

Disease processes of the parasite *Perkinsus marinus* in eastern oyster *Crassostrea virginica*: minimum dose for infection initiation, and interaction of temperature, salinity and infective cell dose

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ABSTRACT: Experiments were conducted to: (1) test the response of oysters to different doses of the oyster parasite *Perkinsus marinus* and to 2 stages, meronts or prezoosporangia; and (2) investigate the synergistic effects of temperature, salinity and infective cell concentration on *P. marinus* infection in oysters. A dose-dependent response of *P. marinus* infection was found in oysters inoculated with 0, 10, 10^2 , 10^4 , and 10^5 meronts or prezoosporangia per oyster and maintained at 22 to 25°C and 14 to 21 ppt for 8 to 12 wk. The minimum dose required to infect oysters was 10^2 meronts or prezoosporangia per oyster through shell cavity inoculation. Interactive effects between temperature, salinity, and infective cell dose on *P. marinus* prevalence was insignificant in the experiment, in which oysters were challenged by 0, 2.5×10^3 or 2.5×10^4 meronts per oyster and held at 9 temperature-salinity regimes (10, 15 and 25°C at 3, 10 and 20 ppt). However, there was a significant positive interaction relevant to infection intensity between temperature and salinity, and between temperature and meront dose. Temperature was the most important factor followed, respectively, by the infective cell dose and salinity in determining the susceptibility to *P. marinus* in oysters. Reduced condition index was observed in moderately to heavily infected oysters and in oysters at 25°C.

KEY WORDS: Oyster · *Crassostrea virginica* · Oyster parasite · Oyster disease · *Perkinsus marinus* · Temperature · Salinity

INTRODUCTION

The protozoan parasite *Perkinsus marinus* (Dermo) has caused significant mortality in the eastern oyster *Crassostrea virginica* from the mid-Atlantic to the Gulf of Mexico in the United States since the 1950s. *P. marinus* is now more prevalent in mid-Atlantic waters, and Chesapeake Bay in particular, than the other common protozoan pathogen, *Haplosporidium nelsoni* (MSX) (Andrews 1988, Ragone-Calvo & Bureson 1995). The meront and prezoosporangia stages of the parasite can cause *P. marinus* infection in oysters (Volety & Chu 1994). The meront stage is more effective than the prezoosporangia stage in inducing infection (Volety &

Chu 1994) and is believed to be the principal stage for transmitting disease in the field (Perkins 1988). The distribution and abundance of *P. marinus* in the field are limited by temperature and salinity (see reviews by Andrews 1988, Andrews & Ray 1988). The dosage of *P. marinus* cells is also considered to be critical in disease transmission (Mackin 1956, 1962). However, thus far no laboratory study has been conducted to examine the interactive effects of these 3 crucial factors on the disease processes in oysters. Similarly, the number of *P. marinus* infective cells that are required to transmit the disease is unclear. In this study, we investigated (1) the response of eastern oysters to different doses of meront and prezoosporangia of *P. marinus*; (2) the minimal dose for initiation of *P. marinus* infections in eastern oysters; and (3) the interaction between tempera-

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ture, salinity and infective cell dose on *P. marinus* infections in the eastern oysters.

METHODS AND MATERIALS

Preparation of meront and merozoite suspensions.

Meronts and merozoites (immature meronts) of *Perkinsus marinus* were isolated and purified as follows: oysters heavily infected with *P. marinus* were rinsed thoroughly with filtered (0.22 μm) York River water (YRW) and subsequently homogenized in 0.22 μm filtered YRW with a blender (Virtis, Model 23) at high speed for 2 min. The tissue suspension was then passed through a series of 100, 50, 35 and 20 μm meshes to remove oyster tissue residues (La Peyre & Chu 1994). Meronts contained in the filtrate were further purified by repeated centrifugation and washing. The number of meronts in suspension was counted using a hemacytometer (Reichert, Buffalo, NY, USA) and adjusted to the desired concentrations. About 99% of the isolated infectives were meront/merozoites and $\leq 1\%$ were schizont (sporangium).

Preparation of prezoosporangia. Prezoosporangia were isolated and purified from *Perkinsus marinus* infected oyster tissues previously cultured in fluid thioglycolate medium (FTM) for 4 to 5 d on the basis of the method described by Chu & Greene (1989).

