hen egg white lysozyme dissolved in appropriate salinity water was used in contructing standard curve.

Serum protein concentration (P)

The concentrations of serum protein were assayed according to Lowry et al. (1951) using bovine albumin as a standard. Ten μl of a cell-free hemolymph sample from individual oysters was used for the serum protein measurement.

Experiments:

EXPERIMENT 1:

Response of oysters to different doses of meronts and prezoosporangia:

Oysters used in this study were collected from Damarsicotta River, Maine, a region beyond the geographical distribution of *P. marinus*. The ambient temperature and salinty at the time of collection were T=15°C and S=30 ppt respectively. The oysters were gradually acclimated to the test temperature and salinity of 25°C and 14 ppt. A total of 150 oysters (2-2.5" size range) were collected from Damarscicotta River. Hemolymph was withdrawn from 15 randomly selected oysters using a syringe fitted with a 27 gauge needle. Total and % granulocytes were counted using a hemacytometer. After the hemolymph withdrawl, a piece of rectal, mantle and digestive gland from individual oysters were excised and used for *P. marinus* infection diagnosis according to Ray (1952). All the oysters were diagnosed to be free of *P.*

marinus infection at that time. Condition index of the oysters were then determined according to Lucas and Beninger (1985). The remaining 135 oysters were divided into 9 groups (groups challenged with 10, 10^2, 10^4, or 10^5 meronts or prezoosporangia and a control group, 15 oysters per group) and oysters randomly arranged in individual plastic chambers with 1μ filtered York River Water (YRW) and aerated. Water was changed every other day and the oysters fed daily with 0.2 gms/oyster algal paste (a mixture of *Isochrysis galbana*, *Pavlova lutheri*, and Tahitian *Isochrysis galbana*). *P. marinus* meronts or prezoosporangia in 100 μl of 1μm filtered YRW containing 10, or 10^2, or 10^4, or 10^5 were injected into the shell cavity of individual oysters. Oysters from the control group received plain filtered YRW. Oysters were sampled for hemolymph after 8 weeks and total and % granulocytes, and condition index (dry meat weight/dry shell weight x 100; Lucas and Beninger 1985) determined. *P. marinus* infection was also determined using tissue assay (Ray 1952).

EXPERIMENT 2:

Comparison of infectivity of meront and prezoosporangia and time required to initiate infection:

Two experiments (trials) were conducted to compare the infectivity and pathogenic effects of meronts and prezoosporangia. Attempts to isolate and culture zoospores in our and several other laboratories were unsuccessful, hence the infectivity of zoospores was not included in the present study.

Trial 1:

Eastern oysters were collected from the Ross's 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 six 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 1μm filtered York River Water (YRW). During the acclimation and the experimental period, oysters were fed daily with algal paste (0.1 gms/oyster), and water was changed every other day. Meronts were partially purified from infected oyster tissue according to LaPeyre and Chu (1994). Prezoosporangia were prepared based on the method described by Chu and Greene (1989). One hundred μl of filtered YRW containing 5 x 10^4 meronts or prezoosporangia cells (meronts cultured in FTM and enlarged to size range of > 100μm) were injected into the shell cavity of each oyster. Controls were injected with 1μ filtered YRW. There were three treatments: control, meront-challenged and prezoosporangia-challenged oysters. To follow infection development, eight oysters were randomly sampled from each treatment at 15, 25, 40 and 65 days post-challenge. Hemolymph samples were withdrawn from the anterior adductor muscle of individual oysters using a syringe with a 27 gauge needle. Serum lysozyme (L) and protein (P) concentration were measured. Hemolymph was also assayed to evaluate *P. marinus* infection (Gauthier and Fisher 1990). After withdrawal of hemolymph samples, oysters were sacrificed and condition index (CI) was determined. *P. marinus* infections in oysters were also diagnosed using rectal and mantle tissue according to the tissue assay described by Ray (1952).

Trial 2:

The experimental conditions were similar to trial 1, with the exception that oysters were collected from the Damarsicotta River, Maine, (ambient salinity and temperature: 32 - 35 ppt and 12 - 14°C respectively). As in trial 1, oysters were gradually adjusted to the test conditions ($T = 21.78 \pm 0.84$°C, $S = 20.5 \pm 1.19$ ppt) in six 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, serum L and P were conducted in individual oysters as previously indicated.

EXPERIMENT 3:

Synergetic effects of temperature and salinity on *P. marinus* meront in oysters:

Five hundred oysters were collected from Damarsicotta River, Maine (ambient temperature 0°C, salinity 32 ppt). All the oysters were gradually acclimated in 250 gallon tanks with 1 μm filtered water at nine combinations of test temperatures and salinities: 3 ppt at 10, 15 and 25°C; 10 ppt at 10, 15 and 25°C; and 20 ppt at 10, 15 and 25°C. Oysters were fed 0.1 gms of algal paste/oyster/day. Before the commencement of the experiment, a random subsample of 30 oysters were sacrificed and examined for condition index and *P. marinus* infection. After the oysters were acclimated to the respective test temperatures and salinities, they were divided into nine groups of different test temperatures and salinities and randomly placed in individual chambers with 1 μm filtered YRW adjusted to test salinities and temperatures and aerated well. *P. marinus* meronts were isolated from infected oyster tissue and adjusted to a concentration of 2.5×10^4 or 2.5×10^5 cells/ml in YRW. One hundred μl of YRW containing 2.5×10^3 or 10^4 meronts was injected into the shell cavity of individual oysters. Control oysters were innoculated with filtered YRW. Hemolymph was withdrawn from oysters after 8 weeks to determine total and % granulocyte counts P and L concentrations. Oysters were sacrificed and examined for *P. marinus* infection. Infection intensities and weighted index were ranked according to Mackin (1962).

<u>Statistical analyses</u>:

In the comparison of infectivity of lifestages experiment (experiment 2), trial 1, 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. Since 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 trial 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 using One-Way ANOVA to determine differences among groups. In trial 2, CI, L and P data were analyzed using One-Way ANOVA without splitting 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 the trials.

A two way ANOVA in dose response experiment (experiment 1), and a three way

ANOVA in synergetic effects of temperature and salinity experiment (experiment 3) were used to determine the differences in TC, PG, L, S and CI. CI data in experiment 1 were SIN transformed. Logistic regression was used to determine the prevalences of infection between doses and lifestage of cells used.

Summary of Results:

1) ~~Response~~ of oysters to different doses of P. marinus trophozoites and prezoosporangia:

Prevalence and intensity of *P. marinus* infection.

A dose dependent response of *P. marinus* infection was observed in oysters. *P. marinus* infection in oysters increased ($p < 0.0001$) with increasing dose of *P. marinus* cells (Fig 1). Significant differences in prevalence of *P. marinus* infection in oysters were observed between oysters challenged by meronts and by prezoosporangia. Meront-challenged oysters had a significantly higher ($p < 0.05$) infection rate compared to prezoosporangia-challenged oysters (Fig 1). No *P. marinus* infection was detected in control oysters. Results indicate that the minimum dose of *P. marinus* cells required to initiate infection in oysters is about 100 cells/oyster (Fig 1).

Intensity of *P. marinus* infection in oysters also increased with increasing number of *P. marinus* cells inoculated into the oyster shell cavity. The infection intensity in oysters challenged with meronts was higher compared to those challenged with prezoosporangia (Fig 2).

Total number of hemocytes and % granulocytes, condition index, lysozyme and protein concentrations:

No significant differences were observed in total hemocyte count and percent granulocytes of oysters challenged by either meronts or prezoosporangia and between challenged doses ($p > 0.05$). Significant differences were found in condition index of oysters challenged with prezoosporangia compared to oysters challenged with meronts. Prezoosporangia-challenged oysters had a significantly lower CI compared to meront-challenged oysters ($p < 0.05$) (Fig 3).

2) Comparison of infectivity of trophozoites and prezoosporangia and time required for each life stage to initiate *P. marinus* infection:

Prevalence and intensity of P. marinus infection.

In trial 1, infection first appeared in oysters 15 days after being challenged with prezoosporangia and 25 days after challenge with meronts (Fig 4a). Prevalence, at 65 days post challenge was higher in oysters challenged by meronts (87.5%), compared to oysters challenged by prezoosporangia (43%) (Fig 4a). 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 - 5) in meront-challenged oysters, whereas no heavy infections were detected in prezoosporangia - challenged oysters (Fig 5a). 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 to oysters challenged with prezoosporangia (0.86).

In trial 2, the first infections appeared after 40 days in both meront and prezoosporangia-challenged oysters. Prevalence (Fig 4b) was significantly ($p < 0.002$) higher in meront-challenged oysters (77.5%) compared to prezoosporangia challenged oysters (57.2%). As in trial 1, infection in both groups increased with time ($p < 0.0001$). Intensities of infections ranged from light to moderate heavy (1 - 4) in oysters challenged with meronts whereas only light infections (1) were observed in prezoosporangia challenged oysters (Fig 5b). Similar to trial 1, weighted incidence (Table 1) at the end of the experiment was higher in meront challenged oysters (0.86) as compared to prezoosporangia challenged oysters (0.5).

Condition index, lysozyme and protein concentrations:

There were no differences in CI, L and P among treatments at different sampling times in trial 1 ($p > 0.05$). In trial 1, within the prezoosporangia-challenged group, CI of infected oysters were lower than uninfected oysters (Fig 6). The CI of infected oysters from the group challenged by meronts was not different from infected oysters from the group challenged by prezoosporangia ($p > 0.05$). Serum P concentrations in infected oysters challenged with prezoosporangia were significantly lower ($p < 0.05$) than the uninfected oysters (Fig 7). However, no significant difference in P concentrations were 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 meront-challenged and prezoosporangia-challenged infected oysters challenged with meronts or prezoosporangia. In oysters challenged by meronts, lysozyme activity was significantly higher ($p < 0.05$) in uninfected than infected oysters (Fig 8). No such differences were observed between infected and uninfected oysters challenged with prezoosporangia.

In trial 2, CI and serum protein 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 CI of the oysters at the end of 50 and 75 days (Fig 9). P concentrations in oysters from all treatments decreased with time (Fig 10). 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 post-challenge (Fig 10). No significant differences were observed in lysozyme activities between treatments at any sampling time.

3) Synergetic effects of temperature and salinity on response of oysters to different doses of P. marinus trophozoites:

<u>Prevalence and intensity of *P. marinus* infection.</u>

P. marinus prevalence in oysters increased with increasing temperature ($p < 0.0001$) and salinity ($p < 0.0003$) and there was a dose dependent response to *P. marinus* meronts challenged ($p < 0.0001$) (Fig 11). Temperature appears to be the most important factor followed by dose of infective particles in influencing *P. marinus* susceptibility and subsequent disease development in oysters. Salinity was the least influencing factor compared to temperature and meront doses. However, the interaction between the three factors on disease prevalence was insignificant.