***Perkinsus marinus* diagnosis.** *P. marinus* infections were diagnosed using a thioglycolate tissue assay (Ray 1952, 1966). At the end of each experiment, one piece each of rectal, digestive diverticulum and mantle tissues were removed from individual oysters and incubated in FTM for 4 to 5 d. Disease intensity was ranked 0, 1, 3, 5 (negative, light, moderate, or heavy) based on the number of prezoosporangia present in the tissues. Weighted prevalence (WP = sum of disease intensity rank/number of oysters examined) was calculated according to Ray (1954), Mackin (1962) and Andrews (1988).

Experiments. Responses of oysters to different doses of meronts and prezoosporangia: Two replicate experiments were performed to test the responses of eastern oysters to different doses of meronts or prezoosporangia using oysters from the Damarsicotta River, Maine, USA, a region beyond the geographical distribution of *Perkinsus marinus*. The ambient temperature and salinity at the time of collection were 1°C and 30 ppt (February 1994) and 20°C and 30 ppt (August 1994) in Expts 1 and 2, respectively. The oysters (size range 5.0 to 6.5 cm) were gradually acclimated in 1000 l tanks over a period of 6 wk to the ambient salinity of YRW and room temperatures (Expt 1: 24.53 \pm 1.39°C and 13.6 \pm 2.56 ppt; Expt 2: 22 \pm 1.19°C and 20.64 \pm 0.55 ppt). Oysters were fed 0.2 g algal

paste (*Thalassiosira weissflogii*) per individual daily and the water was changed 3 times per week during acclimation. A total of 150 oysters were used for each experiment. Before the experimental oysters were challenged with *P. marinus*, 15 randomly selected oysters were diagnosed for possible *P. marinus* infection using tissue thioglycolate assay. All oysters were diagnosed to be negative at that time. Condition indices (CI = dry meat weight/dry shell weight \times 100; Lucas & Beninger 1985) of these oysters were also determined. The remaining 135 oysters were divided into 9 groups (15 oysters per group) and were inoculated into the shell cavity with a dose of 0, 10, 10², 10⁴, or 10⁵ freshly isolated meronts/merozoites or prezoosporangia in 100 μl of 0.22 μm filtered YRW per oyster. These oysters were then randomly arranged in individual 2.0 l plastic chambers containing 1 μm filtered aerated YRW. Water was changed every other day and the oysters fed as before. Oysters of each group were sampled to measure *P. marinus* prevalence and intensities, and condition index (CI) after 92 d (Expt 1) and 60 d (Expt 2) following *P. marinus* challenge.

Interactive effects of temperature, salinity and infective cell dose:

The interactive effects between temperature, salinity and infective cell doses were tested by exposing oysters to 3 different doses (0, 2.5 \times 10³ or 2.5 \times 10⁴) of freshly isolated meronts/merozoites per oyster) at 9 salinity-temperature combinations: 10, 15 and 25°C at 3, 10 and 20 ppt for 60 d. Five hundred oysters were collected from Damarsicotta River (January 1993; ambient temperature = 0°C, salinity = 32 ppt). A random subsample of 30 oysters was examined for *Perkinsus marinus* infection and condition index. No *P. marinus* infection was detected in any of the 30 oysters. Experimental oysters were gradually acclimated over a period of 67 d in 1000 l tanks to the 9 test temperature and salinity regimes indicated above using 1.0 μm filtered YRW adjusted to desired temperature and salinity. After the oysters were acclimated to their respective test temperatures and salinities, they were randomly placed in individual chambers and maintained thereafter in aerated water of the relevant test salinity and temperature. Water was changed every other day and each oyster was fed daily with 0.1 g algal paste. The shell cavities of individual oysters ($N = 15$) were inoculated with 100 l filtered YRW containing 0, 2.5 \times 10³ or 2.5 \times 10⁴ freshly isolated *P. marinus* meronts. Mortality was monitored through the experimental period and dead oysters were examined for *P. marinus* infection.

Statistical analyses. Log linear model and logistic regression (Agresti 1990) were used to determine: (1) the significance of interactive effects among temperature, salinity, and *Perkinsus marinus* infective cell doses on disease prevalence; and (2) differences in

infection prevalence in oysters challenged by different doses of meronts or prezoosporangia. A log linear model was first used to determine the interactive effects of temperature, salinity and dose of *P. marinus* cells on infection prevalence in oysters. Since no interactive effects were observed among temperature, salinity and *P. marinus* cell dose on infection prevalence, individual effects were analyzed by the more parsimonious model, logistic regression. Analysis of variance was used to determine: (1) effects of *P. marinus* cell stages and cell doses (2-way ANOVA) on oyster CI in dose response Expts 1 and 2; (2) effects of temperature-salinity-infective cell dose (3-way ANOVA) on CI in oysters in the interactive effect experiment; (3) difference in CI between infected and uninfected oysters (1-way ANOVA); and (4) interactive effects of temperature-salinity-infective cell dose (3-way ANOVA) on infection intensity. A multiple comparison test (Tukey) was used to evaluate the differences among treatment means.

RESULTS

Dose response in oysters

Infection prevalence and intensity

A dose-dependent response of *Perkinsus marinus* infection prevalence was observed in the 2 replicate experiments, in oysters exposed to 0, 10, 10², 10⁴ and 10⁵ meronts or prezoosporangia ($p < 0.0001$; Figs. 1A & 2A). A similar pattern was shown in infection intensity, expressed as weighted prevalence (Figs. 1B & 2B). The meront stage caused much higher ($p < 0.05$) infection prevalence and intensity in oysters than did prezoosporangia. The infection prevalences in oysters challenged by 10, 10², 10⁴, and 10⁵ meronts was 0, 13, 50 and 71 %, respectively, in Expt 1 and was 0, 13, 67, and 80 %, respectively, in Expt 2. Oysters inoculated with prezoosporangia of concentrations corresponding to meront-challenged groups resulted in prevalences

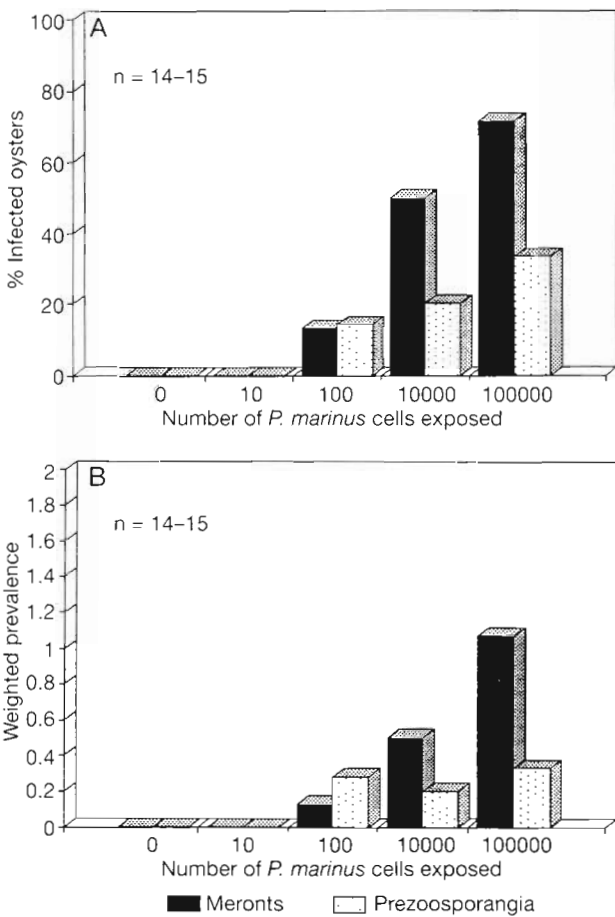


Fig. 1. *Crassostrea virginica*. (A) Prevalence and (B) intensity of *Perkinsus marinus* in oysters 92 d (Expt 1) after inoculation with 0, 10, 10², 10⁴, or 10⁵ meronts or prezoosporangia (n = 14 or 15 per group)