Intensity of *P. marinus* infection in oysters increased with the increase of temperatures ($p < 0.0001$), salinities ($p < 0.01$) and meront doses ($p < 0.0001$) (Figs 12a, b, c). There was a significant effect of interaction between temperature and meront dose ($p < 0.0001$) on intensity of *P. marinus* infection. However, the effect of interaction between salinity and meront dose was insignificant.

<u>Condition index, total hemocyte count and % granulocytes, lysozyme and protein concentrations.</u>

Condition index of oysters was significantly reduced with increasing temperature ($p < 0.0001$) (Fig 13). Salinity and dose of meronts injected into the oysters did not significantly affect the CI of oysters. CI of infected oysters was significantly lower compared to uninfected oysters ($p < 0.05$) (Fig 14).

Total hemocyte count was significantly higher at treatments of lower temperatures than at higher temperatures. Mean total hemocyte count in oysters at 10 and 15°C was significantly greater than in oysters at 25°C ($p < 0.05$) (Fig 15). In contrast, oysters at higher temperatures had higher % granulocyte count. Mean % granulocytes in oysters at 25°C was significantly greater than those at 10 and 15°C ($p < 0.0001$) (Fig 16). Both total hemocyte count and %

granulocytes did not appear to be affected by either salinity or the number of meronts injected into the oysters. There was no synergetic effect of temperature, salinity and infective particles concentration on total hemocyte count in oysters. However, there was a significant effect of the interaction between temperature and *P. marinus* infection on % granulocytes in oysters ($p < 0.05$).

Mean P concentrations were significantly higher in oysters for treatments at higher temperature and salinity (Figs. 17a, b . P concentrations in oysters at 15 and 25°C were similar but significantly higher than oysters at 10°C (Fig 17a). L concentrations in oysters at 15°C was significantly greater ($p < 0.005$) than in oysters at 10°C (Fig 18a). L concentrations decreased with decreasing salinity. Oysters at 20 ppt had the highest lysozyme concentration (Fig 18b). Both L and P concentrations were not affected by the number of meronts the oysters were exposed to. The interaction of salinity and doses of meronts posted a significant effect on P ($p < 0.01$) and there was also a significant ($p < 0.05$) interaction effect of temperature and salinity on L concentrations.

Discussion and conclusions:

Results of the present study show that both meronts and prezoosporangia infect oysters with meronts being more infective than prezoosporangia. This supports the hypothesis (Perkins 1988) 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 the present 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.

Although sporangia divide and release biflagellated-zoospores in sea water (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 sea water 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 infected oysters compared to uninfected ones. Lower CI in infected oysters challenged by prezoosporangia compared to uninfected oysters in trial 1, though not statistically significant (Fig 3), may be because, only a few of the oysters were heavily infected. The decrease in condition index of oysters with time in trial 2, may be due to the stress in the confined environment.

The results from trial 1 indicated that infected oysters challenged by prezoosporangia had significantly lower protein concentrations than uninfected oysters. Lower tissue and hemolymph protein has been observed in oysters heavily infected by *Haplosporidium nelsoni* (Ford 1986 a, b, Barber et al 1988, Ling 1990). However, no significant differences in protein concentrations were observed in oysters lightly infected by *P. marinus* as compared to 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 (Mc Dade and Tripp 1967a, b, Cheng 1981, 1983, Huffman and Tripp 1982, Moore and Gelder 1985, Chu 1988). Lysozyme activity in oysters was observed to be negatively correlated with *P. marinus* infection and temperature (Chu and LaPeyre 1993a). Lysozyme activities of uninfected oysters in trial 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 lysozyme activity which may explain the significantly higher lysozyme activity in uninfected oysters. However, no difference in lysozyme activity was observed between meront challenged and prezoosporangia challenged oysters.

The higher prevalence, intensity and weighted incidence of *P. marinus* infections in trial 1 compared to trial 2 may be due to the higher temperature in the former trial (Table 1).

Temperature is one of the two most important factors (the other being salinity) influencing the geographic distribution of *P. marinus* in oysters. The batches of *P. marinus* meronts used for the challenging the oysters in the two trials 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 lower incidence of *P. marinus* infection. Differences in susceptibility of oysters from different populations to *P. marinus* infection have been reported (Chu and La Peyre 1993 b, La Peyre 1993).

Since only light infections were detected in trial 2 in both oysters challenged with meronts and prezoosporangia, the insignificant differences noted in condition index, lysozyme activity, and protein concentrations 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.

Results from the dose response of *P. marinus* cells experiment reenforce our previous findings (this proposal) that meronts are the principal agents of *P. marinus* disease transmission in oysters. Results also suggest that the minimum number of *P. marinus* infective particles necessary to initiate infection in oysters is about 100 cells and that the prevalence and intensity of Dermo infection is dose dependent. Results of dose response study also demonstrated that CI of oysters infected by prezoosporangia is significantly lower compared to that of oysters infected by meronts.

The results of synergetic effects of temperature and salinity on Dermo dose response in oysters revealed that temperature is the most important environmental factor influencing *P. marinus* infection in oysters. Results in our laboratory indicate that growth rates of *P. marinus* increased with increasing temperature (up to 28°C) (manuscript in preparation). This may explain the higher infection rate in oysters at higher temperatures. Meronts may multiply rapidly at elevated temperatures thus increasing the prevalence in oysters. Dose of *P. marinus* is the next important factor contributing to the increased infection. The increased (challenge) number of *P. marinus* cells may exert increased energy demand on the oysters. Increased salinity also significantly enhances *P. marinus* infection. However, the effect of interaction of these three factors (temperature, dose of *P. marinus* particles and salinity) was not important. The decreased CI of infected oysters and at higher temperatures may suggest that *P. marinus* is exerting high energetic demand on oysters and that high temperatures are stressing the oysters from a cold climate (Damarsicotta River, Maine).

The low L concentration in oysters at 3 ppt suggests that the salinity of 3 ppt may be too stressful for oysters which were used to living in a habitat where the salinity was always 32-33 ppt. High mortality in oysters recorded at this salinity when oysters were adjusted to a temperature of 25°C indicates that Maine oysters, apparently, could not tolerate salinities lower than 10 ppt and temperature higher than 15°C.

In conclusion, we have achieved all the objectives addressed in the proposal. In addition, we have also conducted experiments to compare the acid phosphatase activity in in two lifestages of *P. marinus*, namely, cultured meronts and freshly isolated prezoosporangia, as well as hemocytes and serum of the host *C. virginica* from different regions. Acid phosphatase in some parasites has been psotulated to play a role in avoiding the host defense through dephosphorylation of host proteins and/or inhibition of the superoxide radicals released by the host phagocytes. Results are as follows: Hemocytes of oysters from James River, Virginia, showed significantly ($p < 0.05$) higher activity (4.73 ± 0.17 at 10°C, 6.43 ± 0.13 at 15°C, and 7.62 ± 0.07 units at 25°C) than those from Damarsicotta River, Maine (0.37 ± 0.03 at 10°C, 0.5 ± 0.03 at 15°C, and 0.58 ± 0.03 units at 25°C). Acid phosphatase activity was higher ($p < 0.05$) in hemocytes compared to meronts (0.49 ± 0.04 at 10°C, 0.91 ± 0.2 at 15°C, and 0.94 ± 0.08 units at 25°C) and prezoosporangia (0.03 ± 0.02 at 10°C, 0.05 ± 0.06 at 15°C, and 0.03 ± 0.01 units at 25°C). Only trace amounts of acid phosphatase were detected in sera from both Virginia and maine oysters. The difference of acid phosphatase activity in hemocytes from different regions might be related to differences in food, habitat or genetic factors. Based on these results, the role of acid phosphatase in avoiding host defense is inconclusive. Further study is needed to examine the processes of acid phosphatase secretion by the parasite.

Publications and oral presentations with published abstracts:

1) Aswani K. Volety and Fu-Lin E. Chu. (1994, In Press). Comparison of infectivity and pathogenecity of meront (trophozoite) and prezoosporangiaie stages of the oyster pathogen *Perkinsus marinus* in eastern oysters, *Crassostrea virginica* (Gmelin, 1791). J. Shellfish Research.

2) Fu-Lin E. Chu, Aswani K. Volety, and Georgeta constantin. (1994). Synergetic effects of temperature and salinity on the dose response of oysters (*Crassostrea virginica*) to the pathogen, *Perkinsus marinus*. J. Shellfish Research. 13: 293 (abstract).

3) Aswani K. Volety, and Fu-Lin E. Chu. (1994). A comparative study of acid phosphatase in the parasite *Perkinsus marinus* and its' host *Crassostrea virginica*. J. Shellfish Research. 13: 297 (abstract).

4) Aswani K. Volety and Fu-Lin E. Chu. (1993). Infectivity and pathogenecity of two lifestages, meront and prezoosporangia, of *Perkinsus marinus* in eastern oysters, *Crassostrea virginica*. J. Shellfish Research. 12: 130 (abstract).

Manuscripts in preparation:

1) Fu-Lin E. Chu and Aswani K. Volety. Synergetic effects of temperature and salinity on response of eastern oysters, *Crassostrea virginica* to different doses of *Perkinsus marinus* meronts. (In preparation).

2) Aswani K. Volety and Fu-Lin E. Chu. Response of American oyster, *Crassostrea virginica* to different doses of *Perkinsus marinus* stages. (In preparation).

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TABLE 1:

P.marinus INFECTION:

INFECTIVE CELL	WEIGHTED INCIDENCE		EXPERIMENTAL CONDITIONS	
	EXPT1	EXPT2	EXPT1	EXPT2
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±1.04PPT	S=20.5±1.19PPT

Table 1: Weighted incidence of *P.marinus* infection and experimental conditions.