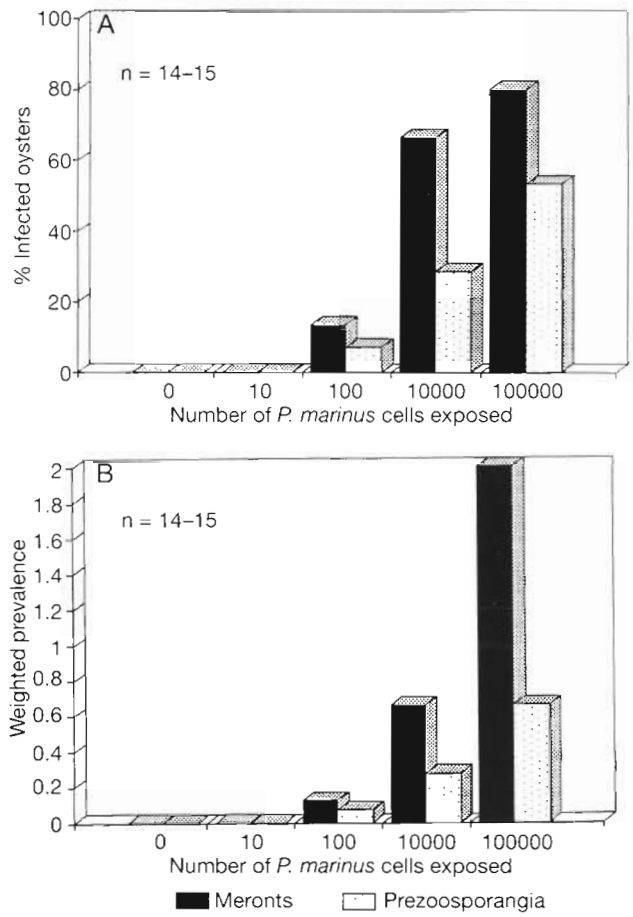


Fig. 2. *Crassostrea virginica*. (A) Prevalence and (B) intensity of *Perkinsus marinus* in oysters 60 d (Expt 2) after inoculation with 0, 10, 10², 10⁴, or 10⁵ meronts or prezoosporangia (n = 14 or 15 per group)

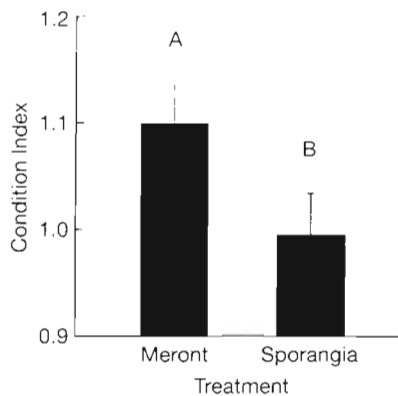


Fig. 3. *Crassostrea virginica*. Mean CI (±SE) in meront- and prezoosporangia-challenged oysters. Different letters denote significance and difference ($p < 0.05$)

of 0, 14, 20 and 33%, respectively, in Expt 1 and 0, 7, 29 and 53%, respectively, in Expt 2. No infections were detected in control oysters. The lowest dose that initiated a detectable *P. marinus* infection was 10^2 meronts or prezoosporangia per oyster. No mortality occurred during the experimental period in either experiment.

Condition index

In Expt 1, when data were pooled from all dose groups regardless of infection, the CI of prezoosporangia-challenged oysters was significantly ($p < 0.05$) lower than meront-challenged oysters (Fig. 3). No significant differences in CI were observed between meront-challenged and prezoosporangia-challenged oysters in Expt 2 ($p > 0.05$). There was no significant difference in CI between infected and uninfected or among dose groups in both experiments.