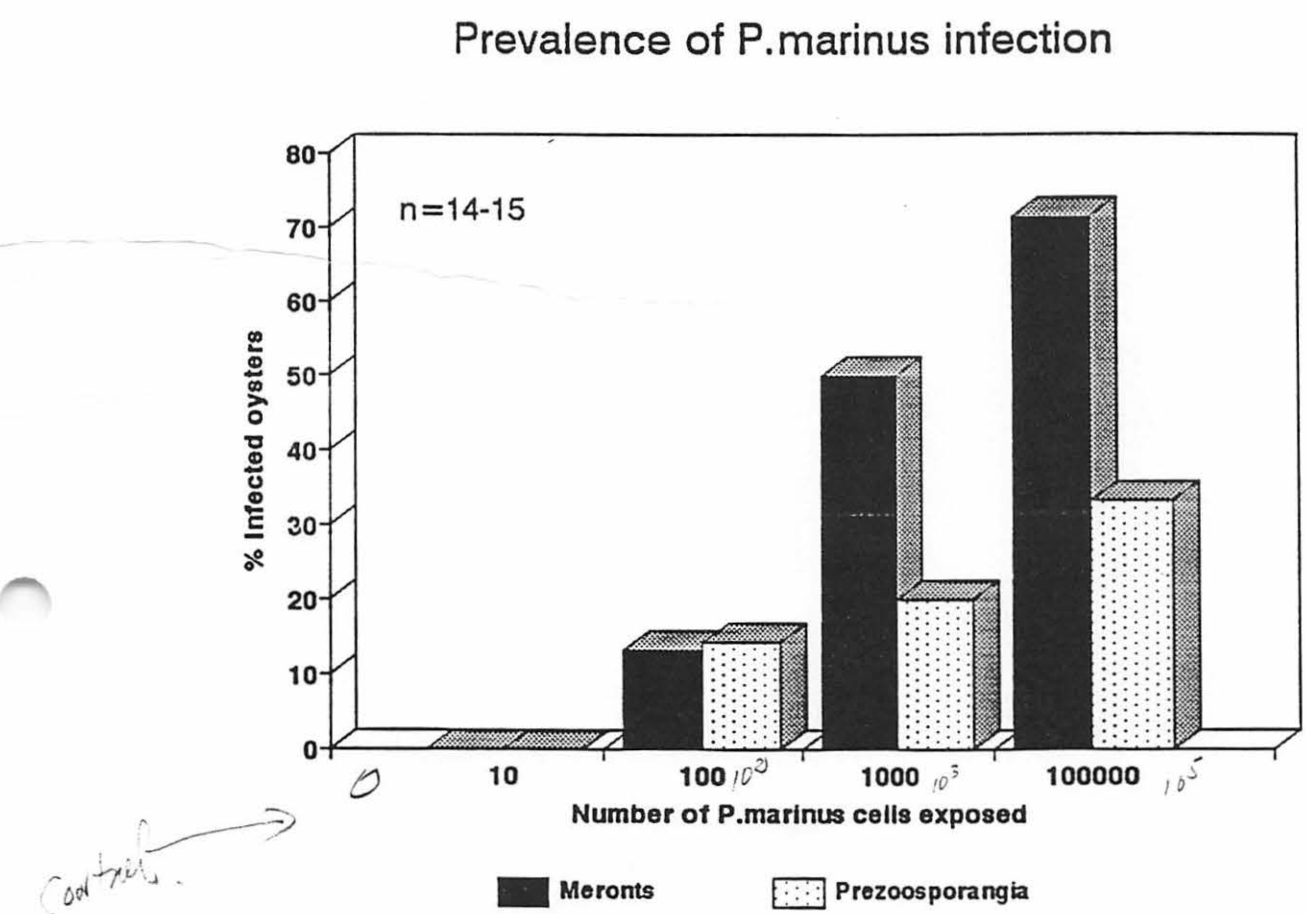

Fig. 1. Disease prevalence of P. marinus infection in oysters challenged with 10, 100, 1000 and 100000 meronts or prezoosporangia.

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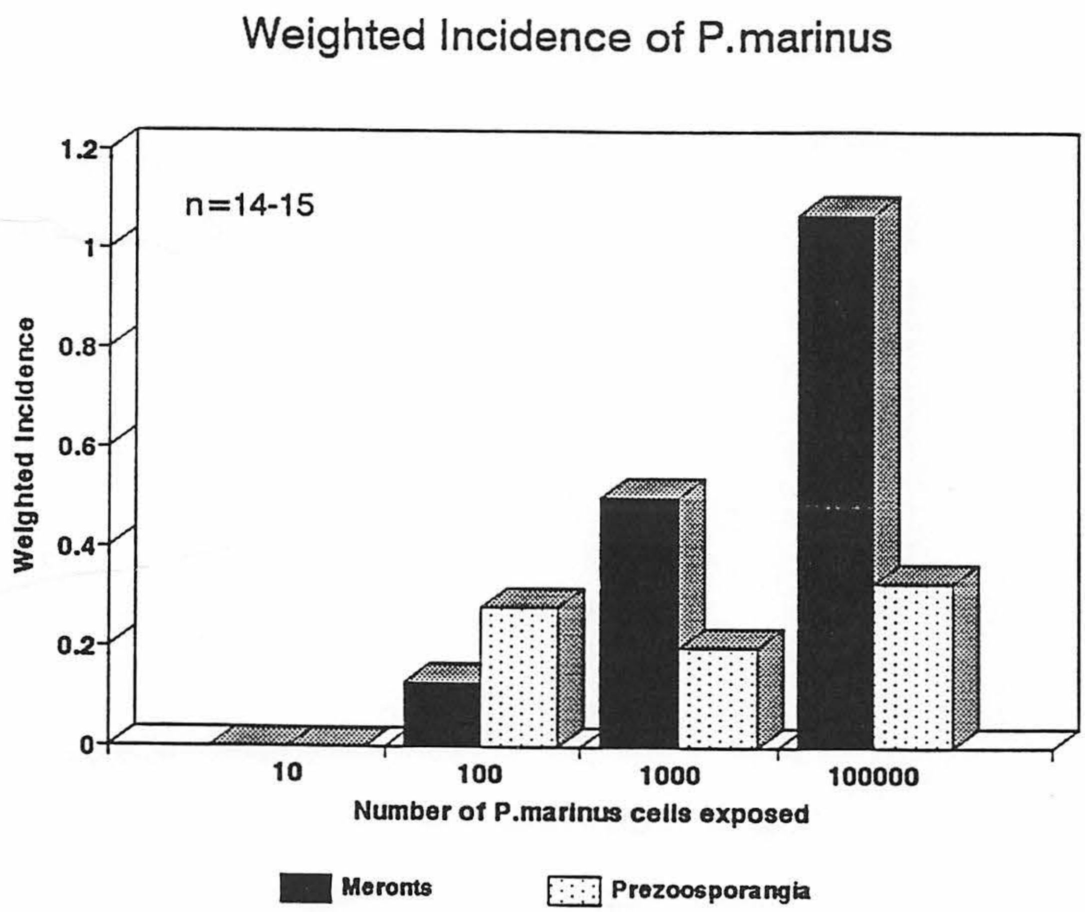


Fig. 2. Mean infection intensity of oysters challenged with 10, 100, 1000 and 100000 meronts or prezoosporangia.

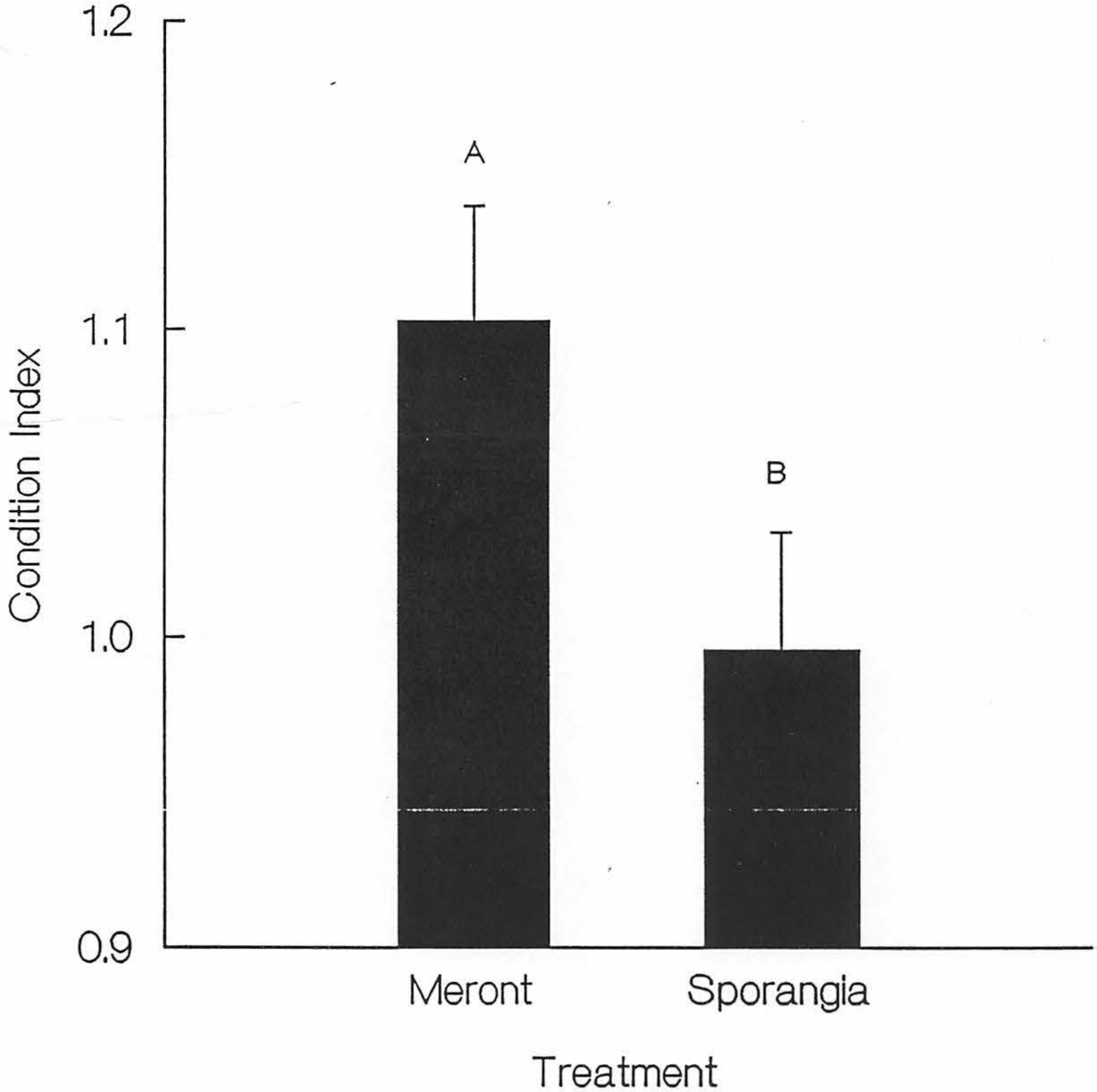
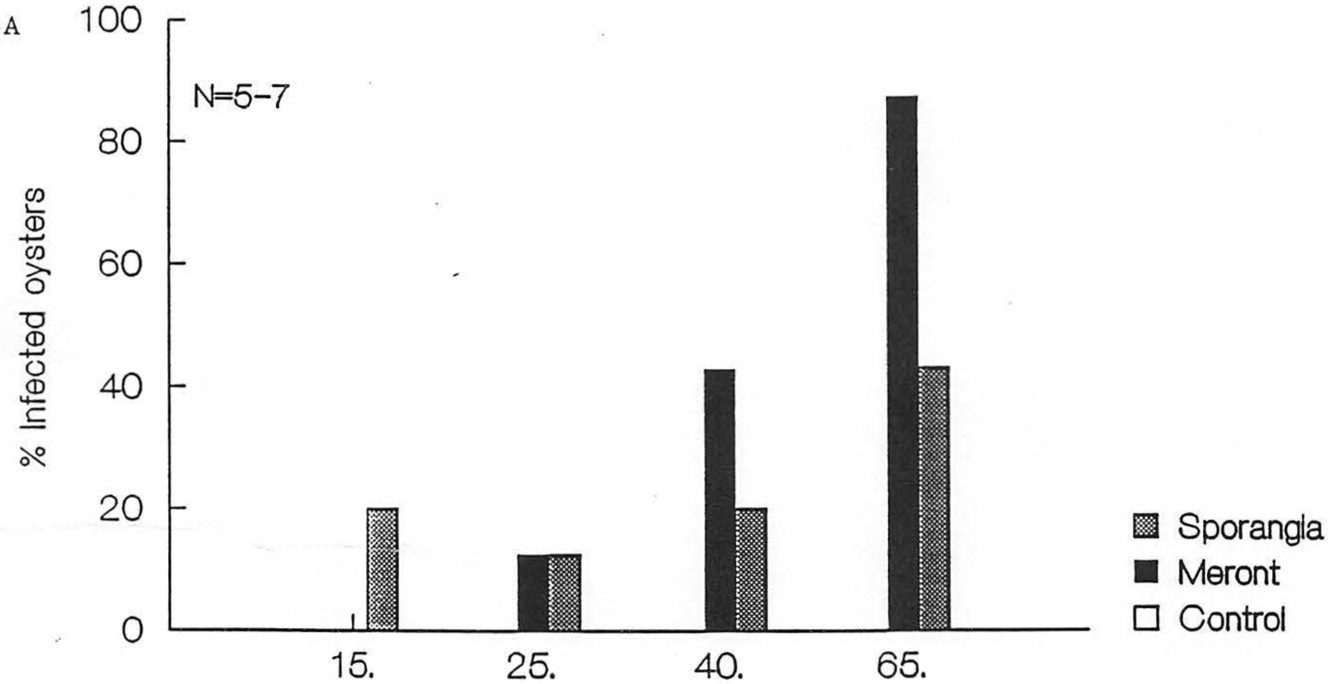
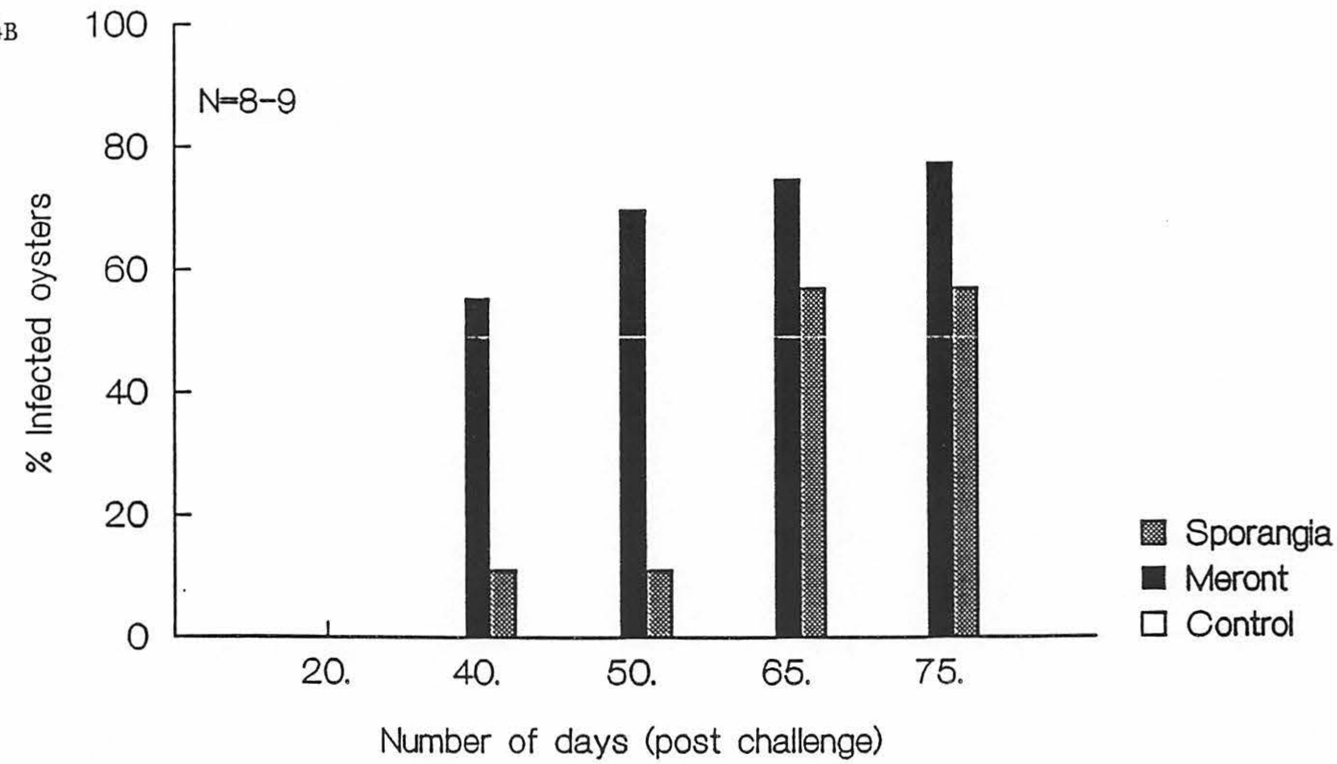


Fig 3: Condition Index of oysters (+ SE) challenged with meronts or sporangia. Bars with similar letters are not significantly differenrt (p>0.05).

Fig 4A



ig 4B



<u>P. marinus</u> prevalence in oysters after 15, 25, 40, and 65 days (Fig. 4A) and 20, 40, 50, 65 and 75 days postchallenge (Fig. 4B) by meronts or prezoosporangia.

Fig. 5A

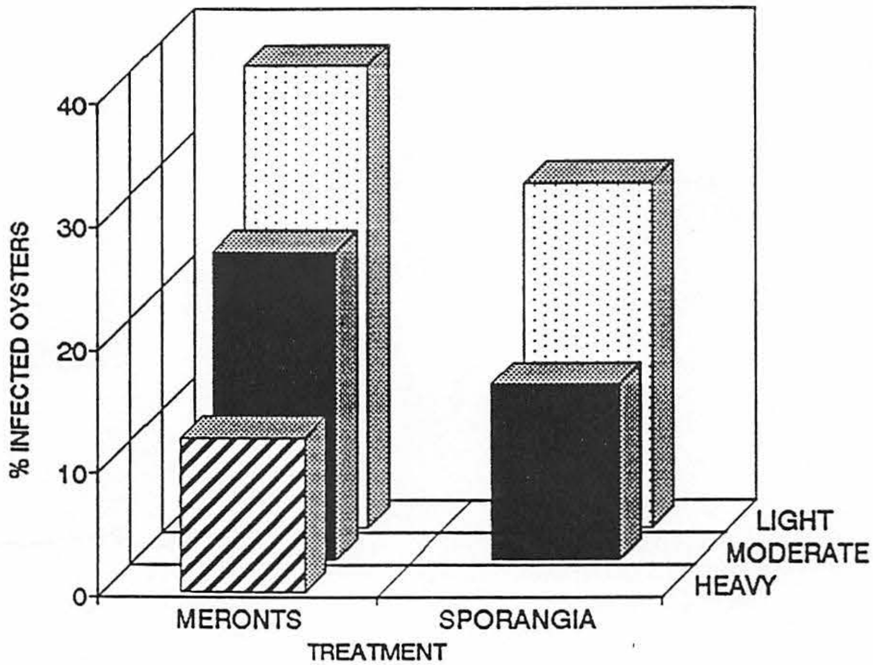
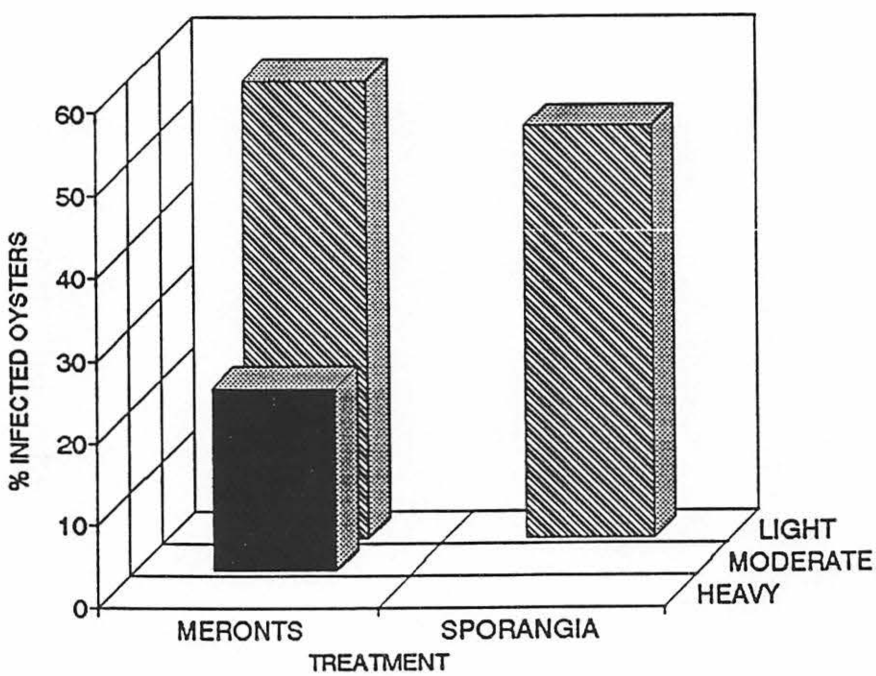


Fig. 5B



P. marinus infection intensity in oysters from trial 1 (Fig. 5A) after 65 days and trial 2 (Fig. 5B) after 75 days postchallenge by meronts and prezoosporangia.