Interaction of temperature, salinity and *Perkinsus marinus* cell doses

Infection prevalence and intensity

Increased infection prevalence and intensity (Fig. 4A, B) occurred at high temperatures ($p < 0.0001$) and salinities ($p < 0.0001$), and there was a dose-dependent response to infective cells ($p < 0.0001$). The results of a log linear model analysis revealed that temperature was the most important factor in determining the sus-

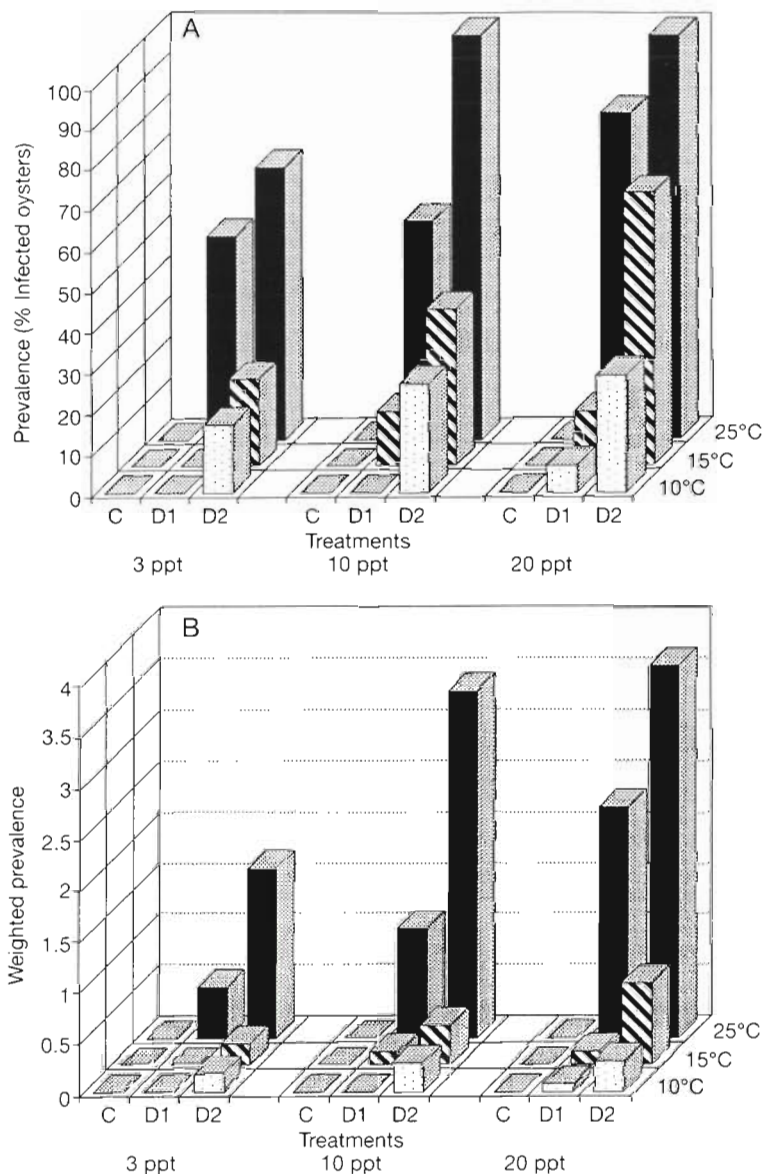


Fig. 4. *Crassostrea virginica*. (A) Prevalence and (B) intensity of *Perkinsus marinus* in oysters at different temperature and salinity regimes after being challenged with 2 different doses of *P. marinus* infective cells. C: control; D1: 2.5×10^3 meronts oyster⁻¹; D2: 2.5×10^4 meronts oyster⁻¹

ceptibility of oysters to *Perkinsus marinus*. This was followed, respectively, by the dose of infective particles and salinity. The effect of the 3-way interaction among temperature, salinity and meront doses on disease prevalence was found to be insignificant ($p > 0.05$). However, there was a significant and positive 2-way interaction between temperature and salinity ($p < 0.0001$), and between temperature and meront dose ($p < 0.01$) on infection intensity. Mortalities occurred in oysters during acclimation. Mortality was particularly heavy when the acclimation salinity

Table 1 *Crassostrea virginica*. % Mortality of oysters during the acclimation period. Numbers in parentheses indicate mortality (no. dead/total no.)

Salinity (ppt)	Temperature (°C)		
	10°C	15°C	25°C
3	22 (10/45)	27 (12/45)	76 (34/45)
10	2 (1/45)	4 (2/45)	0 (0/45)
20	2 (1/45)	0 (0/45)	2 (1/45)

and temperature reached 3 ppt and 25°C (Table 1). However, none of the dead oysters were found to be infected with *P. marinus*. Since the mortalities occurred before *P. marinus* challenge, the number of dead oysters was not included in disease prevalence calculation.