Fig. 6

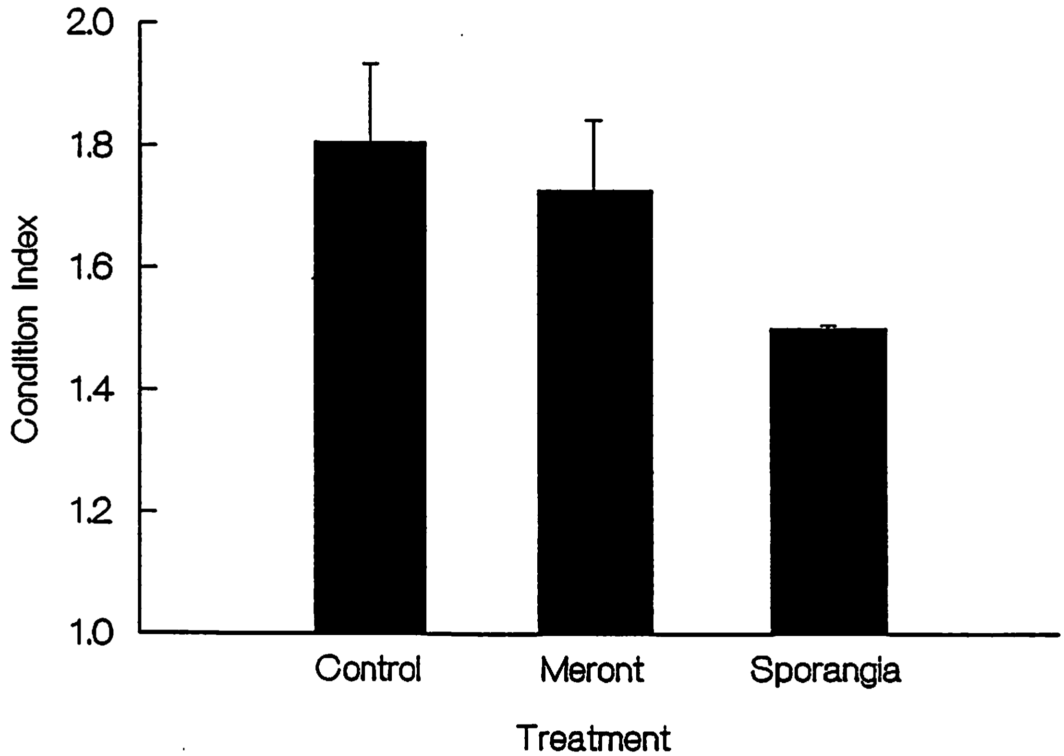


Figure 6. Mean CI (±SE) in uninfected meront-, and prezoosporangia-challenged oysters.

Fig 7

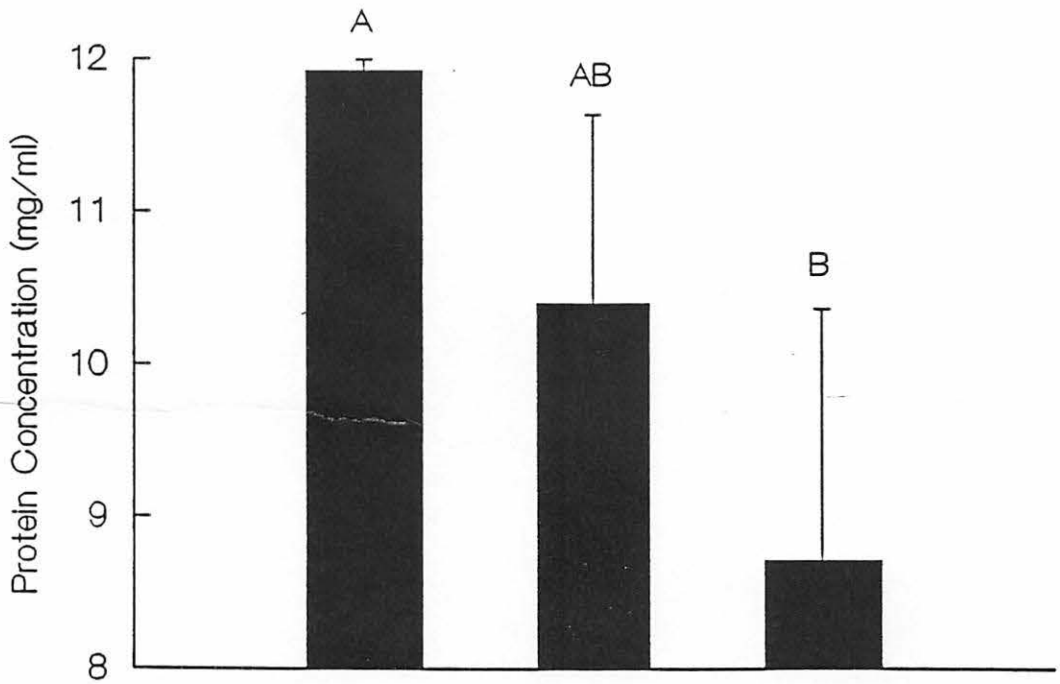
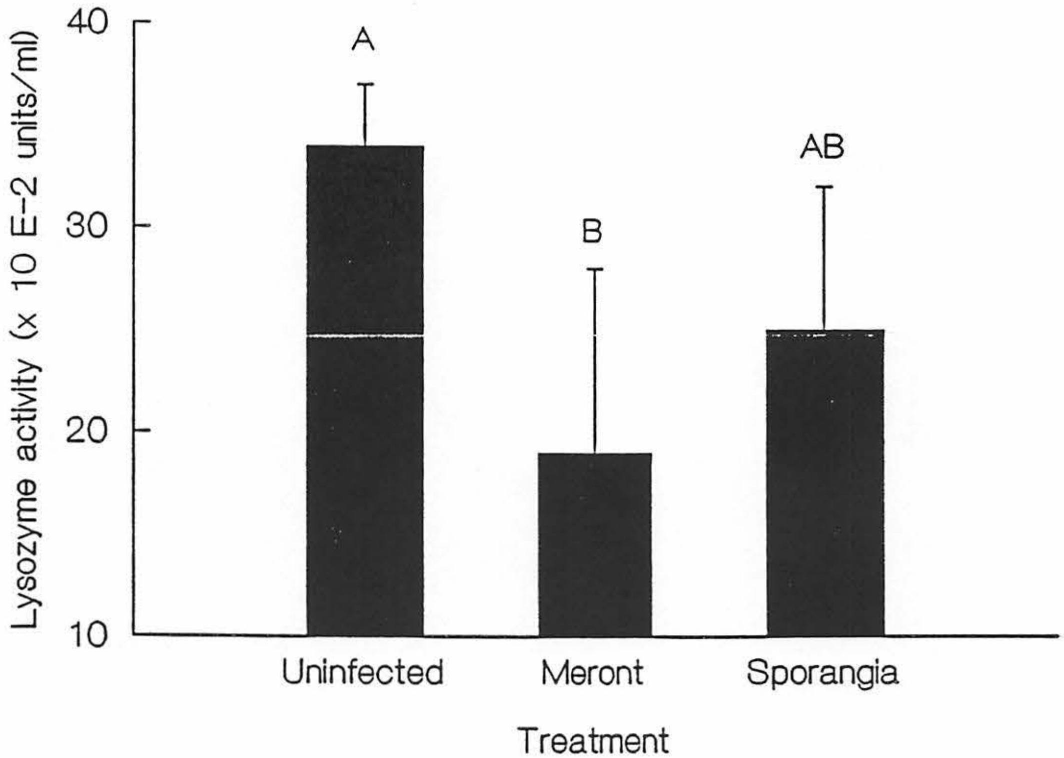


Fig 8



Figures 7 and 8. Mean serum protein concentration (±SE) (Fig. 7) and mean serum lysozyme (±SE) (Fig. 8) in uninfected and infected oysters challenged by meront and prezoosporangia. Bars with similar letters are not significantly different ($p>0.05$).

Fig. 9

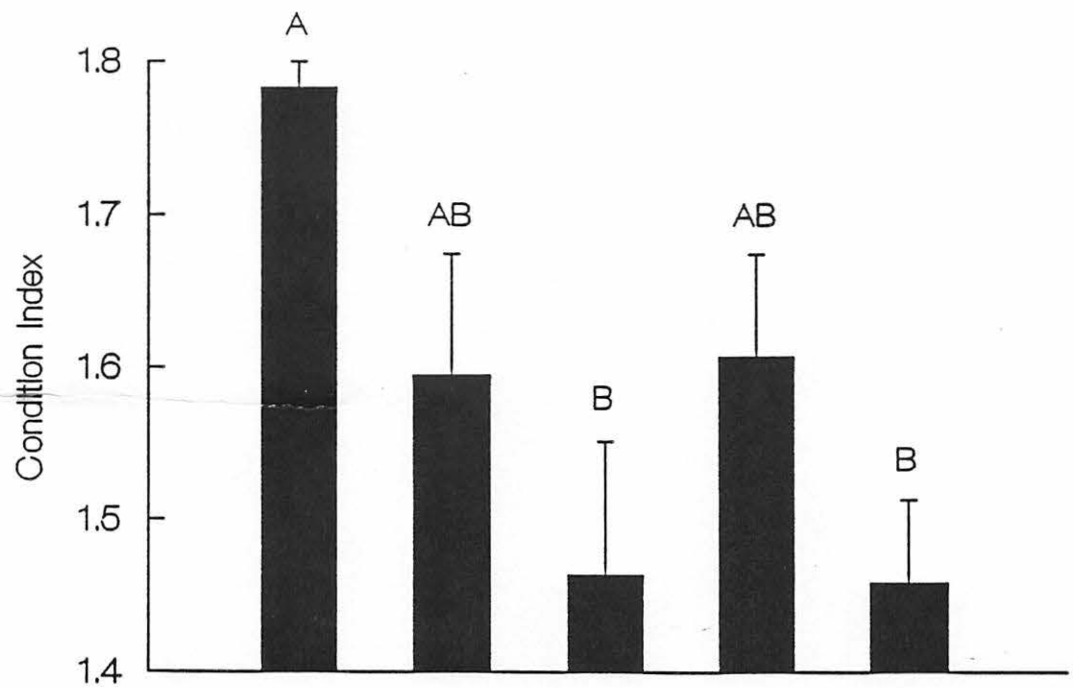
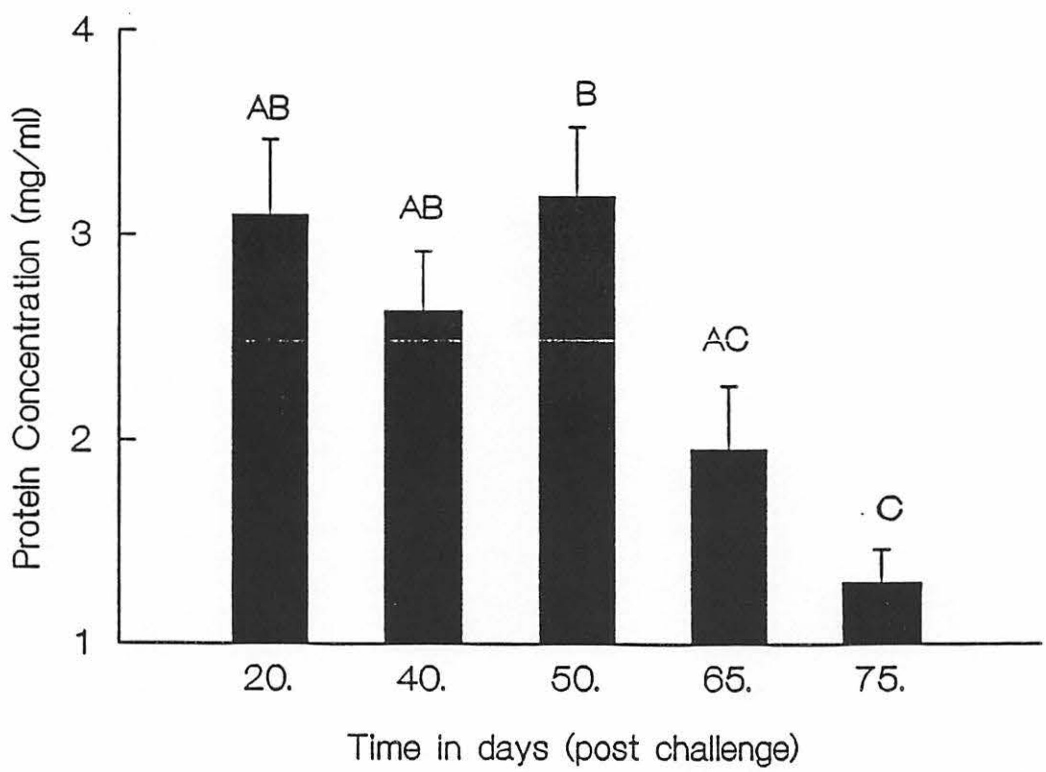