Condition index

Oyster CI was reduced significantly ($p < 0.0001$) with an increase in temperatures (Fig. 5). Neither salinity nor meront concentrations affected ($p > 0.05$) the CI of oysters. When the CI data of infected or uninfected oysters from all treatments were pooled, the CI of the former was significantly lower ($p < 0.0001$) than the latter.

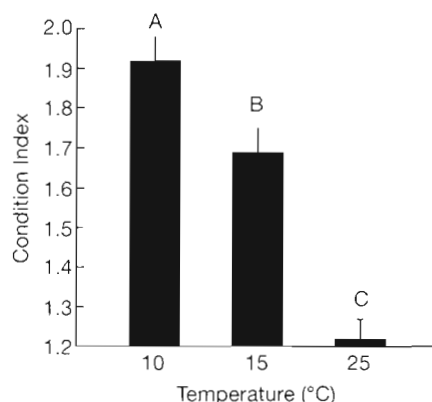


Fig. 5. *Crassostrea virginica*. Mean condition index (\pm SE) of oysters maintained at 10, 15 and 25°C. Different letters denote significance and differences ($p < 0.05$)

DISCUSSION

The relationship between ambient infective cell concentrations and infection rate, and the number of *Perkinsus marinus* cells that are required to initiate an infection, is of great interest. In addition to temperature and salinity, *P. marinus* infective cell concentration has been considered to be a crucial factor for dis-

ease transmission among oysters in the field (Mackin 1956, 1962). Mackin (1962) argued that the observed low *P. marinus* prevalence and intensity in areas with high river or fresh water input is a result of dilution of waterborne infective particles. The dose-dependent responses of oysters to *P. marinus* infection demonstrated in the present study shows that on-site concentration of infective cells is indeed important in *P. marinus* transmission. Dose was the second important factor (after temperature) in contributing to *P. marinus* infections in oysters. The dose response in oysters to *in vitro* cultured *P. marinus* was studied by Bushek (1994). Similar to our results, he observed increased infection prevalence and intensity in oysters as the dose of *P. marinus* cells increased. An earlier study by Mackin (1962) and Valiulis (1973) also showed that there was a relationship between oyster mortality and inoculated infective cell dose. All of these results support Mackin's view that diluting infective particles during the peak infection season (summer-fall) by influx of fresh water or tidal flushing should decrease the rate of new infections in oyster populations although low salinity is also a controlling factor (Mackin 1962).

As shown by the present study, a minimum number of infective cells is required for induction of *Perkinsus marinus* infection: i.e. 10^2 meronts or prezoosporangia per oyster via shell cavity inoculation. While no infection was detected in oysters 92 and 60 d after inoculation with 10 meronts or prezoosporangia in our study, in a previous investigation development of light infection was noted in oysters 105 d after receiving a dose of 10 cells per oyster injected into their shell cavity (Valiulis 1973). We believe that the dosage required for *P. marinus* transmission probably lies between 10 and 10^2 per oyster by shell cavity injection. Mackin (1962) found that direct shell cavity injection of 1.0 to 5.0×10^2 *P. marinus* cells per oyster is necessary to cause disease-related mortality. *P. marinus* is probably acquired by filtering the water and may be eliminated through feces and pseudofeces (Bushek et al. 1994). Thus, the number of cells required to effect transmission may be much greater in the field.

How *Perkinsus marinus* infective cells, discharged from carriers or released from infected gapers, actually enter the host oyster is unclear. Since other alternatives appear to be absent, entry through filtration/feeding is assumed to be the main route of *P. marinus* transmission in nature. However, shell cavity injection appeared to be more effective than feeding in infection induction studies (Bushek 1994, Perkins 1994). Thus, although in nature more than 10 to 10^2 *P. marinus* infectives cells may be required to initiate infection in oysters, once this number of infective cells are trapped in the shell cavity during feeding and/or filtration processes, infection will result.

Consistent with our previous finding (Volety & Chu 1994), the meront stage caused much higher *Perkinsus marinus* infection prevalence and intensity in oysters than prezoosporangia. Volety & Chu (1994) speculated that the higher infectivity/pathogenicity of meronts than prezoosporangia is because of the rapid multiplication of inoculated meronts at warm temperatures (i.e. 21 to 27°C). Exactly what occurs after the prezoosporangia are injected into the oyster shell cavity is unknown. There may be a lag time for the inoculated prezoosporangia to transform to meront stage, or culturing meronts in FTM may affect the infectivity/pathogenicity of the subsequent prezoosporangia. It has been reported that prezoosporangia developed in FTM produced meront/merozoites through bipartition and protoplast cleavage (La Peyre 1993).

Perkinsus marinus is most dominant and abundant in subtropical and tropical regions. Temperature has long been documented in field and laboratory studies to be a factor limiting *P. marinus* infection (Mackin 1951, 1956, Ray 1954, Andrews & Hewatt 1957, Quick & Mackin 1971, Andrews & Ray 1988, Chu & La Peyre 1993a). It is not surprising that among the factors tested, temperature turned out to be the most important. Similarly, Fisher et al. (1992) found temperature to be more influential than salinity on *P. marinus* intensity and mortality in oysters from the Gulf of Mexico, maintained at different test temperatures and salinities. It has also been demonstrated that *in vitro* warm temperatures (i.e. 21 to 28°C) favor *P. marinus* development (Perkins 1966, Chu & Greene 1989) and the proliferation rate increased with increasing temperature (Volety 1995). In the Gulf of Mexico region, fluctuations of disease prevalence in oysters were noted to be more sensitive to changes in temperature than to salinity (Mackin in Ray 1954). In the Chesapeake Bay region, the results of analysis of the last 10 yr data indicate that *P. marinus* activity and annual periodicity is largely correlated with the fluctuations of seasonal temperatures (Burrenson & Ragone-Calvo 1996). However, it should be noted that water along the Gulf coast, unlike Chesapeake Bay, usually stays warm year-round. Between 1986 and 1989, the seasonal shift of *P. marinus* infection was found to be associated with the change of salinity caused by rainfall and river runoff rather than temperature (Powell et al. 1992).

Although under laboratory-controlled conditions salinity is less influential than temperature or *Perkinsus marinus* cell concentration in controlling *P. marinus* infection in oysters, it has been well documented that susceptibility to infection by *P. marinus* in oysters is significantly and positively correlated with salinity (Chu et al. 1993, Chu & La Peyre 1993b). Only light infections were observed in oysters below 10 ppt in the present study. In nature, *P. marinus* infection is more

abundant and intense in areas of elevated salinity (Scott et al. 1985, Andrews 1988, Andrews & Ray 1988, Craig et al. 1989, Soniat & Gauthier 1989, Crosby & Roberts 1990, Ragone-Calvo & Burrenson 1995). *In vitro* development of the parasite has also been reported to be significantly affected by salinity (Perkins 1966, Chu & Greene 1989). Salinity lower than 6 ppt inhibited the sporulation of prezoosporangia (Chu & Greene 1989). Low salinities (<12 ppt) also significantly reduced the viability of *in vitro* cultured *P. marinus* meronts (Burrenson et al. 1994).

To our best knowledge, this is the first laboratory investigation of the potential synergetic effects of temperature-salinity-infective cell dose on *Perkinsus marinus* infection in oysters. Clearly, temperature and salinity each have a significant and direct effect on the outcome of the interaction between the host and the parasite. Infective cell concentration is certainly another crucial factor for initiation of *P. marinus* infection in oysters. However, the interactive effects of these 3 factors is likely more striking than any single factor acting alone. Although this interactive effect on infection prevalence was statistically insignificant, the positive interaction between temperature and salinity, and between temperature and meront dose, significantly intensified the infection. Based on data collected from field samples, temperature and salinity interaction on *P. marinus* infection intensity was noted in a field study conducted on the Gulf coast (Soniat 1985). A recent study by Ragone-Calvo & Burrenson (1994) also suggests that a combination of low temperature and salinity significantly restricts the *P. marinus* activity in oysters. The decline of *P. marinus* prevalence and intensity during winter appears more rapidly in low salinity areas than in high salinity areas in the Chesapeake Bay (Burrenson & Ragone-Calvo 1996). The synergistic effects of high temperature and high salinity should work in favor of the parasite. Several consecutive years of droughts and warm summers resulted in exceptional high oyster mortality in the Chesapeake Bay caused by *P. marinus* infection (Andrews 1988). The combined effects of temperature and salinity on *in vitro* *P. marinus* development were assessed by Perkins (1966) and by Chu & Greene (1989). High temperature-salinity treatment shortened the time for prezoosporangia developing to sporulation. The positive interaction between temperature and infective cell concentration may also explain why *P. marinus* infection in oysters is greatest in the summer months in mid-Atlantic water. During summer, overwinter infections generally progress towards moderate to heavy infections and high disease-associated mortality usually occurs during the warmest time (late July and August) of the year. At that time, infective cells discharged from live and dead infected oysters in the water col-