Fig. 10



Figures 9 and 10. Mean CI (±SE) (Fig. 9) and serum protein concentration (±SE) (Fig. 10) in oysters at the end of 20, 40, 50, 65 and 75 days postchallenge. Bars with similar letters are not significantly different.

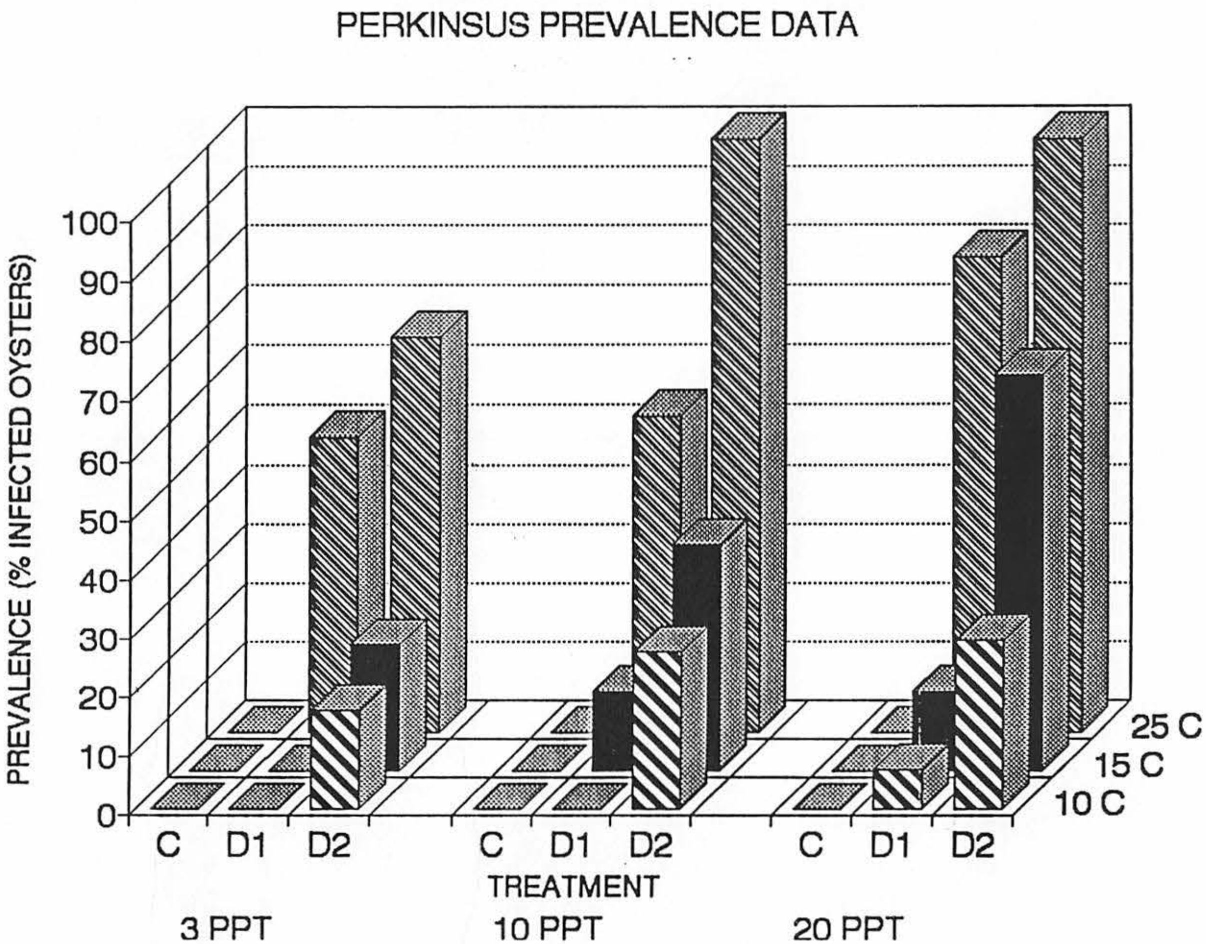
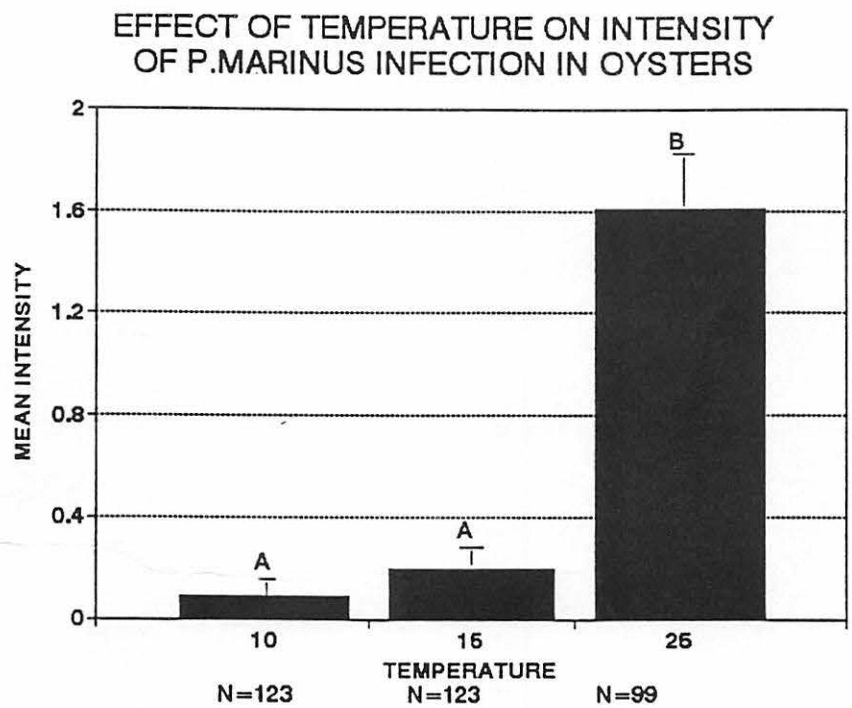


Fig. 11

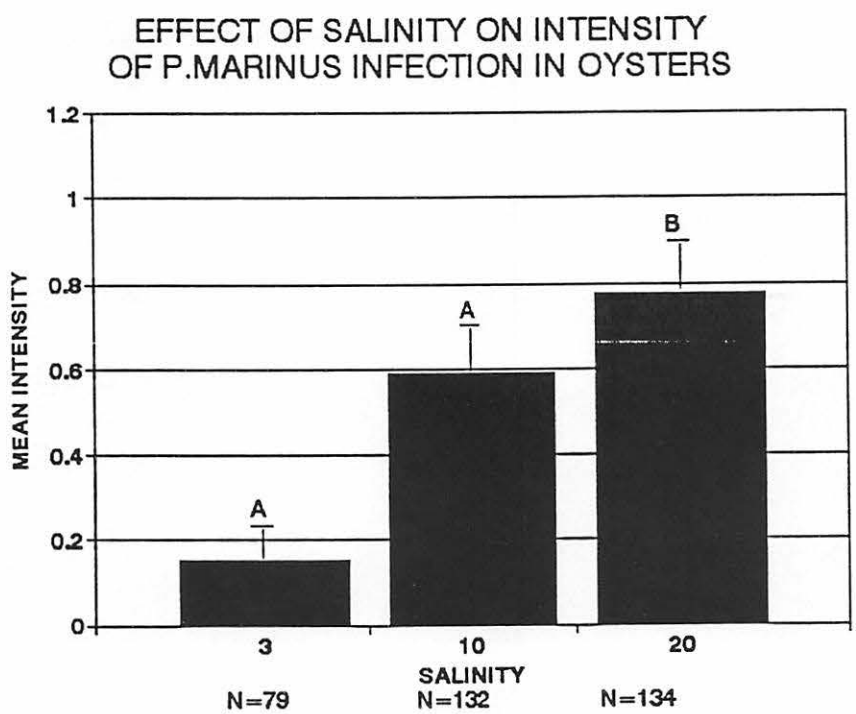
Disease prevalence of P. marinus infection. (C = control, D1 = 2.5×10^3 meronts, D2 = 2.5×10^4 meronts / oyster).

Fig. 12a



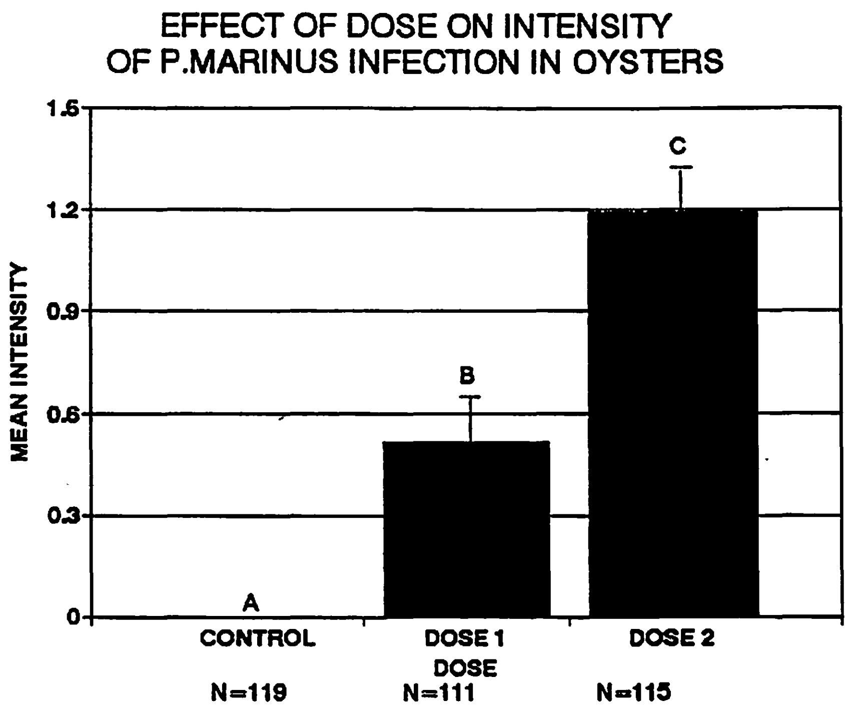
Arrow bars represent ± 1 SE.
Bars with similar letters are not significantly different. (P<0.05).

Fig. 12b



Arrow bars represent ± 1 SE.
Bars with similar letters are not significantly different. (P<0.05).

Fig. 12c



Arrow bars represent $\pm$ 1 SE.
Bars with similar letters are not significantly different. (P<0.05).

Fig. 13

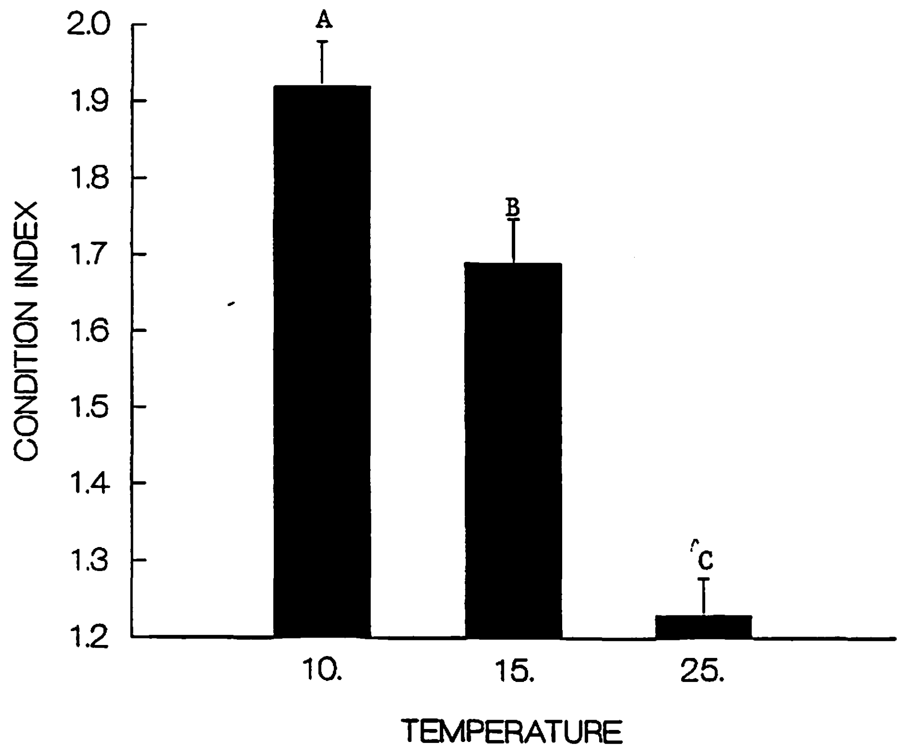


Fig. 14

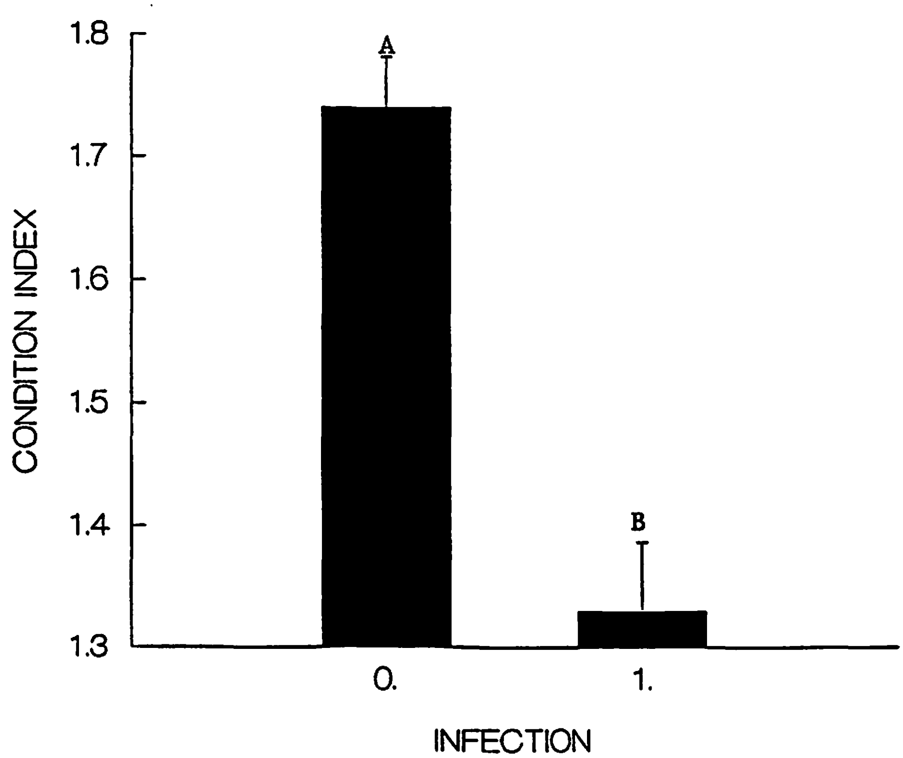


Fig. 13: Condition Index (CI) of oysters at 10, 15 and 25°C. Arrow bars represent ± 1 SE. Bars with similar letters are not significantly different ($p>0.05$).

Fig. 14: Condition Index (CI) of infected and uninfected oysters. 0 = Negative infection, I = Infected. Arrow bars represent ± 1 SE. Bars with similar letters are not significantly different ($p>0.05$).

Fig. 15

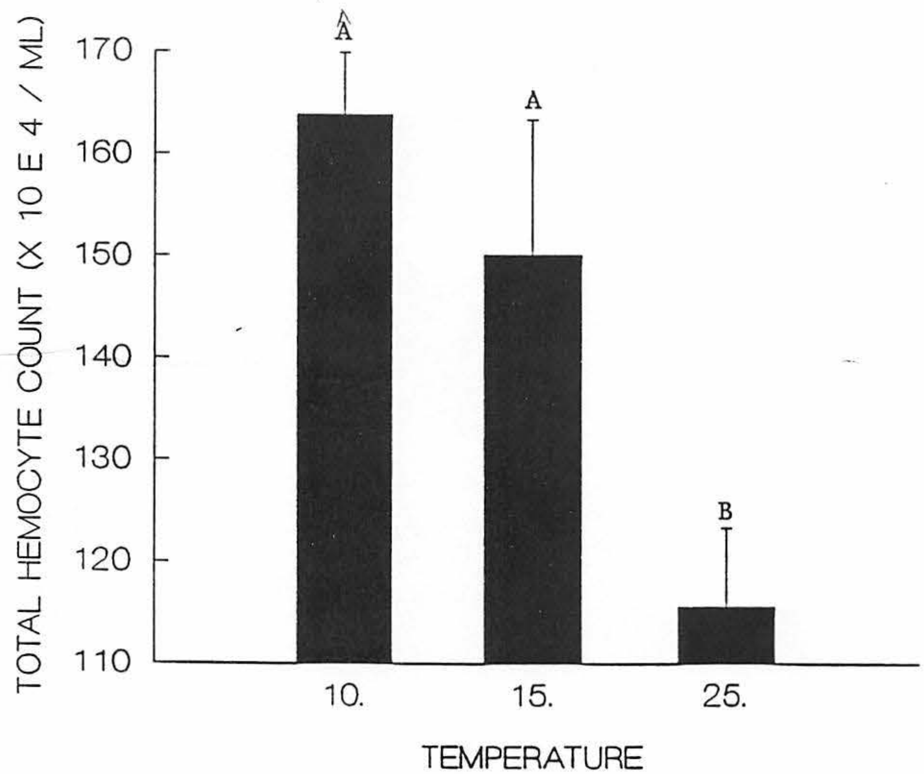
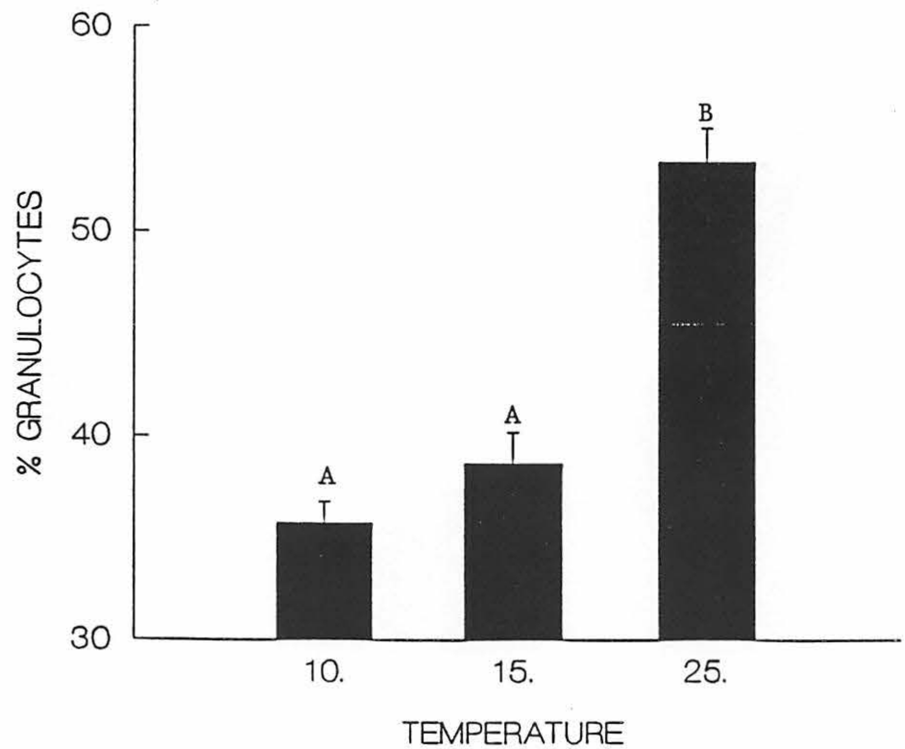


Fig. 16



Total hemocyte counts (TC) (Fig. 15) and % granulocyte (PG) (Fig. 16) in oysters at 10, 15 and 25°C. Arrow bars represent ± 1 SE. Bars with similar letters are not significantly different ($p>0.05$).