umn should increase significantly. Concentrations of *P. marinus*-like cells resembling the meront stage were reported in the range 3000 to 9000 cells l⁻¹ in the upper Chesapeake Bay during the warm months (March to October) between 1992 and 1993 (Dungan & Roberson 1993). Assuming an average of 6000 cells l⁻¹ present in ambient water and an oyster filtration rate of ≥ 8 l water h⁻¹ (Galtsoff 1964) results in an encounter rate ≥ 48 000 cells h⁻¹. Chronic feeding of high levels of *P. marinus* may be necessary to cause infection in oysters in the field (Bushek 1994). Given that the dose required for infection initiation lies between 10 and 10² infective cells per oyster by shell cavity inoculation and that an oyster can encounter ≥ 48 000 cells h⁻¹ in the warm months of a year, it is easy to see why infection rate reaches its peak in the summer months: a positive interactive effect of temperature and infective cell concentration. It is not understood though why high concentrations of infective cells were found as early as March and April in the Dungan & Roberson (1993) study. It is very unlikely that infections acquired in the previous year would develop to moderate or heavy intensity during the spring months and release large amount of *P. marinus* cells. The methodology of employing *P. marinus* polyclonal antibodies to quantify *P. marinus* infective cells has the disadvantage of cross-reactivity; thus the reported *P. marinus* concentrations (Dungan & Roberson 1993) may be substantially overestimated.

The reduced CI noted in infected oysters in the temperature-salinity dose experiment is an effect of advanced infection. Most of the infected oysters at higher temperatures, salinities, and dose were moderately to heavily infected (Fig. 4B). In contrast, the intensity of infection in the dose response experiments were light. Reduction of CI was previously reported in oysters heavily infected by *Perkinsus marinus* (Paynter & Bureson 1991, Dittman 1993) and MSX (Newell 1985, Barber et al. 1988a, b). In Expt 1, the CI of prezoosporangia-challenged oysters was significantly lower than meront-challenged oysters and is similar to our previous findings (Volety & Chu 1994). However, such effect was not seen in Expt 2. It is not known why oysters challenged by prezoosporangia had lower CI than oysters challenged by meronts.

High mortality recorded in oysters at temperatures above 15°C and salinity less than 10 ppt during acclimation suggests that 3 ppt and 25°C are stressful for oysters adapted to cold and high salinity environment. Overall, the decreased CI in oysters at higher treatment temperature (i.e. 15 and 25°C) may be due to the combined effects of the following: (1) higher metabolism; (2) infection advancement; and (3) stressing cold-adapted oysters (Damariscotta River) by high temperature treatments.

In summary, *Perkinsus marinus* susceptibility and disease progression is positively correlated with temperature, salinity and the number of infective cells the oyster encountered. Although no synergistic effects was demonstrated among the 3 factors, temperature in combination with infective particle or with salinity significantly affect disease progression. Temperature was determined to be the most important factor, followed by infective cell dose and then salinity, in determining the subsequent disease development in oysters. A minimum number of infective cells (i.e. between 10 and 10² cells per oyster by shell cavity inoculation) was required to induce *P. marinus* infection in oysters. Therefore, culture of oysters in tributaries with high river water input and/or fresh water run off could effectively dilute *P. marinus* infective elements, thus providing some level of protection to oysters from infection. Disease progression and temperature stress caused condition index reduction in the host.

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