Fig. 17a

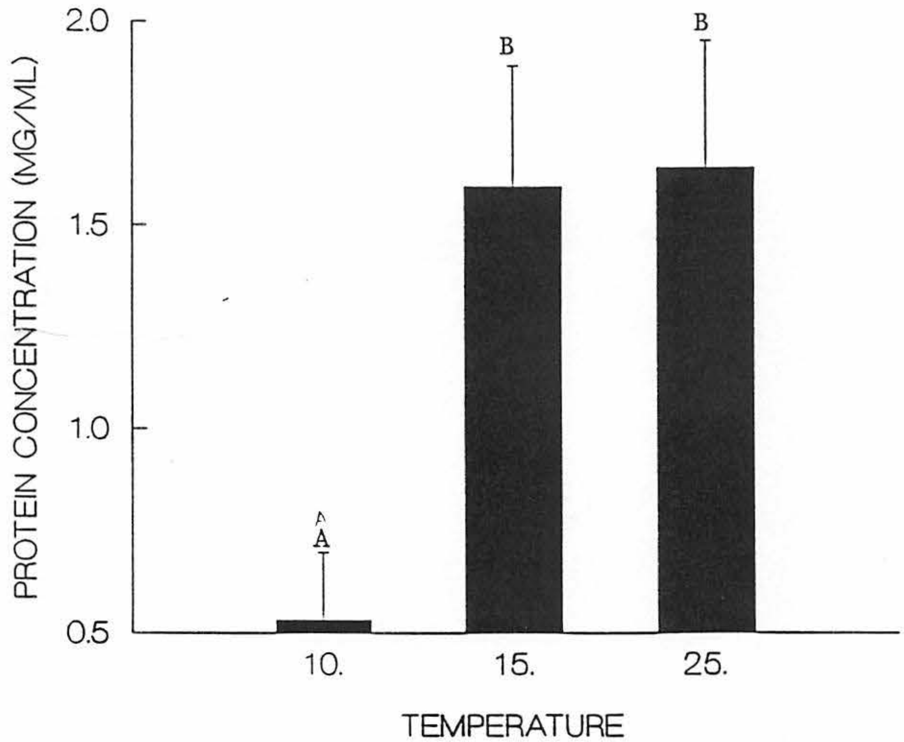
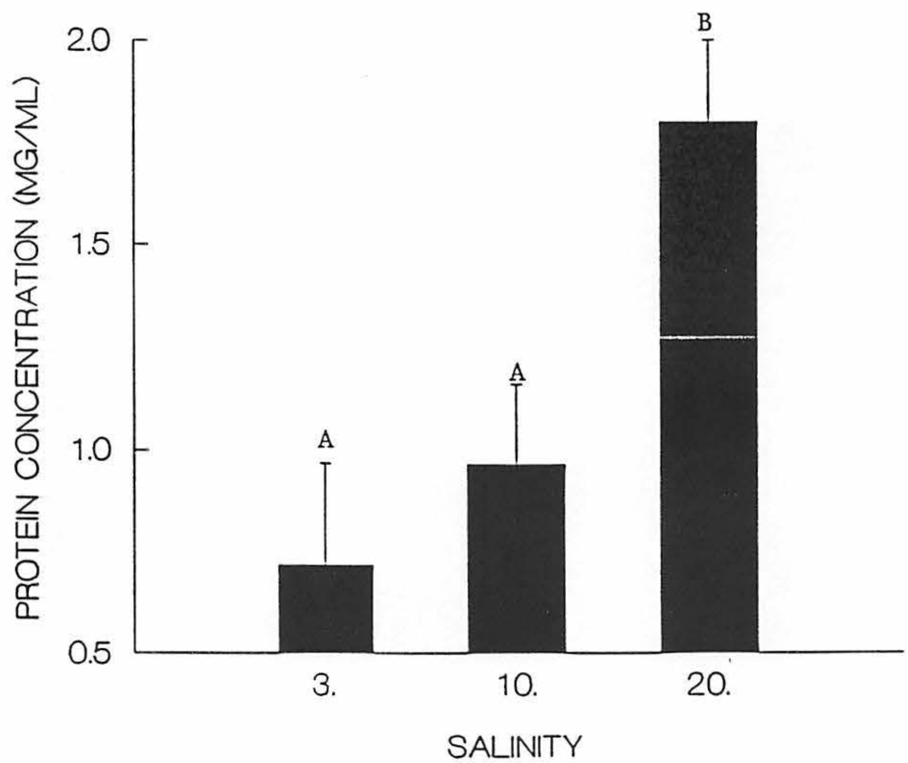


Fig. 17b



Protein concentrations in oysters at 10, 15 and 25°C (Fig. 17a) and at 3, 10 and 20 ppt (Fig. 17b). Arrow bars represent ± 1 SE. Bars with similar letters are not significantly different ($p>0.05$).

Fig. 18a

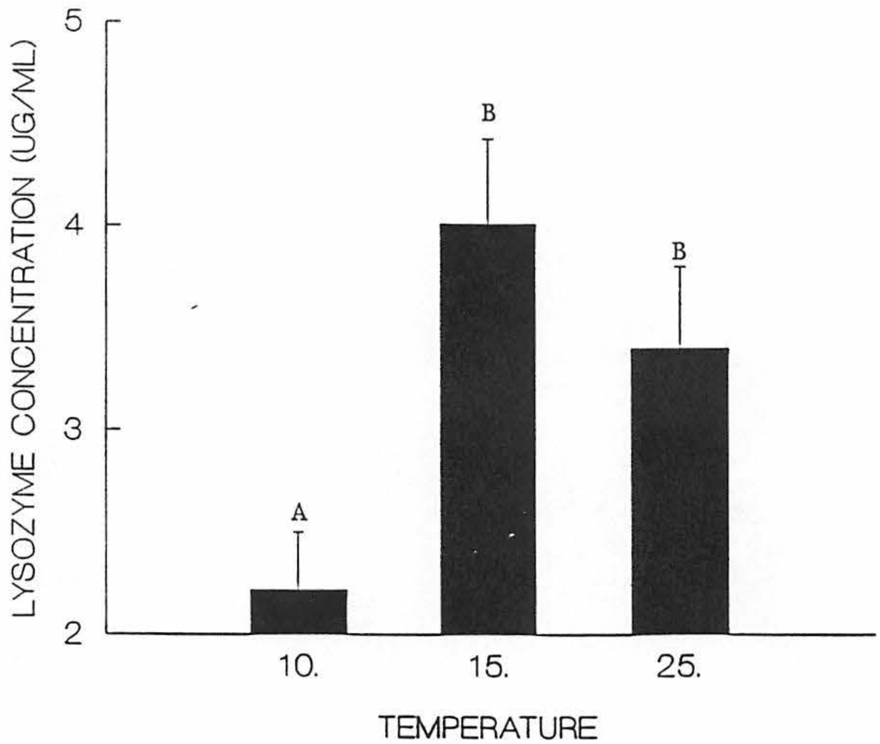
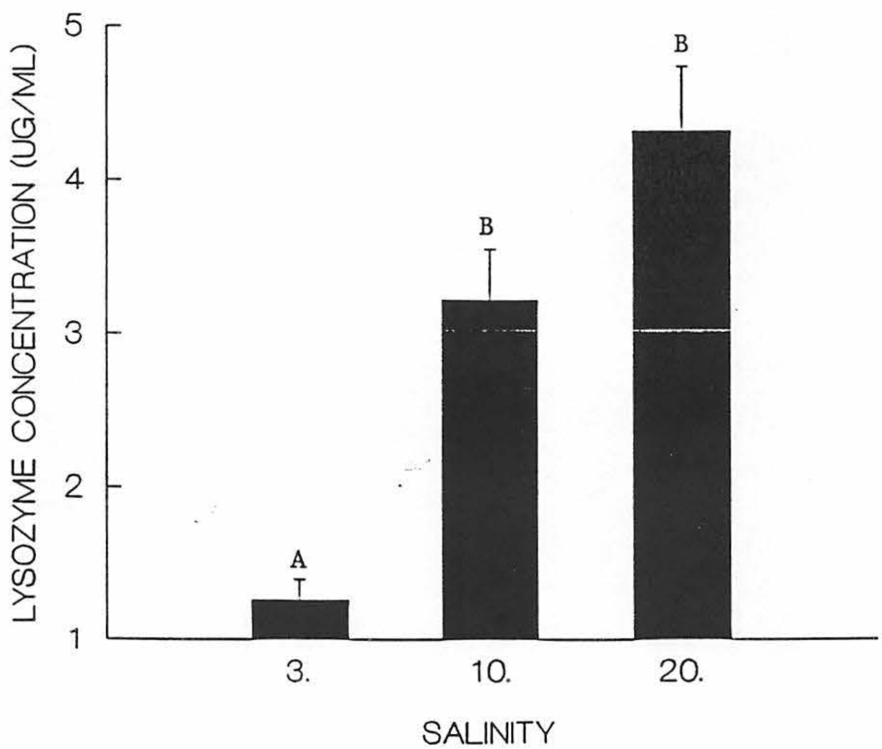


Fig. 18b



Lysozyme concentrations in oysters at 10, 15 and 25°C (Fig. 18a) and 3, 10 and 20 ppt (Fig. 18b). Arrow bars represent ± 1 SE. Bars with similar letters are not significantly different ($p>0.05$).

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